TOLERABLE UPPER INTAKE LEVELS FOR VITAMINS AND MINERALS

Scientific Committee on Food
Scientific Panel on Dietetic Products, Nutrition and Allergies

February 2006

European Food Safety Authority

About EFSA

Following a series of food scares in the 1990s (e.g. BSE, dioxins) which undermined consumer confidence in the safety of the food chain, the European Union (EU) concluded that it needed to establish a new scientific body charged with providing independent advice on food safety issues associated with the food chain. Its primary objective was to contribute to a high level of consumer health protection in the area of food safety. To voice concerns about food safety and the ability of regulatory authorities to fully protect consumers, the result was the establishment of the European Food Safety Authority (EFSA) was established funded by the European Community as an independent agency in 2002.

In close collaboration with national authorities and in open consultation with its stakeholders, EFSA provides independent scientific advice on all matters linked to food and feed safety - including animal health and welfare and plant protection - and provides scientific advice on nutrition in relation to Community legislation.

EFSA's work falls into two areas: risk assessment and risk communication. In particular, EFSA's risk assessment provides risk managers (EU institutions with political accountability, i.e. the European Commission, European Parliament and Council) with a sound scientific basis for defining policy-driven legislative or regulatory measures required to ensure a high level of consumer protection with regards to food and feed safety.

EFSA communicates to the public in an open and transparent way on all matters within its remits.

Collection and analysis of scientific data, identification of emerging risks and scientific support to the Commission, particularly in case of a food crisis, are also part of EFSA's mandate, as laid down in the founding Regulation (EC) No 178/2002 of 28 January 2002.

For more information about the European Food Safety Authority, visit the EFSA home page at: http://www.efsa.eu.int

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Foreword

The scientific opinions presented in this compilation were developed at the request of the European Commission by the Scientific Committee on Food (SCF) (up to April 2003) and the Scientific Panel on Dietetic Products, Nutrition and Allergies (NDA) of EFSA (May 2003 to 2005). The context of this request was the need for scientific advice on the safety of vitamins and minerals to support the implementation of impending harmonized EU legislation for food supplements and fortified foods, and particularly to assist with the setting of maximum limits for micronutrients in these products.

These opinions present comprehensive evaluations of possible adverse health effects of individual micronutrients at intakes in excess of dietary requirements and, where possible, establish Tolerable Upper Intake Levels (UL) for different population groups. The approach taken was based on the principles of scientific risk assessment.

Both the SCF and the NDA Panel, in turn, established a working group to prepare draft opinions which were then considered, revised and adopted at plenary meetings of the SCF or the NDA Panel. This working group, which I was privileged to chair, comprised of experts in toxicology and nutrition drawn from the SCF or NDA Panel, complemented by additional experts.

Sincere appreciation is due to the many scientists whose dedication and commitment made the completion of these reports possible. In particular, I would like to thank those who prepared draft reports - Jan Alexander, Wulf Becker, Maxine Bonham, Veronique Azaïs Braesco, Angelo Carere, Hans Classen, Peter Elias, Ibrahim Elmadfa, Werner Grunow, Alan Jackson, Andreu Palou, Hildegard Przyrembel, Andy Renwick, Wim Saris, Eberhard Schmidt, Klaus Schümann, Gerrit Speijers, Sean Strain, Henk van den Berg and Ron Walker.

I also thank the EFSA and EC staff who supported this work, in particular Pilar Rodríguez Iglesias and Leng Heng (Secretariat of the NDA Panel), Miguel Ángel Granero Rosell, (Secretariat of the SCF), and Helen Lee, Basil Mathioudakis and Sabine Osaer (DG Health and Consumer Protection).

These opinions will find immediate application by the regulatory agencies that oversee addition of micronutrients to foods and food supplements in the EU. They also represent a valuable scientific reference on the safety of micronutrients which will be used by scientists and policy makers for many years.

Albert Flynn, *Chair*
EFSA Scientific Panel on Dietetic Products, Nutrition and Allergies

December, 2005

History

ABOUT THE SCF

The Scientific Committee for Food (SCF) was originally established by Commission Decision 74/234/EEC of 16 April 1974, replaced by Commission Decision 95/273/EC of 6 July 1995, to advise the European Commission on matters relating to the protection of the health and safety of persons arising or likely to arise from the consumption of food, in particular on nutritional, hygienic and toxicological issues.

Following the reorganisation of the Commission's Scientific Committees in 1997, the previous Decisions were replaced by Commission Decision 97/579/EC of 23 July 1997 setting up eight Scientific Committees, including the Scientific Committee on Food (SCF).

The members of the SCF were independent persons, highly qualified in the fields associated with medicine, nutrition, toxicology, biology, chemistry, or other related disciplines.

The Secretariat of the Scientific Committees was provided by the services of the Commission. Most of Community Directives and Regulations related to foodstuffs, required the Commission to consult the Committee on provisions which may have an effect on public health falling within the scope of these directives and regulations.

The SCF mandate consisted of advising the European Commission on scientific and technical questions concerning consumer health and food safety associated with the consumption of food products and in particular questions relating to toxicology and hygiene in the entire food production chain, nutrition, and applications of agrifood technologies, as well as those relating to materials coming into contact with foodstuffs, such as packaging.

ABOUT THE NDA PANEL

With the establishment of EFSA by the European Parliament and Council Regulation (EC) No 178/2002, the scientific advice provided by the previous Scientific Committees was handed over to EFSA. In accordance with its founding Regulation, EFSA is required to provide scientific advice and scientific and technical support for the Community's legislation and policies in all fields which have a direct or indirect impact on food and feed safety. It is required to provide independent information on all matters within these fields and communicate on risks.

The Scientific Committee and Scientific Panels of EFSA are responsible for providing the scientific opinions of the Authority, each within their own spheres of competence. EFSA is required to contribute to a high level of protection of human life and health, and in this respect take account of animal health and welfare, plant health and the environment, in the context of the operation of the internal market.

Under Article 18 of the Decision concerning the establishment and operations of the Scientific Committee and Panels, adopted by the Authority's Management Board on 17 October 2002, the Scientific Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) deals with questions relating to dietetic products, human nutrition and food allergy, and other associated subjects such as novel foods.

Background

In accordance with the 1998 working programme of the European Commission, active consideration was given to the subject of harmonising legislation for food supplements containing vitamins and minerals and to the addition of vitamins and minerals to foods. In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In 2003, the Commission published a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods[2].

The SCF was therefore asked to advise the European Commission in accordance with the following terms of reference. With a view to provide scientific support to the Commission's legislative work in this field, the SCF issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals.

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within its mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements, the European Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, in 2003 EFSA was asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the following terms of reference.

Terms of Reference

In 1998 the European Commission requested the SCF 1) to review the upper levels of daily intakes of individual vitamins and minerals that are unlikely to pose a risk of adverse health effects; and 2) to provide the basis for the establishment of safety factors, where necessary, for individual vitamins and minerals which would ensure the safety of fortified foods and food supplements containing these nutrients.

These terms of reference were handed over to EFSA with respect to the outstanding 12 vitamins and minerals in 2003.

1 - *Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.*

2 - *Proposal for a Regulation of the European Parliament and of the Council on the addition of vitamins and minerals and of certain other substances to foods. COM(2003) 671 final. 2003/0262 (COD). Brussels, 10.11.2003.*

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GUIDELINES OF THE SCIENTIFIC COMMITTEE ON FOOD FOR THE DEVELOPMENT OF TOLERABLE UPPER INTAKE LEVELS FOR VITAMINS AND MINERALS

(ADOPTED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

These guidelines outline a framework of general principles for evaluation of the adverse effects of micronutrients in humans and for establishing upper levels of intake of micronutrients which are unlikely to result in adverse effects in the general population. It is recognised that these principles may have to be reconsidered in the light of experience obtained in the evaluation of individual micronutrients and of interactions with other micronutrients.

Vitamins and (essential) minerals are micronutrients which are essential components of the human diet and the human body. Like other chemical substances, micronutrients may have adverse effects if consumed in excessive amounts. However, when evaluating the adverse effects of micronutrients it is necessary to take into account that, in contrast to non-essential chemical substances, there is a (lower) level of intake below which risk of deficiency conditions or sub-optimal functioning arises. This aspect has been addressed by the Scientific Committee on Food in establishing the recommended daily intakes (SCF, 1993). The focus of this report is the evaluation of 'risk' although it is recognised that nutritional requirements will need to be taken into consideration when setting upper levels of intake. This will be done on a nutrient by nutrient basis.

A number of reports on upper levels of intake of nutrients have been consulted in the development of these guidelines (Bernier, 1995; Nordic Council of Ministers, 1995; Anon, 1996; FNB, 1997, 1998, 2000; Hathcock, 1997; Shrimpton, 1997; WHO, 1996).

2. DEFINITIONS

Tolerable upper intake level (UL) - the maximum level of total chronic daily intake of a nutrient (from all sources) judged to be unlikely to pose a risk of adverse health effects to humans. 'Tolerable intake' in this context connotes what is physiologically tolerable and is a scientific judgement as determined by assessment of risk, i.e. the probability of an adverse effect occurring at some specified level of exposure. ULs may be derived for various lifestage groups in the population.

The UL is not a recommended level of intake. It is an estimate of the highest level of intake which carries no appreciable risk of adverse health effects. To establish whether an exposed population is at risk requires a risk assessment to determine what is the fraction (if any) of the population whose intake exceeds the UL and the magnitude and duration of the excessive intake.

To whom does it apply? – all groups of the general population (excluding those receiving the nutrient under medical supervision), including sensitive individuals, throughout the life stage - except in some cases discrete, identifiable sub-populations (e.g. those with genetic predisposition or certain disease states) that may be especially vulnerable to one or more adverse effects (FNB, 1997). The exclusion of such sub-populations will be considered on a nutrient by nutrient basis.

Adverse effect - change in morphology, physiology, growth, development or life span of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increase in susceptibility to the harmful effects of other environmental influences (WHO, 1994). Decisions on whether or not any effect is adverse require expert judgement.

3. APPLICATION OF RISK ASSESSMENT TO NUTRIENTS

3.1. Special considerations for nutrients

Nutrients possess some characteristics which distinguish them from other food chemicals for the purpose of risk assessment. Nutrients are essential for human well-being within a certain range of intakes and there is a long history of safe consumption of nutrients at the levels found in balanced human diets. Additionally, for some nutrients there may be experience of widespread chronic consumption (e.g. from dietary supplements) at levels significantly above those obtained from endogenous nutrients in foods without reported adverse effects. Data on adverse effects of nutrients are also often available from studies in humans which helps to reduce the uncertainty factors. Furthermore, many nutrients are subject to homeostatic regulation of body content through adaptation of absorptive, excretory or metabolic processes, and this provides a measure of protection against exposures above usual intakes from balanced diets.

Where possible, ULs should be derived for total intake of nutrients from all sources. It should be noted that added nutrients may sometimes differ from endogenous nutrients in foods in a number of ways, e.g. chemical form, timing of intake and amount consumed in a bolus dose, and effect of the food matrix and interaction of the nutrient with other constituents of the diet.

3.2. Basic concepts

In general, the same principles of risk assessment apply to nutrients as to other food chemicals, but it must be recognised that nutrients possess some distinguishing characteristics, as outlined above.

Risk assessment is a systematic means of evaluating the probability of occurrence of adverse health effects in humans from an excess exposure to an environmental agent (FAO/WHO, 1995) (in this case nutrients in food and water, nutrient supplements and medicines). The hallmark of risk assessment is the requirement to be explicit in all of the evaluations and judgements that must be made to document conclusions.

A generic model for carrying out risk assessment for biological and chemical agents was agreed upon at the FAO/WHO Expert Consultation 'Application of risk analysis to food standards issues' in 1995 (FAO/WHO, 1995) and this model now constitutes the basis of discussions on risk assessment by the Codex Alimentarius Commission and the European Commission. A similar model for risk assessment of nutrients has been used recently in the US and Canada and has been described in detail (FNB, 1997, 1998, 2000).

The process of the risk assessment may be divided into a number of steps (FAO/WHO, 1995; FNB, 1997, 1998, 2000):

Step 1. Hazard identification - identification of known or potential adverse health effects of a given nutrient. It involves the collection, organisation and evaluation of all information pertaining to the adverse effects of a given nutrient. It concludes with a summary of the evidence concerning the capacity of the nutrient to cause one or more types of adverse effect in humans.

Step 2. Hazard characterisation – the qualitative and quantitative evaluation of the nature of the adverse effects associated with a nutrient; this includes a dose response assessment, i.e. determining the relationship between nutrient intake (dose) and adverse effect (in terms of frequency and severity).

Based on these evaluations, an UL is derived, taking into account the scientific uncertainties in the data. ULs may be derived for various life-stage groups within the population.

Step 3. Exposure assessment - evaluates the distribution of usual total daily nutrient intakes among members of the general population.

Step 4. Risk characterisation - analyses the conclusions from steps 1 through 3 and characterises the risk. Generally, risk is considered to be the probability of an adverse effect (and its severity). The risk will depend on the fraction of the population exceeding the UL and the magnitude and duration of the excessive intake. Scientific uncertainties associated with both the UL and the intake estimates are described so that risk managers understand the degree of scientific confidence they can place in the risk assessment.

3.3. Thresholds

For nutrients, no risk of adverse effects is expected unless a threshold dose (or intake) is exceeded (FNB, 1997).

Thresholds for any given adverse effect vary among members of the population. In theory, ULs could be established by defining some point in the distribution of thresholds that would be protective for some specified fraction of the population. However, in general, for nutrients there are insufficient data to establish the distribution of thresholds for individual adverse effects.

Nevertheless, it is possible to derive ULs for which there is confidence that it lies very near the low end of the theoretical distribution of thresholds, thus protecting most of the general population, including the most sensitive (but excluding discrete sub-populations that may be especially vulnerable to one or more adverse effects).

3.4. Variability in the sensitivity of individuals to adverse effects

Adverse effects of nutrients are influenced by physiological changes and common conditions associated with growth and maturation that occur during an individual's lifespan. Therefore, where necessary, and to the extent possible, ULs are derived for each separate life-stage group, e.g. infants, children, adults, the elderly, and women during pregnancy or lactation. Even within relatively homogenous life-stage groups, there is a range of sensitivities to adverse effects, e.g. sensitivity is influenced by body weight and lean body mass.

The derivation of ULs for the normal healthy population, divided into various life-stage groups accounts for normally expected variability in sensitivity, but it excludes sub-populations with extreme and distinct vulnerabilities due to genetic predisposition or other considerations (including these would result in ULs which are significantly lower than are needed to protect most people against adverse effects of high intakes). Sub-populations needing special protection are better served through the use of public health screening, health care providers, product labelling, or other individualised strategies. The extent to which a sub-population becomes significant enough to be assumed to be representative of a general population is an area of judgement and of risk management and will be considered for individual nutrients.

3.5. Bioavailability

Bioavailability of a nutrient relates to its absorption and may be defined as its accessibility to normal metabolic and physiological processes. Bioavailability determines a nutrient's beneficial effects at physiological levels of intake and the nature and severity of adverse effects at excessive intakes. Because of the considerable variation in nutrient bioavailability in humans, bioavailability data for specific nutrients must be considered when deriving ULs. In particular, the chemical form of a nutrient may have a large influence on bioavailability and should be specified in deriving the UL. Other modulating factors include: nutritional status of the individual, nutrient dose, interaction with other dietary components and the food matrix (e.g. consumption with or without food).

4. STEPS IN THE DEVELOPMENT OF THE UL

4.1. Hazard identification

This step outlines the adverse health effects that have been demonstrated to be caused by the nutrient.

Human studies provide the most relevant data for hazard identification and, when they are of sufficient quality and extent, are given the greatest weight. Other experimental studies (*in vivo* and *in vitro*) may also be used. Six key issues that can be addressed in the data evaluation of human and animal studies are:

- ***evidence of adverse effects on humans:*** all human, animal and in vitro published evidence addressing the likelihood of a nutrient eliciting an adverse effect in humans is examined. Not all demonstrable structural or functional alterations represent adverse effects; some alterations may be considered of little or self-limiting biological importance. Decisions on which observed effects are 'adverse' are based on scientific judgements.

- ***causality:*** it is important to determine whether there is a causal relationship established by the published human data. Criteria for judging the causal significance of an exposure-effect association

indicated by epidemiological studies have been adopted in various reports (e.g. NRC, 1982, 1989; Department of Health, 1998). These include demonstration of a temporal relationship, consistency, strength of association (narrow confidence intervals for risk estimates), a dose-response relationship (a biological gradient), specificity, biological plausibility, and coherence.

- ***relevance of experimental data:*** for example, animal data - all animal data should be considered, taking into account interspecies differences, and explicit reasons given for excluding data not considered relevant to human risk; route of exposure - ingestion exposure is more relevant than other routes; duration of exposure and relevance of exposure to dietary intakes by human populations (e.g. chronic daily versus short-term bolus exposure).

- ***mechanisms of adverse effects:*** knowledge of the molecular or cellular events underlying the adverse effect can assist in dealing with the problems of data interpretation.

- ***quality and completeness of the data base.***

- ***identification of distinct and highly sensitive sub-populations:*** these may or may not be included in the derivation of the UL, subject to judgement applied on a case by case basis.

4.2. Hazard characterisation

This step includes dose response assessment which addresses the relationship between nutrient intake (dose) and adverse effect (in terms of intake and severity) and involves a number of key components (FNB, 1997):

- ***data selection:*** the data evaluation process results in the selection of the most appropriate or critical data set(s) for deriving the UL. Selecting the critical data set includes the following considerations:

==> human data are preferable to animal data. Human studies should be considered in relation to hazards identified in animal studies.

==> in the absence of appropriate human data, information from an animal species whose biological responses are most like those of humans is most valuable.

==> if it is not possible to identify such a species or to select such data, data from the most sensitive animal species, strain, or gender combination are given the greatest emphasis.

==> the route of exposure that most resembles the route of expected human intake is preferable. This includes considering the digestive state (e.g. fed or fasted) of the subjects or experimental animals. Where this is not possible, the differences in route of exposure are noted as a source of uncertainty.

==> the critical data set defines the dose-response relationship between intake and the extent of the adverse effect known to be the most relevant to humans. Data on bioavailability need to be considered and adjustments in expressions of dose response are made to determine whether any apparent differences in dose response between different forms of a nutrient can be explained.

==> the critical data set should document the route of exposure and magnitude and duration of intake, and the intake that does not produce adverse effects as well as the intake which produces adverse effects.

- ***identification of NOAEL (or LOAEL) and critical endpoint:*** the no observed adverse effect level (NOAEL) is the highest intake of a nutrient at which no adverse effects have been observed. The NOAEL can be identified from evaluation of the critical data set. If there are not adequate data demonstrating a NOAEL, then a lowest observed adverse effect level (LOAEL - the lowest intake at which an adverse effect has been demonstrated) can be used. Where different adverse effects (or endpoints) occur for a nutrient the NOAELs (or LOAELs) for these endpoints will differ. The critical endpoint is the adverse effect exhibiting the lowest NOAEL (e.g. the most sensitive indicator of a nutrient's adverse effects). The derivation of a UL based on the most sensitive endpoint will ensure protection against all other adverse effects.

- ***uncertainty assessment:*** there are usually several scientific uncertainties associated with extrapolating from the observed data to the general population and several judgements must be made in deriving uncertainty factors to account for the individual uncertainties. The individual uncertainty factors may be combined into a single composite uncertainty factor for each nutrient and applying this (composite) uncertainty factor to a NOAEL (or LOAEL) will result in a value for the derived UL that is less than experimentally derived NOAEL, unless the uncertainty factor is 1.0. The larger the uncertainty, the larger the uncertainty factors and the lower the UL, which represents a lower estimate of the threshold above which the risk of adverse effects may increase. In the application of

uncertainty factors there should be cognisance of nutritional needs, e.g. the derived UL should not be lower than the recommended intake.

- Because imprecision of the data, lack of data and adequacy of the data on variability are major limitations of risk assessment, uncertainty factors are used. Considerable scope must be allowed for the application of scientific judgement in making the final determination of uncertainty factors. Since data are generally available in human populations, and since studies on human populations may cover part of the variability inherent in the population, the data on adverse effects of nutrients may not be associated with the same uncertainties as with non-essential chemical substances resulting in uncertainty factors for nutrients typically less than 10. The uncertainty factors are lower with higher quality data and when the adverse effects are extremely mild and reversible. The availability of toxicokinetic data in humans may permit a lower uncertainty factor. In general, when determining an uncertainty factor, the following potential sources of uncertainty are considered:

==> *interindividual variation and sensitivity:* a small uncertainty factor is used if it is judged that little population variability is expected for the adverse effect, and a larger uncertainty factor (close to 10) may be used if variability is expected to be great (NRC, 1994).

==> *experimental animal to human:* an uncertainty factor is generally applied to the NOAEL to account for the uncertainty in extrapolating from animal data to humans. A larger uncertainty factor may be used if it is believed that the animal responses will underpredict average human responses (NRC, 1994).

==> *LOAEL to NOAEL:* if a NOAEL is not available, an uncertainty factor may be applied to account for the uncertainty in deriving a UL from the LOAEL. The size of the uncertainty factor involves a judgement based on the severity and incidence of the observed effect at the LOAEL and the steepness (slope) of the dose response.

==> *subchronic NOAEL to predict chronic NOAEL:* when data are lacking on chronic exposures, scientific judgement is necessary to determine whether chronic exposure is likely to lead to adverse effects at lower intakes than those producing effects after subchronic exposures.

- ***derivation of an UL:*** the UL is derived by dividing the NOAEL (or LOAEL) by the (composite) uncertainty factor. ULs are derived for different life-stage groups using relevant data. In the absence of data for a particular life-stage group, extrapolations are made from the UL for other groups on the basis of known differences in body size, physiology, metabolism, absorption and excretion of a nutrient. When data are not available for children and adolescents, extrapolations are made on the basis of body weight using the reference weights in the Appendix. It should be noted that derivation of a UL does not take into account possible adverse effects of acute bolus dosages. This issue will be addressed separately for individual nutrients, where relevant.

4.3. Characterisation of risk

This may include a description of the scientific uncertainties associated with the UL estimates in order to indicate the degree of scientific confidence that can be placed in these estimates. It may also include an estimate of intake for population groups, where data are available, as well as an indication of the margin between recommended or actual intakes and the UL, and an indication of circumstances, if any, in which risk is likely to arise.

It should indicate whether sub-populations having distinct and exceptional sensitivities to the adverse effects of the nutrient have been excluded, and whether more research is needed. For nutrients for which there are no, or insufficient, data on which to base the establishment of a UL, an indication should be given on the highest level of intake where there is reasonable confidence in data on the absence of adverse effects.

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APPENDIX

Reference body weights of population groups in Europe (SCF, 1993)

Age (years)	Mean weight (kg)	
	Male	Female
1-3	13.0	12.5
4-6	20.0	19.0
7-10	28.5	29.0
11-14	44.5	45.0
15-17	61.5	53.5
18-29	74.6	62.1
30-59	74.6	62.1
60-74	73.5	66.1
≥75	73.5	66.1

OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF BETA CAROTENE

(EXPRESSED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

ß-Carotene and carotenoids in general are isoprenoid compounds which are not synthesised in animals but biosynthesised by plants and micro-organisms. About 700 naturally occurring carotenoids have been identified so far. About 10% of them can be found in the human diet, and about 20 of these compounds have been found in plasma and tissues of the mammal. The predominant carotenoids observed in the plasma are ß-carotene, lycopene, lutein, ß-cryptoxanthin and α-carotene, accounting for more than 90% of the circulating carotenoids in humans (see Rock, 1997, for specific references).

Some dietary carotenoids, such as ß-carotene, serve as an important source of vitamin A, which is the major known function of carotenoids in humans. ß-Carotene is a hydrocarbon $C_{40}H_{56}$ that has a ß-ionone structure as the terminal ring system at each side of the poliene chain. Carotenoids containing at least one unsubstituted ß-ionone ring and a poliene chain are potential precursors of vitamin A. The preformed vitamin A is only present in animal products (e.g. liver, eggs, milk products), thus, in countries where the intake of animal products is low, carotenoids have to meet (i.e. by 80% or more in Asia and Africa) the vitamin A requirements. Even in developed countries carotenoids usually contribute to vitamin A supply by more than 40% (Woutersen *et al*, 1999).

The best-characterised natural functions of carotenoids are to serve as light-absorbing pigments during photosynthesis and protection of cells against photosensitization. Carotenoids provide considerable coloration and identification for many species, from vegetables to animals. In addition, carotenoids serve several other functions, such as radical quenching, antioxidant and anticarcinogenic activities in different animal sites and are regulators of cell function.

A number of reviews, monographs and comments on the safety of ß-carotene have been published during the last decade (e.g. Bauernfeind *et al*, 1981; Heywood *et al*, 1985; Rock, 1997; IARC, 1998; Omenn, 1998; Palozza, 1998; SCF, 1998; Woutersen *et al*, 1999). Very recently the SCF has set out the scientific data relevant to the safety of use of β-carotene from all dietary sources but limited their conclusions only to food additive uses (SCF, 2000).

2. NUTRITIONAL BACKGROUND AND METABOLISM

In the majority of industrialised countries, fruits and vegetables provide an estimated 2-3 mg/day of pro-vitamin A carotenoids, of which β-carotene is the principal component (Granado *et al*, 1996). An approximate β-carotene intake of 1.8 mg/day in a randomly selected population of women in the USA has been reported (Chug-Ahuja *et al*, 1993), the main dietary sources being carrots, orange juice, oranges, tomatoes and dark green leafy vegetables. The average intake in the German National Food Consumption Survey was 1.81 mg/day (Pelz *et al*, 1998), mainly from carrots. An average β-carotene intake of 1.7-2.1 mg/day has been reported in Finland (Heinonen, 1991), and of 3.0 mg/day in The Netherlands (Goldbohm *et al*, 1998). Levels of fruit and vegetable consumption, however, vary greatly between individuals and β-carotene intake may be much higher than average in people who regularly consume substantial amounts of foods such as carrots (Gregory *et al*, 1990; Scott *et al*, 1996). Some authors have reported that β-carotene intake varies according to seasonal factors, perhaps due to the differing availability of specific fruits and vegetables, or because of factors such as light and heat that may affect the carotenoid content of foods (Olmedilla *et al*, 1994; Rautalahti *et al*, 1993; Takagi *et al*, 1990). The SCF has not recommended the consumption of β-carotene and carotenoids in general, beyond what is needed to supply vitamin A (SCF, 1993).

The Committee has not received detailed information on how much the use of ß-carotene as an additive contributes to the overall intake. However, unpublished data from a Danish and Austrian survey showed that present use levels (Elmadfa *et al*, 1996; SCF, 1997), combined with the knowledge of the eating habits of the population, suggest an average exposure from ß-carotene, used as food additive, of about 1-2 mg/person/day.

The general mechanism of intestinal ß-carotene absorption in mammals is by passive diffusion of mixed micelles, which are formed during fat digestion in the presence of bile acids. In general, the types and amounts of carotenoids in the plasma reflect those in the diet. Depending on specific conditions, the extent of absorption for ß-carotene reported in the literature varies between 10% and 90% (see Woutersen *et al*, 1999). Absorption appears to be linear up to intakes of 20-30 mg, but becomes limited at higher intakes. Limiting factors are dependent on the formulation or food matrix, the amount and type of fat co-ingested with the carotenoid and the presence of bile acids. Release from the food matrix into the lipid phase and solubilisation within mixed micelles appears to be the most critical steps in ß-carotene absorption. Dietary fibre and other meal components, together with a number of metabolic factors and subject characteristics (Rock, 1997) may also affect ß-carotene absorption. Important differences in the rates of absorption and intestinal cleavages have been demonstrated between man and laboratory animals (see section 2.1).

The main site of carotenoid metabolism is the intestinal mucosa, at least in rodents, but peripheral tissues such as lung, kidney, liver and fat of several mammals, including humans and rodents, can also convert ß-carotene to retinoic acid (RA) (Wang *et al*, 1992; Redlich *et al*, 1996).

ß-carotene can be cleaved in mammalian tissues mainly at the central double bond (C-15,15') yielding two molecules of retinal which may either be reduced to retinol (vitamin A) or further oxidised to RA; an alternative pathway (which can also yields RA, with or without the involvement of intermediate retinal) is the non-central (eccentric) cleavage at eccentric double bonds (e.g. C-13',14', C-11',12', C-9',10' and C-7',8') (Krinsky *et al*, 1990; Wang *et al*, 1992; Wang *et al*, 1999) to form retinoids and apo-ß-carotenoids, which have structures that are similar to retinoids, the function of these being largely unknown.

Carotenoids are transported in association with the lipoproteins, with a distribution highly correlated to that of cholesterol. Liver and adipose tissue are the main sites of carotenoid deposition. After absorption retinyl esters formed in the enterocyte are incorporated into chylomicrons, before they are secreted into the intestinal lymph and move into the blood stream. In the fasted state about 75% of the ß-carotene is bound to LDL and about 25% to HDL and VLDL. Circulating carotenoid concentrations are found to be lower in smokers than in non-smokers, due in part to the depletion of these compounds by components of cigarette smoke (Handelman *et al*, 1996).

Carotenoids can act as antioxidants and free radical/reactive species scavengers (Tsuchiya *et al*, 1993; Everett *et al*, 1996; IARC, 1998; Omenn, 1998). *In vitro*, carotenoids efficiently quench excited molecules such as singlet oxygen and can scavenge peroxyl radicals; interactions with several other radicals have also been reported and a synergistic antioxidant protection by carotenoids with vitamins E and C has been shown (Edge and Truscott, 1997). The role *in vivo* and in humans is less clear (IARC, 1998; Palozza, 1998; Lambert, 1999). The switch from antioxidant to pro-oxidant behaviour can be, for example, a function of oxygen concentration (Edge and Truscott, 1997; Palozza, 1998). The pro-oxidant activity of ß-carotene has been demonstrated at a high partial pressure of oxygen; because this is highest in the outermost cells of the lung, these cells might be particularly subject to the pro-oxidant effect of ß-carotene (cited in Paolini *et al*, 1999).

Other effects of carotenoids, which can be related to cancer prevention, are the enhancement of the immune response observed in some experimental models, which may be due to production of tumour specific antigens (IARC, 1998). In addition, carotenoids have been reported to modulate cytochrome P450 metabolism, inhibit arachidonic acid metabolism, inhibit chromosome instability and chromosome damage, influence apoptosis, and affect several other biological processes (see IARC, 1998, and text later on)

Part of the effects of ß-carotene can be mediated by the formation of retinoic acid (RA) that has a key function as a regulator of gene expression, morphogenesis, and growth in vertebrate embryos. Cellular responses to retinoids are generally mediated by two families of nuclear receptors (RARs and RXRs) (Chambon, 1996). Different retinoic acid receptor isotypes display a characteristic pattern of tissue distribution, RARα being the most ubiquitously distributed (Chambon, 1996). RARß plays an important role in lung development and has been proposed to have a tumour suppresser function in lung (Houle

et al, 1993). Primary lung tumours and lung cancer cell lines lack RARß expression, and such loss of expression may be an early event in lung carcinogenesis. RARß2 is the most abundant isoform in normal human lung tissue and restoration of RARß2 in a RARß-negative lung cancer cell line has been reported to inhibit tumorigenicity in nude mice (see references in Wang *et al*, 1999).

2.1. Species differences in ß-carotene metabolism

Most laboratory animals break down ß-carotene in their intestine and thus absorb almost none intact. Hence, rodents have low serum carotenoid levels (about 1/1000 of human levels) that are not related to dietary intake due to very active dioxygenase cleavage to retinal. In man, roughly 20-75% of the ß-carotene is absorbed intact (Wang *et al*, 1992; Rock, 1997).

Studies have thus indicated that the rat and mouse are not suitable models for studying the uptake of β-carotene into the plasma, with the possible exception of experiments using very high doses or a non-oral way of administration. Similarly, studies in hamsters showed that β-carotene concentrations remained low in animals given dietary β-carotene supplementation, although retinol levels increased, indicating that hamsters are also efficient converters of β-carotene to retinol (cited in IARC, 1998). Rabbits do not appear to absorb β-carotene well and these animals when fed a carotenoid-rich diet showed no carotenoids in the blood and only small increases in liver vitamin A concentrations (cited in IARC, 1998). Strict carnivores obtain a diet rich in pre-formed vitamin A and thus do not depend on provision via carotenoids in the diet. Indeed, cats reportedly lack the enzyme β-carotene-15,15'-dioxygenase and, thus, have a requirement for pre-formed vitamin A in the diet (Bauernfeind *et al*, 1981).

Ferrets (Gugger *et al*, 1992; Wang *et al*, 1992; White *et al*, 1993a; Rock, 1997; Wang *et al*, 1999), the pre-ruminant calf (Poor *et al*, 1992) and the Mongolian gerbil (Krinsky *et al*, 1990; Mathews-Roth, 1993) have been proposed as useful models for human ß-carotene absorption and cleavage as these animals also absorb and release intact ß-carotene from the enterocyte. The ferret studies are particularly relevant to the present report. This animal model partially mimics the absorption and tissue metabolism of ß-carotene in humans. It has been used for studies of tobacco smoking and inhalation toxicology (Sindhu *et al*, 1996), and also it has been used to test the hazard associated with a high dose of ß-carotene and tobacco smoking on lung (Wang *et al*, 1992; Wang *et al*, 1999). Although serum β-carotene levels are normally very low in these animals, dietary supplementation has been shown to increase concentrations to levels similar to those detected in human serum, and also to increase levels in the liver, adipose and other tissues (Ribaya-Mercado *et al*, 1989; Gugger *et al*, 1992; Ribaya-Mercado *et al*, 1992; Ribaya-Mercado *et al*, 1993; White *et al*, 1993a; White *et al*, 1993b; Wang *et al*, 1999). It has to be recognised that no single species provides a good model for studying all aspects of β-carotene in humans (van Vliet, 1996; IARC, 1998), but ferrets are particularly interesting as an example which allows reproducing (to some extent) the problem in the particular tissue (the lungs) pointed out by human trials.

3. HAZARD IDENTIFICATION

A number of epidemiological studies in humans and several animal studies developed during the last third of the past century support the idea that ß-carotene can prevent cancer, cardiovascular diseases and other diseases in humans. However human chemoprevention trials developed the last decade (see Section 3.2) have shown that supplemental ß-carotene actually increases both lung-cancer incidence and mortality in human smokers and, more recently, mechanisms which offer likely explanations of these adverse effects have been derived from experimental studies in appropriate animal models (see Section 3.3).

3.1. Animal studies

3.1.1. Standard toxicological studies

In summary, no adverse effects of high-dose oral β-carotene supplementation have been observed in several standard toxicological studies in various experimental animals (rat, mice, rabbits) (IARC, 1998; Woutersen *et al*, 1999). These studies included acute toxicity, up to 5000 mg/kg bw/day in Sprague Dawley rats (Woutersen *et al*, 1999) and up to 2000 mg/kg bw/day in Wistar rats (Buser, 1992; Strobel, 1994), chronic toxicity/carcinogenicity up to 1000 mg/kg bw/day for life in rats (Hummler and Buser, 1983; Heywood *et al*, 1985) or mice (Buser and Hummler, 1983a; Heywood *et al*, 1985), teratogenicity and reproductive toxicity (up to 1000 mg/kg bw/day for 3 generations, or during days 7 to 16 of gestation, in rats; up to 400 mg/kg bw/day during days 7 to 19 of gestation in rabbits) (Komatsu, 1971, cited in Kistler, 1981; Buser and Hummler, 1982; Heywood *et al*, 1985, and Woutersen *et al*, 1999).

In beagle dogs (Buser and Hummler, 1983b; Heywood *et al*, 1985) no toxic effects (up to 250 mg/kg bw/day for 2 years) were observed. However, this study, in addition to the problem of using a hydrosoluble formula, had a non-explained episode, at week 88 of the study (Buser and Hummler, 1983b), when a dramatic weight loss in dogs after withdrawing ß-carotene was observed.

However, the above studies were not aimed at investigating specific effects in the lung, which now we know appears to be the more sensitive tissue. In addition, species used are particularly unsuitable for oral studies, due to the high efficiency of conversion to vitamin A, such that no significant levels of unaltered β-carotene are absorbed and incorporated into the systemic circulation.

Genotoxicity and modulation of genotoxic effects of β-carotene has been previously reviewed (SCF, 2000), most studies giving negative findings. However there are no good experimental studies especially addressing the genotoxicity of ß-carotene *in vivo* and negative findings in studies designed to asses the anticlastogenic activity of ß-carotene do not provide conclusive evidence on the lack genotoxicity *in vivo*. Positive results obtained in a limited study with synthetic ß-carotene should be evaluated with caution but not dismissed, in view of the pro-oxidant activity of ß-carotene and the evidence of micronucleus induction *in vitro* by synthetic ß-carotene (Xue *et al*, 1998). The latter study also suggests that the genotoxicity *in vitro* of ß-carotene formulations can be modulated by their relative stereoisomer composition. These findings should be taken into account also in the evaluation of studies *in vivo*, which used samples of ß-carotene of different and/or unspecified composition. In summary, the data available are insufficient for an adequate evaluation of the genotoxicity of ß-carotene *in vivo.*

The majority of studies relating to carcinogenicity effects by β-carotene have indeed shown either preventive, or no effect, as it has been previously reviewed (SCF, 2000). However, in contrast to studies relating to tumours at other sites, only one report (Furukawa *et al*, 1999) has described an inhibitory effect of β-carotene supplementation on carcinogen-induced respiratory tract tumourigenesis. Supplementation of the diet with β-carotene at levels up to 0.25% (approximately 250 mg/kg bw/day), for 12 weeks, resulted in a significant reduction of the incidence of benign respiratory tract changes (hyperplasia and papillomas) in hamsters exposed to cigarette smoke (Furukawa *et al*, 1999). The majority of studies have shown no effect of β-carotene supplementation on experimentally induced respiratory tract tumourigenesis in mice (Murakoshi *et al*, 1992; Nishino, 1995; Yun *et al*, 1995), or hamsters (Beems, 1987; Moon, 1994; Wolterbeek *et al*, 1995).

Two reports in hamsters (Beems, 1987; Wolterbeek *et al*, 1995) and one in ferrets (Wang *et al*, 1999), which have been recently reviewed by the SCF (2000), describe potential enhancement of chemically-induced respiratory tract tumourigenesis, although statistically significant increases in the incidences of malignant tumours have not been reported. The study in ferrets, which was specifically designed to mimic the human trials, is described below.

Study in ferrets

Wang *et al* (1999) used a ferret model to assess the single or combined effects of cigarette smoke and/or β-carotene supplementation on lung histopathology/biochemistry.

To mimic human trials, by correcting for species differences in ß-carotene absorption, Wang *et al* (1999) fed ferrets with 2.4 mg ß-carotene/kg per day (15 times higher than the 0.16 mg of ß-carotene/kg per day for the control group fed a low ß-carotene diet). This dose mimics an intake equivalent to 30 mg of ß-carotene per day in a 70-kg human. It was shown that: 1) the plasma level of ß-carotene in the ferrets had a similar increase (17-22 fold) to that observed in human trials (see Section 3.2.2); 2) tissue levels of ß-carotene, retinol and RA in control ferrets were within the range found in the normal human, although this was not the case for the higher plasma levels of retinyl esters in the ferret; 3) the lung architecture and formation of oxidative metabolites from ß-carotene were considered similar in both species (references listed in Wang *et al*, 1999), and 4) the concentration of urinary cotinine equivalents in the smoke-exposed ferrets was similar to that found in humans smoking 1.5 packs of cigarettes per day.

Four groups of 6 males were treated with either 2.4 mg/kg bw/day β-carotene supplementation (in corn oil, fed orally), cigarette smoke exposure (smoke from 10 cigarettes, in a chamber, for 30 minutes, twice daily), both, or neither, for a period of 6 months, at which point they were killed (Wang *et al*, 1999). Histopathological analysis revealed that all β-carotene treated animals showed an increase in cell proliferation and squamous metaplasia in lung tissue, and this was further enhanced in the animals that were also exposed to cigarette smoke. Animals exposed to cigarette smoke alone did not show these changes. The assessed histopathological endpoint, squamous metaplasia, may not be directly related

to carcinogenesis, but this study did reveal interestingly related molecular/biochemical changes in the lungs of the animals tested which are discussed later in this report (see Section 3.3).

3.2. Human studies

In humans, doses of 20-180 mg/day ß-carotene have been used to treat patients with erythropoietic photoporphyria, with no evidence of toxicity and without the development of abnormally elevated blood vitamin A.

A substantial amount of epidemiological information linking higher carotenoid intake with lower cancer incidence was accumulated in the 1970s and 1980s. Also noted was the apparent lack of toxicity of ß-carotene in high-dose clinical use against erythropoietic photoporphyria (doses of 20-300 mg/day given for many years) (Mathews-Roth, 1993; Meyers *et al*, 1996). Thus, these facts, together with the known biological properties of ß-carotene (see above), combined to justify large-scale, cancer prevention trials in humans. However, these trials did not confirm the positive expectations.

3.2.1. Epidemiological studies

3.2.1.1. ß-carotene and incidence of cardiovascular disease

A number of descriptive, cohort and case-control studies have been reviewed (IARC, 1998; Woutersen *et al*, 1999), suggesting that carotenoid and/or ß-carotene rich diets may prevent cardiovascular disease. Recently, the Rotterdam 1999-Study in the elderly (Klipstein-Grobusch *et al*, 1999) confirmed a protective association. However, the finding in numerous observational studies that increased intake of carotenoid-containing diets and higher blood concentrations of carotenoids are associated with reduced risks for cardiovascular disease cannot be interpreted as a specific protective effect of ß-carotene or other carotenoids *per se*.

In the ATBC study (ATBC Study Group, 1994), 11% more total cardiovascular death was seen in men taking ß-carotene. When the analysis was restricted to the 1862 participants who had previously had an MI, men who received ß-carotene alone had relative risks of 1.75 for fatal coronary heart disease and 3.44 for fatal MI. Similarly, an increased number of deaths from cardiovascular disease was seen in the CARET study (Omenn *et al*, 1996a; Omenn, 1998) among men taking supplemental ß-carotene plus retinol (relative risk of 1.26).

ß-carotene and cancer incidence

A number of reviews published in the 1990s have summarised the research on diet and lung cancer during the preceding 25 year period (see Steinmetz *et al*, 1996, and Ziegler *et al*, 1996). The consensus was that observational studies of diet and lung cancer, whether prospective or retrospective, consistently demonstrated reduced risk with increased intake of carotenoids from vegetables and fruits. Further, high levels of ß-carotene in the blood were consistently associated with reduced incidence of lung cancer in prospective studies. The simplest explanation of the epidemiology was that ß-carotene was protective, although other carotenoids or other compounds from vegetables and fruits, and associated dietary patterns had not been adequately explored.

The observational data suggesting cancer preventive effects are most consistent for some types of cancer -lung, oral, pharyngeal and stomach- (IARC, 1998; Woutersen *et al*, 1999), the incidence of which tends to be inversely related to ß-carotene intake or blood concentrations. A review that summarised results from 206 human epidemiological studies confirmed the evidence for a protective effect of greater vegetable and fruit consumption against cancers of the stomach, oesophagus, lung, oral cavity and pharynx, endometrium, pancreas and colon (Steinmetz *et al*, 1996). The types of vegetables or fruit that most often appeared were raw vegetables, allium vegetables, carrots, green vegetables, cruciferous vegetables and tomatoes. A number of interesting substances present in these foods include dithiolthiones, isothiocyanates, indol-3-carbinol, allium compounds, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, vitamin C, D-limonene, lutein, folic acid, ß-carotene, lycopene, selenium, vitamin E, flavonoids and dietary fibre (Steinmetz *et al*, 1996).

A recent case-control study in Greece (Bohlke *et al*, 1999) involved 820 women with histologically confirmed breast cancer who were compared with 1548 control women. Among postmenopausal women there were no associations between any of the micronutrients evaluated and the risk of breast cancer. Among premenopausal women, ß-carotene, vitamin C and vitamin E were each inversely associated with breast cancer, but after mutual adjustment among the three nutrients only ß-carotene remained significant.

In conclusion, the general assumption is confirmed that individuals who eat more fruits and vegetables, rich in carotenoids, and/or have high levels of serum ß-carotene, have a lower risk for cancer and cardiovascular diseases. However, a possibility could be that ß-carotene may be only a marker of the intake of other beneficial substances in fruits and vegetables, or perhaps other life-style habits. Actually (see below) no clinical trial of ß-carotene as a single agent, has shown a reduction in the risk of cancer at any specific site. On the contrary there is evidence of an increase in the risk for lung cancer among smokers and asbestos workers receiving ß-carotene supplements at high doses, which resulted in blood concentrations an average of 10-15 times higher than normal.

ß-carotene and other diseases

Erythropoietic protoporphyria (EP) (Mathews-Roth, 1993) is a genetic disease of porphyrin metabolism, characterised by abnormally elevated concentrations of protoporphyrin, which acts as an endogenous photosensitizer. As carotenoids can interact and quench photosensitizer triplet states and single oxygen, their efficacy in this disorder appears to be a consequence of the quenching of excited species. Most patients with EP or other photosensitivity diseases benefit from recommended doses for adults of about 180 mg/day, with no serious side effects and no long-term toxicity reported. These photosensitivity diseases are the only current therapeutic use of carotenoids.

Dietary carotenoids have been suggested to reduce the risk of age-related macular degeneration (Seddon *et al*, 1994; Cooper *et al*, 1999), the most common cause of irreversible blindness in people over age 65 in western countries.

Senile cataract is another ocular condition potentially related to oxidation, and ß-carotene has been studied for a possible role in the prevention of this disorder. However the available results are somewhat inconsistent. Carotenoids have also been suggested to be of benefit for several other health outcomes (such us ageing, impaired cognition, rheumatoid arthritis and cystic fibrosis), however the data are scant (IARC, 1998).

3.2.2. Selection of critical data. Prevention trials in humans with ß-carotene supplementation

Six major prevention trials with ß-carotene supplementation have been completed so far (Greenberg *et al*, 1990; Blot *et al*, 1993; ATBC Study Group, 1994; Greenberg *et al*, 1994; Hennekens *et al*, 1996; Omenn *et al*, 1996a). Short-term trials using sputum as a presumed intermediate endpoint were conducted as well with some preliminary promising results (see Omenn, 1998). However, results from the majority of clinical trials reported are not in support of using ß-carotene supplementation as a mean to reduce cancer and cardiovascular disease rates.

The first study (Greenberg *et al*, 1990) showed that supplementation with 50 mg ß-carotene/day for 5 years had no effect on the occurrence of new basal-cell or squamous-cell carcinoma in well nourished patients who had skin cancer previously. However, a 12-year latency period for these cancers diminished the value of these results.

In a second study (Greenberg *et al*, 1994), ß-carotene (25 mg/day), with or without vitamin C (1g/day) and α-tocopherol (400 mg/day) for 5-8 years, was not found to reduce the occurrence of colorectal adenoma in patients who had a prior history of adenomas.

A lack of effect of long term supplementation with ß-carotene on the incidence of malignant neoplasms and cardiovascular disease was reported in 1996 (Hennekens *et al*, 1996).

The Tyler asbestos cohort studied 755 randomised asbestos workers (McLarty, 1992) at Tyler (Texas), receiving 50 mg of ß-carotene together with 25,000 IU retinol/day or placebos. There was no difference in the two groups by criteria of sputum atypia. The ß-carotene was obtained from BASF and it is thought that the 50 mg dosage is almost equivalent to 30 mg of Roche-ß-carotene.

Two notable trials (Blot *et al*, 1993; Li *et al*, 1993) were conducted in China (The Linxian Trials) but they were very complex in design and difficult to compare with the findings (see below) in western populations: the observed effects cannot be directly attributed to ß-carotene supplementation as a combined supplementation was given and low population nutrient intake was interfering.

3.2.2.1. The Alpha-Tocopherol/Beta-Carotene (ATBC) Trial in Finland

The ATBC trial (ATBC Study Group, 1994) involved 29,133 male smokers (age 50-59) with a smoking history averaging one pack/day for 36 years. The 2x2 factorial design evaluated 20 mg ß-carotene

(from Roche) and/or 50 IU alpha-tocopherol (vitamin E) daily for 6.5 years. These doses represent a 10-fold and 5-fold excess over the median intake of ß-carotene and α-tocopherol, respectively, in this population. After 2 years of treatment, median serum ß-carotene levels had increased 17.5-fold in the ß-carotene treatment groups.

Results were unexpected. Vitamin E supplementation did not reduce the incidence of lung cancer (relative risk (RR) was 0.98). Participants receiving ß-carotene alone or in combination, had significantly higher lung cancer incidence (RR 1.18; 95%CI 1.03-1.36) and higher mortality (RR 1.08; CI 1.01-1.16) than subjects receiving placebo.

The excess lung cancer incidence was not apparent in the initial 18 months, but the incidence curves significantly diverged thereafter. Subsequent subgroup analysis (see Albanes *et al*, 1996) revealed a higher risk in heavy smokers (20 or more cigarettes/day) (RR 1.25, CI 1.07-1.46) than in light smokers (5-19 cigarettes/day) (RR 0.97, CI 0.76-1.23). Associations with alcohol intake and with non-small-cell histology were also noted. The risk was confined to the heavier drinkers (more than 11 g ethanol per day).

Interestingly, in agreement with earlier observational studies, both dietary intake and serum ß-carotene levels at baseline (before treatment) were found to be inversely related to risk of lung cancer during the trial (Albanes *et al*, 1996).

3.2.2.2. The ß-carotene and Retinol Efficacy Trial (CARET) in the USA

The CARET study (Omenn *et al*, 1996a; see also Omenn *et al*, 1996b, and Omenn, 1998) successfully randomised 18,314 participants. 30 mg ß-carotene and 25,000 IU vitamin A (retinyl palmitate) were administered daily to 14,254 smokers and former smokers (45% female) aged 50-59 at enrolment, and to 4,060 asbestos-exposed males (age 45-74). After five years of study the median serum ß-carotene levels in the active treatment group was increased by 12-fold (170 ng/ml *versus* 2100 ng/ml).

A total of 388 new cases of lung cancer were diagnosed during the 73,135 person-years of follow- up (mean 4.0 years). The active treatment group had a RR of lung cancer of 1.28 (CI 1.04-1.57), compared with the placebo group. The differences (significant from 24 months of treatment onwards) were greater as the intervention progressed. There were no statistically significant differences in the risks of other types of cancers.

In the active group the RR of death from any cause was 1.17, of death from lung cancer, 1.46, and of death from cardiovascular disease, 1.26.

As in a further analysis from ATBC published in the same issue (Albanes *et al*, 1996), there was an association (less clear trend than in ATBC study) of the excess lung cancer incidence between treatment groups with the highest quartile of alcohol intake, but no association with baseline serum ß-carotene concentrations.

In the CARET study it is not possible to distinguish the ß-carotene effects from those of the vitamin A, since the two compounds were administered in combination.

3.2.2.3. Physicians Health Study

This trial was to test the effect of aspirin on cardiovascular disease incidence (Steering Committee of the Physicians' Health Study Research Group, 1989). ß-carotene was added in a 2x2 design, using 50 mg BASF ß-carotene on alternate days. 22,071 male physicians were followed for a mean of 12.5 years. Those assigned to receive ß-carotene had significantly higher serum concentrations than those given placebo (2240 nmol/l *vs* 560 nmol/l) (4-fold). It has to be noted that this increase is lower compared with that obtained in the two previously considered trials, a situation that could be related to higher basal levels in the PHYS population and/or to a lower bioavailability of ß-carotene compared with the other trials.

In this healthy population, with 50% never-smokers and only 11% current smokers, 170 lung cancers were accumulated over the follow up period. The relative risks were 1.02 (CI 0.93-1.11) for overall mortality, 0.98 (CI 0.91-1.06) for all malignant neoplasms, and 0.93 for lung cancer.

In summary there was no effect of ß-carotene supplementation on total cancer, on total mortality, or on heart disease. Neither was an effect on lung cancer observed, but due to the lower number of cases, the power of the statistical analysis underlying this conclusion is rather weak.

3.3. Mechanisms

In light of the adverse findings in human intervention trials, in which β-carotene supplementation was associated with a promotional effect on lung tumourigenesis in smokers, studies in animals have been carried out to elucidate potential mechanisms by which these effects may have occurred.

Mechanisms have been proposed, which are related to effects in the same target tissue, the lungs, where the adverse effects have been observed in humans.

3.3.1. Effects on P450-related activities. A mechanism for a hypothetical co-carcinogenic effect of ß-carotene

Perocco *et al* (Perocco *et al*, 1999) first reported that β-carotene enhanced the transforming effect of benzo(α)pyrene (B[α]P) and cigarette-smoke condensate (tar) on mouse BALB/c 3T3 cells in an *in vitro* cell transformation assay, although β-carotene alone was not transforming in this system. The authors suggested that β-carotene may exert its effects by inducing P450 activities (in particular CYP 1A1/2), with a consequent increase in the metabolism of cigarette smoke constituents. Interestingly, however, β-carotene showed no capacity to enhance the transforming activity of 3-methylcholanthrene (3-MCA), which also requires metabolic activation by CYP1A1.

The same group (Paolini *et al*, 1999) found that dietary supplementation of rats with 500 mg/kg bw/day β-carotene for 5 days significantly increased lung enzyme activities associated with CYP1A1 and 1A2 (activating aromatic amines, polychlorinated biphenyls, dioxins and PAHs), CYP2A (activating butadiene, hexamethyl phosphoramide and nitrosamines), CYP2B1 (activating olefins and halogenated hydrocarbons) and CYP3A (activating aflatoxins, 1-nitropyrene and PAHs). The authors postulated that these powerful booster (stimulating) effect on phase I carcinogen-bioactivating enzymes might explain why ß-carotene supplementation increases the risk of lung cancer in smokers, probably due to the co-carcinogenic properties of β-carotene and it's capacity to generate oxidative stress.

Other studies (Astorg *et al*, 1994; Astorg *et al*, 1997, Basu *et al*, 1987, Gradelet *et al*, 1996) showed no ß-carotene enhanced effects on phase I or phase II xenobiotic-metabolising enzymes, but measurements were made in the liver and not in lung tissue (SCF, 2000).

3.3.2. Altered retinoid signalling: a mechanism to enhance lung tumourigenesis after high dose ß-carotene supplementation in smokers

When ferrets (animals that metabolise ß-carotene in much the same way as humans) were given ß-carotene doses equivalents to those used in the clinical trials, changes in ß-carotene metabolism were induced that may promote rather than inhibit tumourigenesis (Wang *et al*, 1999). This may explain why high-dose ß-carotene supplements unexpectedly increased lung cancer rates in the two cancer human prevention trials.

Ferrets were given a ß-carotene supplement, exposed to cigarette smoke, or both for 6 months (see also Section 3.1.2). Cell proliferation and squamous metaplasia in lung tissue were assessed by examination of proliferating cell nuclear antigen expression and histopathological examination, respectively. ß-carotene and retinoid concentration in lung tissue and plasma were analysed. Expression of genes for retinoic acid receptors (RARs) and activator protein-1 (encoded by c-jun and c-fos genes) in lung tissue specimens was examined. The results clearly showed that a strong proliferative response in lung tissue was observed in all ß-carotene-supplemented animals, and this response was enhanced by exposure to tobacco smoke. The treatment groups had statistically significant lower levels of retinoic acid in lung tissue, and they exhibited 18-73% reductions in RARß-gene expression, without reduction of RARα and RARγ. Ferrets given a ß-carotene supplement and exposed to tobacco smoke had threefold to fourfold elevated expression of the c-jun and c-fos genes.

Decreased lung concentration of retinoic acid may cause diminished retinoic signalling, enhanced lung cell proliferation, and potential tumour formation. Results showed that localised keratinised squamous metaplasia (a precancerous lesion) was observed in all ferrets in the high-dose of ß-carotene, with or without exposure to smoke. Retinoic acid levels are lowered in lung tissue as a result of ß-carotene supplementation, in spite of having increased levels of ß-carotene (by 300 fold). The possibility that some of the eccentric cleavage products of ß-carotene could act as a ligand and interfere with RA requires further investigation. Thus it is possible that ß-carotene supplementation in itself might modify ß-carotene metabolism. Reduction of retinoic signalling could occur after induction of cytochrome P450 enzymes (see section 3.3.1), perhaps by the ß-apo-8'-carotenal (increased by 2.5-fold by smoke exposure).

It can be deduced from the preceding study that diminished retinoid signalling, resulting from suppression of RARß gene expression and overexpression of activator protein-1 could be a mechanism to enhance lung tumourigenesis after high dose ß-carotene supplementation and exposure to tobacco smoke. However, a relationship between the endpoint studied (squamous metaplasia) and lung cancer has not been demonstrated, and the dose-response relationship was not studied. Nevertheless, lung carcinogenesis is associated with an alteration in retinoid signalling involving the AP-1 complex, which mediates the signal from growth factors, inflammatory peptides, oncogenes, and tumour promoters, usually resulting in cell proliferation. AP-1 (c-fos, c-jun) transcriptional activity can be inhibited by RA treatment, thus contributing to the suppression of human bronchial epithelial squamous metaplasia.

In contrast to what occurs with high doses of ß-carotene (even more when smoking), if low levels of ß-carotene are ingested, eccentric cleavage products are produced by the cells (as would be the case when one consumes ß-carotene from a carotenoid enriched diet). This form of carotenoid intake could be beneficial by giving rise to some RA.

Adverse effects of high dose supplemental ß-carotene (alone) cannot be ruled out. The intervention trial (Hennekens *et al*, 1996) which did not include many smokers, and that did not reveal any increase in incidence of cancer or death, can not be considered conclusive, because precancerous lesions (analogous to those observed in ferrets under high ß-carotene intake) were not considered. They should be further analysed to deduce more definitive conclusions.

3.3.3. The pro-oxidant activity of ß-carotene

The pro-oxidant activity of β-carotene has also been considered as an hypothetical mechanism in lung toxicity (SCF, 2000), taken into account that the relative high partial oxygen pressure in the lung may shift the antioxidant activity of carotenoids into pro-oxidant activity. However, further studies of the pro-oxidant role of carotenoids *in vivo* and *in vitro* will help in testing hypothesis relating to the influence of these compounds in the development of human chronic diseases. In any case, the pro-oxidant activity of β-carotene can be part of the other mechanisms as they are not mutually exclusive.

4. DOSE-RESPONSE ASSESSMENT

No dose-response relationship for ß-carotene effects is available from the intervention trials in humans, as single doses were used in each study, and the conditions were different in the different studies.

The study in ferrets also used a single daily dose. Further studies in ferrets using a range of different ß-carotene doses and a wider range of selected parameters would be appropriate to assist in future toxicological evaluation.

It can be presumed that the effects of ß-carotene are dependent on the specific source of exposure, and that differences will not be unexpected with different matrices or different formulations containing ß-carotene, depending on the composition of accompanying antioxidants and of other components, and also depending on the relative proportion of isomers of ß-carotene. Natural ß-carotene preparations differ from synthetic all-*trans*-ß-carotene in the relative proportion of *trans*/*cis* isomers. From preliminary studies (see Section 3.1.3), the isomeric form appears important in the genotoxicity and antigenotoxicity of ß-carotene. However, at present there is insufficient information to establish the role of all these factors.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Existing evidence from human trials indicates that supplemental ß-carotene (20 mg/day or more) is contraindicated for use in current, heavy smokers. However, there is insufficient scientific basis to set a precise figure for an UL of isolated ß-carotene as no dose-response relationship for ß-carotene effects is available either from the intervention trials in humans or from appropriate animal models. Moreover, it is not possible to be more specific in distinguishing different isomeric forms of ß-carotene or specific formulations.

6. CHARACTERISATION OF RISK

Three general ß-carotene sources can be considered (SCF, 1997): a) natural food sources that may contribute around 2-5 mg/European person/day, b) food additives (1-2 mg/person/day), and c) supplements. The combination of a) and b) sources represents about 3-7 mg/day (or up to 10 mg/day depending on seasonal and diet variations) of ß-carotene exposure. Thus, there may be a very small

difference between the levels that may confer health benefits (up to 10 mg/d, mainly from natural sources) and those that may produce adverse effects in smokers in the general population (20 mg/day in the ATBC study). In this situation it seems that the use of ß-carotene as a supplement should be regarded cautiously.

On one hand, human chemoprevention trials carried out in the last decade have shown that all-*trans*-ß-carotene actually increases both lung-cancer incidence and mortality in human smokers and, more recently, mechanisms which offer likely explanations of these adverse effects have been derived from experimental studies in appropriate animal models.

On the other hand, a number of reviews have summarised the research on diet and lung cancer in humans during the preceding 30 year period. The consensus is that they consistently demonstrated reduced risk of lung cancer, with increased intake of vegetables and fruits rich in carotenoids. Further, high levels of ß-carotene in the blood were consistently associated with reduced incidence of lung cancer. However, these effects can not be attributed to β-carotene as the role of other carotenoids or other compounds from vegetables and fruits, and associated dietary or life style patterns, has not been adequately explored in the epidemiological studies.

Thus, the general assumption that individuals who eat more fruits and vegetables, rich in carotenoids, and/or have high levels of serum ß-carotene have a lower risk for cancer and cardiovascular diseases cannot be extended to specific formulations of ß-carotene.

7. RECOMMENDATIONS FOR FURTHER WORK

The lung appears to be the target tissue for future investigations to address the adverse effects of ß-carotene. This tissue is were the tumourigenic effect of ß-carotene was observed in human trials, it depends on ß-carotene metabolites for the regulation of its growing cells, and is where a highest oxygen partial pressure is present, thus potentially enhancing the pro-oxidant properties of ß-carotene.

The ferret (and perhaps other animals that are able to absorb ß-carotene in its intact form), could be suggested as an appropriate model for studying the role of oral ß-carotene in lung carcinogenesis, provided that differences in the total absorption of ß-carotene are taken into account. This would allow establishing dose-response-related effects and/or likely mechanisms for the effects of ß-carotene.

The beneficial effects of diets rich in vegetables and fruits containing carotenoids and other compounds need to be further studied, to be able to set out specific effects of any of single chemical or combination of compounds.

Further study of the data already collected in the developed and ongoing human trials is recommended. Also studies on the biological effects of supplemental ß-carotene from different sources appears of particular interest.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN B_6

(EXPRESSED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Vitamin B_6 is a mixture of 6 inter-related forms pyridoxine (or pyridoxol), pyridoxal, pyridoxamine and their 5'-phosphates. Interconversion is possible between all forms (Bender, 1989). In this assessment the terms "vitamin B_6" and "pyridoxine" have been used interchangeably, with "pyridoxol" used when this particular form is discussed.

Pyridoxal phosphate plays an essential role in the metabolism of many aminoacids, and deficiency of this coenzyme can lead to many manifestations. Clinical signs include retarded growth, acrodynia, alopecia, skeletal changes and anaemia, while changes in neurotransmitters, such as dopamine, serotonin, norepinephrine (noradrenaline), tryptamine, tyramine, histamine, GABA and taurine, affect brain function and can lead to seizures and convulsions.

2. NUTRITIONAL BACKGROUND

The active form of the vitamin is pyridoxal phosphate, which is a coenzyme that is recognised as being required for the function of more than 60 enzymes involved with transamination, deamination, decarboxylation or desulfuration reactions.

Pyridoxine is present in food in the free form and as the glucoside. The glucoside may undergo partial hydrolysis in the gut lumen, or may be absorbed intact, following which it is largely excreted in the urine without hydrolysis (Gregory, 1990). Within cells pyridoxol and pyridoxamine are phosphorylated by a kinase enzyme and then oxidised to pyridoxal-5'-phosphate, which is the major intracellular form. All cells have the kinase but the liver is the major site for oxidation to pyridoxal phosphate; the liver is the main source of circulating pyridoxal, which is formed by the action of alkaline phosphatase on the hepatic pyridoxal phosphate (Merrill and Henderson, 1990). Excess pyridoxine is metabolised to 4-pyridoxic acid, which is eliminated primarily in the urine. The plasma concentrations of pyridoxal and its phosphate rise rapidly after a single oral dose of pyridoxol, followed by a rapid decrease in pyridoxal due to tissue uptake and phosphorylation. The acid metabolite is formed rapidly and blood levels increase and then decrease to baseline levels within 12 hours (Speitling *et al*, 1990).

Tryptophan metabolism is dependent on vitamin B_6 status, because the enzyme kynureninase, requires pyridoxal phosphate. This enzyme is especially sensitive to vitamin B_6 depletion. Loading doses of tryptophan are given to establish vitamin B_6 status (the tryptophan load test). Determination of the excretion of kynurenic and xanthurenic acids indicates vitamin B_6 nutritional status (Bender, 1989).

Vitamin B_6 is involved in the metabolism of sulphur-containing amino acids (methionine, taurine and cysteine (Sturman, 1986). The disease states homocystinuria and cystathioninuria are due to inborn errors of metabolism involving the enzymes cystathionine β-synthase (EC 4.2.1.22) and gamma-cystathionase (EC 4.4.1.1). Both diseases are characterised by wide ranging clinical signs and mental disturbances, and can be treated with large doses of pyridoxine, although some individuals are unresponsive to this treatment.

The majority of the body's vitamin B_6 is associated with the enzyme glycogen phosphorylase in muscle. When muscle glycogen reserves are depleted due to prolonged fasting, the vitamin is released from muscle, however, muscle pyridoxal phosphate is not released in response to a vitamin B_6 deficient diet, so that muscle reserves cannot be regarded as a storage form of the vitamin (Reports of the Scientific Committee for Food, 1993).

There are several studies that have examined pyridoxal phosphate plasma concentrations in relation to age. In 1964, Hamfelt reported that the plasma concentrations were lower in subjects greater than 60 years old, and the author speculated that this fall could be due to a nutritional defect, such as defective absorption, defective phosphorylation, or increased urinary excretion. Rose and co-workers (1976) also reported that plasma levels of pyridoxal phosphate decline with age. These results have been confirmed in women (Lee and Leklem, 1985), since middle-aged women (55 ± 4.0 years) had lower plasma pyridoxal phosphate and a higher urinary 4-pyridoxic acid than younger women (24.4 ± 3.2 years). Age-related changes in metabolism and tissue distribution of pyridoxine and its metabolite pyridoxal-5'-phosphate have been reported in rats (van den Berg *et al*, 1990).

Lewis (1995) suggested that pyridoxine-related neurotoxicity (see later) may occur when the capacity of the liver to phosphorylate pyridoxine to the active pyridoxal phosphate is exceeded, and that the high circulating concentrations of pyridoxal give rise to the toxicity.

Daily requirements of vitamin B_6 have been determined and are affected by protein intake. Vitamin B_6 levels decline more rapidly in individuals with a high protein intake in comparison with those with a lower protein intake (Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy, 1991). In consequence, the daily requirement is related to protein intake rather than body weight; an intake of 15 µg/g dietary protein is recommended for adults, which is equivalent to about 2-3 mg per day (Reports of the Scientific Committee for Food, 1993). Since there are no storage facilities for vitamin B_6, a continuous daily intake is essential.

The Dietary and Nutritional Survey of British Adults (HMSO, 1990), which studied over 2000 people, reported that the majority of the intake by men was from food sources, whereas supplements represented a significant source (about 50% of total intake) for women more than 24 years old. The use of supplements by some women resulted in extremely wide inter-subject variability and a skewed distribution with the highest 97.5th percentile intake being 16 mg/day in women aged 35-49 years.

Table 1. Pyridoxine intake in EU countries (mg/day).

	Population	n	Method	Supplements	Mean	97.5%
Austria[a]	individual	2488	24 h recall	?	1.68	3.43
Ireland[b]	men women men women	662 717 662 717	7-day record	- - + +	3.2 2.4 3.5 3.6	5.9 4.1 7.6 30.3
Italy[c]	household	2734	7-day record	+	2.0	3.3
The Netherlands[d]	household	5958	2-day record	-	1.59	3.01
UK[e]	men women men women	1087 1110 1087 1110	7-day record	- - + +	2.48 1.57 2.68 2.84	4.47 2.62 5.35 10.46

[a] Elmadfa *et al* (1998) [b] IUNA (2000) [c] Turrini (INRAN)
[d] Hulshof and Kruizinga (1999) [e] HMSO (1990)

It has been suggested that individuals taking high-oestrogen contraception may have an additional requirement for vitamin B_6. Whilst there is some evidence that these oral contraceptives affect tryptophan metabolism, there is no evidence to suggest that they cause changes in vitamin B_6 status of the individuals (reviewed by Bender, 1987). In addition, there is no evidence that the combined oral contraceptive (oestrogen plus progestagen) affects the requirements for pyridoxine.

High doses of vitamin B_6 have also been used for the treatment of premenstrual syndrome, depression, Down's syndrome, hyperkinesis, autism, neurosis, Hodgkin's disease and Parkinson's disease (Sturman, 1986).

3. HAZARD IDENTIFICATION

The principal toxicity of concern associated with excessive intakes of vitamin B_6 is neuronal damage, and sensory and motor effects. The initial observations were from studies in experimental animals, but

more recent studies in volunteers and patients, and case reports of patients have shown that the effects can be produced also in humans.

3.1. Neurotoxicity

3.1.1. Studies in animals

It has been known since 1940 that very large doses of pyridoxine (1-7 g/kg) in rats and dogs result in pronounced ataxia and weakness and degeneration of the spinal cord roots, posterior ganglia and peripheral nerves (Unna, 1940; Unna and Antopol, 1940a, b; Antopol and Tarlov, 1942). More recent studies have indicated species differences in the sensitivity to vitamin B_6 toxicity (Xu *et al*, 1989), and have examined neuronal abnormalities in rats, guinea pigs and mice administered high doses of pyridoxine. Rats were administered 600-1200 mg/kg/day for 6-10 days, guinea pigs 1800 mg/kg/day for 10 days and mice 1800 mg/kg/day for 7 days or 1200 mg/kg/day for 6 weeks (as well as higher doses for shorter time periods). Neuropathy with necrosis of sensory neurons in dorsal root ganglia, accompanied by axonal atrophy and breakdown of peripheral and central sensory axons, was observed in rats. Mice were resistant to such changes. Lower doses in rats (150-300 mg/kg/day) for up to 12 weeks also produced minor effects to the dorsal root ganglia and neuropathy with axonal atrophy and degeneration. The authors concluded that multiple factors including rate of administration, differential neuronal vulnerability and other factors influence species susceptibility.

There are limited data available on the underlying mechanism of toxicity of pyridoxine. Neuropathological changes in dogs have been correlated to changes in electrophysiological and functional tests at doses of 3 g per day (Schaeppi and Krinke, 1982). Two dogs were dosed until signs of morbidity appeared (after only 8 and 26 days), with ataxia and loss of reflexes observed in both dogs. The somatosensory maximum nerve conduction velocity was reduced. The neuropathological changes included lesions in the dorsal spinal column, dorsal spinal roots and ganglia, selected fibres in peripheral nerves and in the sensory spinal trigeminal roots. Nerve fibres located more deeply, and the spinocervical and spinocerebellular fibres were unaffected. Peripheral nerves showed signs of degeneration (demyelination, misformed Schwann cells, missing axons). Gross examination of the nervous systems of dogs treated with 150 mg pyridoxine/kg/day for 100-112 days, revealed abnormal opaque areas in the dorsal funiculus (Hoover and Carlton, 1981b). Histological examination showed degenerative lesions of varied severity in all dogs to which pyridoxine was administered. Lesions in the CNS were limited to the dorsal funiculus, the trigeminal nerves and the spinal tract of the trigeminal nerves. In these areas, the number of axons was reduced and the myelin was irregular and fragmented. The severity of the lesions in the rostal dorsal funiculi varied considerably among dogs from minimal to marked, with up to 70-80% of the nerves affected in some animals. The lesions were more severe in the rostal aspects of the dorsal funiculus than in the caudal. The lateral funiculi, the ventral funiculus and the gray matter of all segments were histologically normal.

Subtle effects on the central nervous systems, as measured by an attenuation of startle response, have been reported in rats fed diets containing between 7-2100 mg/kg of diet (equivalent to approximately 0.28-84 mg vitamin B_6/kg body weight/day) for 7 weeks (Schaeffer, 1993).

3.1.2. Studies in humans

The evidence for neurotoxicity in humans due to vitamin B_6 administration is largely related to a series of case reports of patients with severe effects associated with extremely high intakes. An important aspect of these cases is the duration of intake prior to the development of symptoms. Duration of intake is critical in the interpretation of the results of clinical trials that used vitamin B_6 for premenstrual syndrome (see later). Because both dose and duration of intake are important for B_6 neurotoxicity, the various case reports are described individually in the text and summarised in Table 2. The data (Table 2) indicate that cases of clinical neuropathy occur after about 12 months or longer treatment with doses of 2 g/day or less, whereas neuropathy can develop in less than 12 months at doses greater than 2 g/day.

Schaumburg *et al* (1983) reported gradual progressive sensory ataxia and profound distal limb impairment in 7 adult patients following massive doses of vitamin B_6 (see Table 2). Unstable gait and numb feet were usually the first signs, and numbness and clumsiness of the hands followed. Profound distal limb impairment of position and vibration sense developed, while the senses of touch, temperature and pain were less affected. All tendon reflexes were absent. The clinical profile was similar in all cases. The neurologic disability slowly improved once the patients stopped taking pyridoxine, and those examined after a prolonged period had made a satisfactory recovery. The transport of vitamin B_6 across the blood:brain barrier is saturable, and the authors speculated that the peripheral sensory neuropathy

reflected the vulnerability of the neurons of the dorsal root ganglia because of the absence of the blood:brain barrier (which would protect neurons within the CNS from excessive circulating levels of the vitamin).

The single case reported by Berger and Schaumburg (1984) (Table 2) showed an improvement of her symptoms on stopping vitamin B_6 supplementation, and the authors attributed these symptoms to the administration of pyridoxine.

Table 2. Case reports of neuropathy in patients taking high doses of vitamin B_6.

Dose (g/day)	Duration (months)	Reference
0.1-0.2	36	Parry and Bredersen (1985)
0.1-2.5	9	Parry and Bredersen (1985)
0.1-4.0	72	Parry and Bredersen (1985)
0.2-0.5	24	Berger and Schaumburg (1984)
0.5	8	Parry and Bredersen (1985)
0.5	24	Parry and Bredersen (1985)
0.5-2.0	3	Parry and Bredersen (1985)
1.0	12	Waterson and Gilligan (1987)
1.0-2.0	36	Parry and Bredersen (1985)
1.5-2.0	24	Parry and Bredersen (1985)
1.5-2.5	>12	Parry and Bredersen (1985)
2.0	24	Friedman *et al* (1986)
2.0	12	Parry and Bredersen (1985)
2.0	12	Parry and Bredersen (1985)
2.0	4	Schaumburg *et al* (1983)
2.0	34	Schaumburg *et al* (1983)
2.0	40	Schaumburg *et al* (1983)
2.0-3.5	10	Parry and Bredersen (1985)
2.0-4.5	>12	Parry and Bredersen (1985)
2.0-5.0	2	Parry and Bredersen (1985)
2.0-5.0	4	Parry and Bredersen (1985)
3.0	4	Schaumburg *et al* (1983)
3.5	1	Parry and Bredersen (1985)
4.0	10	Schaumburg *et al* (1983)
5.0	2	Schaumburg *et al* (1983)
6.0	3	Schaumburg *et al* (1983)

Neuropathy associated with pyridoxine intake was described in 16 patients in another report (Parry and Bredersen, 1985 –see Table 2). All patients had symmetric distal sensory loss and the sensory nerve action potentials were absent or severely reduced in amplitude. Central peripheral degeneration confined to sensory axons strongly suggested that toxicity was directed against the dorsal root ganglia. Sural nerve biopsy in two patients showed that the myelinated fibre density was reduced and there was some myelin debris indicating axonal degeneration. The conditions of all patients improved after stopping vitamin B_6 treatment for between 3-18 months, but the symptoms were not fully resolved.

Waterston and Gilligan (1987) reported a case of a young woman who had been taking pyridoxine at a dose of 1000 mg/day for 12 months (Table 2), and whose symptoms resolved on cessation of pyridoxine intake.

Other studies have reported effects at higher doses. A mild motor neuropathy combined with a severe sensory neuropathy was reported in a patient who had taken 10 g/day for 5 years (Morra *et al*, 1993). This case does not fit into the time-trend apparent in Table 2, and was not included because the time to the development of sensory effects was not reported. A case of by bilateral numbness after taking 2 g of vitamin B_6 daily for a period of 2 years (Friedman *et al*, 1986 -see Table 2) improved within 32 weeks of cessation of B_6 intake.

A case-report of severe sensory neuropathy in a middle-aged man treated with 600 mg per day of pyridoxine for 8 months was not included in Table 2 because the subject was also receiving isoniazid (which also produces peripheral neuropathy) (Santoro *et al*, 1991).

Neurological effects were detected when a 1 year old patient was treated with 1 g pyridoxine daily for hyperoxaluria, and these effects were reversed when the dose was reduced to 400 mg per day (de Zegher *et al*, 1985).

3.2. Other adverse effects reported in humans and animals

Photosensitivity has been described as an adverse effect associated with pyridoxine intake. A 35 year old patient who had taken 200 mg pyridoxine per day as part of a multivitamin preparation showed erythema following exposure to UVA irradiation which the authors ascribed to the pyridoxine present in the multivitamin preparation (Morimoto *et al*, 1996). Skin lesions were reported in a woman who had taken massive doses (4 g/day) for a period of 4 years (Baer, 1984). Coleman *et al* (1985) treated 400 patients with Down's syndrome with vitamin B_6 at pharmacological doses (35 mg/kg/day). Reported side effects included skin blisters that were related to sun exposure, vomiting and peripheral neuropathy; all patients who developed blisters did so after a minimum of four and a half years of treatment. Two patients developed motor and sensory polyneuropathy after 9 years administration of doses up to 50 mg/kg and their condition improved once vitamin B_6 administration had stopped.

The report of a high frequency of extrapyramidal dysfunction in a small group of patients with homocysteinuria, and who received pyridoxine, is difficult to interpret because of underlying differences between these patients and normal individuals (Ludolph *et al*, 1991).

Administration of doses of 100 or 500 mg B_6 per day for 10 days to a group of 58 medical students resulted in significantly impaired memorisation at 500 mg/day, and a non-significant decrease at 100 mg/day (Molimard *et al*, 1980). This is a potentially important observation, given the dosage and the short duration of intake. The study was designed to investigate further an earlier unpublished observation of a decrease in "brain performance" from a double-blind study in medical students conducted in 1961. The study of Molimard *et al* (1980) recruited 69 first year medical students who were randomly allocated to receive identical tablets of 50 mg or 250 mg pyridoxine or placebo to be taken twice per day for 10 days. Those who declared that they did not take the tablets were treated as a separate group. The subjects were given a simple digit coding test prior to treatment, immediately after treatment and 14 days later. In addition the subjects underwent a test on the medical physiology that had been taught during the treatment period, plus some simple numerical problems at the end of the treatment period. A total of 58 subjects completed all 3 digit coding tests, which showed a highly significant improvement with time (a learning effect) in all groups. There were no significant differences in the uncorrected scores, but evidence of a dose-related decrease in the learning effect, with a highly significant difference between the placebo group and 500 mg/day group ($P<0.002$) but a smaller difference between the placebo and 100 mg/day group ($P<0.07$). There were no differences in the other tests of performance. In a second trial as part of the same publication, a group of 30 obese patients were randomly allocated to receive placebo or 20 mg or 1000 mg of pyridoxine per day for 15 days, with subjects given a number of tests before and immediately after treatment. An adverse dose-related effect was found for word recognition ($P<0.05$) but not for word or visual memorisation, together with a decrease in the results for a visual retention test in the pyridoxine treated group after treatment. These studies reported effects after short-term treatment, and no studies have investigated the relationship between dose and duration of treatment for such effects.
Many studies have described the effects of vitamin B_6 administration on spermatogenesis in animals (Mori *et al*, 1989; 1992; Kaido *et al*, 1991; Ide *et al*, 1992). Administration of vitamin B_6 (125-1000 mg/kg/day) injected i.p. to rats for 6 weeks resulted in a decrease in the weight of the epididymides and the number of sperm was also decreased (Mori, *et al*, 1989; 1992). Similar results have been reported by other workers (Kaido *et al*, 1991; Ide *et al*, 1992).

Daily oral doses of between 20-80 mg pyridoxine/kg to pregnant rats over days 6-15 of gestation produced no evidence of teratogenicity in the offspring (Khera, 1975). These doses of vitamin B_6 did not affect the number of implantations, corpora lutea or number of live pups. At higher doses (100-800 mg/kg), the number of implantations, live pups and corpora lutea in treated animals were increased in comparison with controls. However, doses of either 400 or 800 mg/kg significantly reduced the body weights of the pups.

4. DOSE-RESPONSE ASSESSMENT

Assessment of the dose-response relationship for pyridoxine-induced neurotoxicity is difficult because of the nature of the available data and potential inverse relationship between the duration of exposure and the doses that can be tolerated without adverse effects.

4.1. Studies in animals

Although pyridoxine is a water-soluble vitamin, which does not accumulate, the animal data indicate that there is a progressive development of the neurological lesion with time. The animal toxicity database on pyridoxine has been reviewed by Cohen and Bendich (1986) and was summarized by Munro (1997) in a paper presented to a symposium discussing the safety of vitamin B_6 (Shrimpton and Holmes, 1997). Krinke and Fitzgerald (1988) have shown that the type of neurotoxicity produced by pyridoxine in the rat is a function of the dose and duration of administration. Rats administered single high doses (1200 mg/kg) of pyridoxine were observed to have neuronopathy (damage to the cell body), those administered lower chronic doses (200 mg/kg for 12 weeks) were observed to have axonopathy to the distal portion of sensory nerves. These workers also reported that animals administered pyridoxine for 5 days a week had considerably less damage than those dosed every day.

There is an extensive toxicity database on the effects of vitamin B_6 in dogs, which supports both the clinical symptomatology in patients and the inverse relationship between duration and dosage. Krinke *et al* (1980) reported on the effects of the oral administration of pyridoxine (300 mg/kg/day for 78 days) on beagle dogs. Animals developed swaying gait within 4 to 9 days of start of treatment and severe ataxia between 8-30 days. Morphological examination on sacrifice, revealed widespread neuronal degeneration in the dorsal root ganglia and the Gasserian ganglia, degeneration of sensory nerve fibres in peripheral nerves, in dorsal columns of the spinal cord and in the descending spinal tract of the trigeminal nerve. Phillips *et al* (1978) administered pyridoxine hydrochloride orally in gelatin capsules (0,50 or 200 mg/kg/day) to 3 groups of female beagle dogs (4 in the control group, and 5 per treatment group) for 100-112 days. Four of the 5 animals in the high dose group (200 mg/kg/day) showed ataxia and loss of balance after 45 days of treatment, whilst the other animal showed clinical signs after 75 days: histological examination of tissues at termination showed bilateral loss of myelin and axons in the dorsal funiculi and loss of fibres in the dorsal roots. Animals in the low dose group (50 mg/kg/day) showed no clinical signs, but histological examination revealed loss of myelin in the dorsal nerve roots in all five dogs.

Hoover and Carlton (1981a) reported that all dogs (5 male and 5 female) treated with 150 mg pyridoxine/kg/day for 100-112 days developed neurologic disease characterised by ataxia involving predominantly the hind limbs at first, but with time, the fore limbs were also affected. Tests of postural reactions reflected proprioceptive abnormalities. Hind limb flexor reflexes were mildly reduced in two dogs and pain perception (pinprick) was mildly reduced in four. However, all dogs remained alert and cranial nerve and ophthalmologic tests were normal.

Comparison of the data of Phillips *et al* (1978), Hoover and Carlton (1981a) and Krinke *et al* (1980) indicates a possible inverse relationship between dose and time to effect.

4.2. Studies in humans

Interpretation of the data from investigations in humans and case reports (summarised above) indicate that adverse neurological effects are detected after very high doses (>500 mg/day which is equivalent to about 8 mg/kg/day). Because of the severity of the adverse effects, there have been few studies designed to define the dose-response relationship in humans. The most important clinical studies are summarised in Table 3 and discussed below.

4.2.1. Clinical studies that reported neurological effects

Berger *et al* (1992) studied only extremely high doses, and did not define a non-effect level. Either 1 or 3 g of pyridoxine was given daily to 5 healthy volunteers until signs of either clinical or laboratory abnormality were present. Sensory symptoms and QST abnormalities were detected in all patients, and the subjects receiving the higher doses became symptomatic earlier than those receiving lower doses. The duration of treatments in the 5 subjects associated with the onset of symptoms was >14, 7, 4.5, 3.5 and 1.5 months in subjects receiving 12, 12, 19.6, 26.5 and 56.9 mg/kg/day respectively. The data demonstrated a clear inverse relationship between the dosage and the duration of consumption prior to the onset of symptoms.

Bernstein (1990) reviewed available data and concluded that women taking 500-5000 mg vitamin B_6/day as self-treatment for premenstrual tension have shown peripheral neuropathy within one to three years. The author stated that his own studies did not find neurological effects in 70 patients at doses of 100 or 150 mg/day for up to 5 years. However there is a discrepancy between this statement and the publications (Del Tredici *et al* (1985) and Bernstein and Lobitz (1988)) cited to support this conclusion, in relation to the numbers of patients, the dosage (150-300 mg/day) and most importantly the duration (mostly less than 6 months). Bernstein (1990) hypothesised that there may be predisposing factors which may make some individuals more sensitive.

The development of peripheral neuropathy has been reported in patients taking lower doses. A short report (Dalton, 1985) stated that 40% of women who had been taking vitamin supplements for premenstrual tension and who had plasma vitamin B_6 levels above normal (3-18 ng/ml), developed various clinical signs consistent with peripheral neuropathy. The signs included shooting and tingling pains, paraesthesia of limbs, clumsiness, ataxia or peri-oral numbness. The vitamin B_6 intake of these women ranged from 50-300 mg per day and involved a variety of multivitamin preparations. Two months after stopping all supplements, 27 of the women were reassessed and all showed improvement.

In a subsequent study (Dalton and Dalton, 1987), vitamin B_6 intake and clinical signs were monitored in women attending a private clinic specialising in the treatment of premenstrual tension. Of 172 women who were found to have elevated vitamin B_6 serum levels (>18 ng/ml), 103 (60%) complained of neurological symptoms, while the other 69 had no symptoms. The neurological symptoms included paraesthesia, bone pains, hyperaesthesia, muscle weakness, fasciculation and numbness. Symptoms were symmetrical. The daily dosages in both groups ranged from <50 mg to >500 mg and the average daily dose was 117 ± 92 mg in the group described by the authors as the “neurotoxic” group and 116 ± 66 in the “controls”. Those complaining of the symptoms had been taking the supplement for 2.9 ± 1.9 years, while those who had no symptoms had a duration of intake of 1.6 ± 2.1 years ($P<0.01$). Three months after stopping vitamin B_6 intake, 55% of the women reported partial or complete recovery from the neurological symptoms and at 6 months, all reported complete recovery and the areas of hyperaesthesia and numbness noted at the initial examination had disappeared. Seven women who had inadvertently not stopped vitamin B_6 intake all reported a continuation of their symptoms. Three women had subnormal serum B_6 levels on cessation of supplement intake, and restarted B_6 at a daily dose of 50 mg; however, symptoms returned and they stopped the supplement intake. In one case, a woman who had taken 75 mg of B_6 daily together with multivitamins, zinc and magnesium, for 2 years, and had serum B_6 level of >34 ng/ml, complained of paraesthesia of the hands, electric shock pains in her head, numbness of the finger tips and itching between her shoulder blades. Examination revealed patchy areas of hypersensitivity on her back and lower limbs, especially her shins. On stopping vitamin B_6 treatment, all symptoms eased within 3 months. However, on restarting B_6 intake at 50 mg per day for 3 months, the same neurological symptoms returned. The appearance of neurological symptoms at lower doses appears to be related to duration of intake which is compatible with the conclusion from Table 1. This study has been severely criticised because of its design; all subjects received vitamin B_6 and the comparisons were between those who did, and those who did not report adverse effects. The adverse effects may have predated treatment with B_6. The only evidence for cause and effect relates to the consequence of stopping or not stopping intake, and correlations with duration of intake. Individuals, who had reported adverse effects, had been taking B_6 for longer than those who did not have symptoms, and a higher proportion (70% compared with 55%) had serum B_6 levels >34 ng/ml.

Table 3. Clinical studies on pyridoxine in relation to neurological effects (see Table 2 for individual/anecdotal evidence)

Authors	Subjects	Number	Dosage (mg/day)	Duration of treatment	Findings	Conclusions
Baker and Frank (1984)	Elderly	6	225	up to 1 year	Adverse effects not reported	Too few subjects to provide useful data; full data not published
Berger *et al* (1992)	Adults	5	1000-3000	up to 7 months	All subjects developed abnormal measurements of vibration and/or thermal thresholds; 4 subjects developed clinical symptoms	Clear evidence of adverse effects at high doses with an inverse relationship between dose and symptom-free duration
Bernstein and Lobitz (1988)	Diabetic patients with pre-existing neuropathies	16	150	up to 6 months	No changes in motor conduction velocity at 5 months	Limited duration and high drop-out rate (only 5 subjects studied at 5 months)
Bonke and Nickel (1989)	Men (marksmen)	18	60 (n = 8) 600 (n = 10)	8 weeks	Shooting performance (a reflection of tremor); significant improvement in score over the 8 week period compared to placebo	Duration too short to assess neurological effects
Brush (1988)	Patients with premenstrual syndrome	cohort 1 = 630	40-200	mostly less than 1 year; 76 for 1-5 years	No neurological effects reported 6 subjects reported mild tingling/ numbness described as "definite side-effects"	Only 7% of patients were treated for 3 or more years. 140 subjects received >100 mg per day but their duration of treatment is not defined No details given about duration of treatment
Brush *et al* (1988)	Patients with premenstrual syndrome	cohort 2 = 336	40-200	duration not stated		
Dalton (1985)	Patients with premenstrual syndrome	58	50-500	Not defined	Significant reductions in symptoms such as headache tiredness and "neuropathy" 2 months after stopping B_6	Insufficient details to assess data; no control group data
Dalton and Dalton (1987)	Patients with premenstrual syndrome	172	<50-<500	<6 months->5 years	Patients with neurological symptoms had similar daily intakes but greater duration (2.9 years); reversal of symptoms on cessation of intake	A selected group of patients with high serum B_6 which showed a high incidence of "neurological" symptoms. There was no control group and the evidence of causality is the relationship with duration of intake and reversibility
Day (1979)	Patients with premenstrual syndrome	67	100	1 month	No assessment of side effects	Duration too short to assess neurological effects

Table 3 (Cont.). Clinical studies on pyridoxine in relation to neurological effects (see Table 2 for individual/anecdotal evidence)

Authors	Subjects	Number	Dosage (mg/day)	Duration of treatment	Findings	Conclusions
Del Tredici *et al* (1985)	Patients with carpal tunnel syndrome	24	150 or 300	4 months	No changes in distal motor latency or self-assessment questionnaire	Duration too short to assess neurological effects
Ellis *et al* (1979) (and studies cited); Ellis (1987)	Patients with carpal tunnel syndrome	35	100-300	up to 12 weeks	No adverse neurological effects reported	The condition may be due to pyridoxine deficiency; typical treatment schedule of 12 weeks (Ellis, 1987) is of too short duration to access neurological effects
Kerr (1977)	Patients with premenstrual syndrome	70	40-100	2 months	No assessment of side effects	Duration too short to assess neurological effects
Mitwalli *et al* (1984)	Patients with hyperoxaluria (kidney stones)	22	250-500	1-6 years	No neurological complications in 22 patients or nerve conduction abnormalities in 7 patients studied in detail	Small group size but very extended duration; influence of the disease on pyridoxine handling, requirements and neurological response are not known
Mpofu *et al* (1991)	Patients with homocysteinuria	17	200-500	10-24 years	No abnormalities of motor or sensory nerve conduction velocities	Small group size but very extended duration; the absence of effects at doses equivalent to 10-90 mg/kg/day suggests that these patients may show reduced responsiveness to the neuronal adverse effects of pyridoxine
Pauling (1984) cites Hawkins	Undefined patients	>5000	200	not given	No details	No details available; cannot be assessed
Pullon *et al* (1989)	Women	410	not described	not described	No assessments of possible neuropathy	Results from a survey of 1826 women; no data on tolerability and side effects
Tolis *et al* (1977)	Women	9	200 or 400	2 months	No effects on growth hormone or prolactin	Small number of subjects and very limited duration of treatment; no assessment of neurological effects
Williams *et al* (1985)	Women with premenstrual syndrome	204 (out of 434)	100	3 months	No difference in side effects between treatments and placebo groups	Duration too short to access neurological effects
Wyatt *et al* (1999)	Women with premenstrual syndrome	526 (out of 910)	50-600	up to 4 months	Data assessment for reports of side effects; 1 reported case of neurological side effects	A systematic review of published and unpublished randomised placebo controlled trials; all studies of inadequate duration

Brush (1988) reported retrospective data on a group of 630 women with premenstrual syndrome who were treated with pyridoxine either alone or in combination with other medications. This cohort appears to have been the same as that described by Brush *et al* (1988), and mentioned by Brush and Perry (1985) in their letter concerning the Dalton study.

Neuropathy was not reported in the group of 630 women who received between 80 and 200 mg pyridoxine daily for premenstrual syndrome (Brush, 1988; Brush and Perry, 1985). This same cohort of patients was described by Brush *et al* (1988), and it is clear that the data are limited by the duration of the study because 80% of the subjects were treated for 12 months or less, and 93% for 24 months or less: neurological assessment was not performed and the tolerability of the treatment was assessed by the patients. Although the authors state that there were no adverse effects in the 1976-1983 cohort of 630 patients, Brush (1988) tabulated "definite side-effects" with 5 cases of dizziness and 6 cases of mild tingling in the 1983-1986 cohort of 336 patients; the authors ascribed this to the higher dose (200 mg/day) in this cohort and/or the adverse publicity at the time.

4.2.2. Clinical studies that did not report neurological effects

In contrast to the "positive" studies described above, there are a number of publications that did not find evidence of adverse effects in humans receiving high doses of vitamin B_6. Interpretation of the various studies is complicated by differences in the duration of treatment. A large number of the studies are in women taking high doses of vitamin B_6 for premenstrual syndrome. The various "negative" studies are described below, followed by a summary of the dose, duration and outcome for both positive and negative studies.

No adverse effects were reported in a small study by Bonke and Nickel (1989) in which small numbers of healthy volunteer marksmen were given a mixture of vitamin B1 (90 or 300 mg), B_6 (60 or 600 mg) and B12 (120 or 600 µg) daily for a period of 8 weeks; however, there was an increase in shooting accuracy, indicating a reduction in tremor, at both doses. Adverse effects were not reported in a group of 6 elderly individuals given 225 mg pyridoxine per day for one year (Baker and Frank, 1984). In a letter to the New England Journal of Medicine, Pauling (1984) stated that a similar dose (200 mg pyridoxine daily) had been given to more than 5000 subjects without reports of side-effects (but these data cannot be evaluated because the duration of intake is not known and the cited reference (Hawkins, 1973) is not available in peer-reviewed literature).

A cohort of 434 patients with premenstrual syndrome was divided into two groups: one group received 100 mg pyridoxine per day and the other group were given a placebo. The patients were allowed to increase the dose to 200 mg/day if they considered they were receiving no benefit from the initial treatment (Williams *et al*, 1985). Patients were studied over 3 menstrual cycles only and therefore the duration of intake and dose were inadequate to allow assessment of any possible neurotoxicity associated with pyridoxine.

Two preliminary reports (Kerr, 1977; Day, 1979) studied the potential value of pyridoxine in the treatment of premenstrual syndrome. Neither study was of sufficient duration (2 months and 7 months respectively) or assessed symptoms sufficiently rigorously to be of value in relation to establishing possible adverse effects of vitamin B_6. A large group of women with premenstrual symptoms (n = 1826) was studied by Pullon *et al* (1989) largely in relation to the syndrome and its management, but this large study provided no data on possible pyridoxine neuropathy. A recent systematic review of studies on the use of vitamin B_6 in premenstural syndrome (Wyatt *et al*, 1999) showed an improvement compared to placebo. The review considered 9 published studies that involved 940 women. Adverse effects were limited to one case of neuropathy that could be attributed to pyridoxine. Dosages ranged from 50-600 mg/day, but the studies were too short in duration (usually 2-4 months) to exclude the possibility of neuropathy after prolonged intake at such intakes.

A small study in patients with carpal tunnel syndrome by Del Tredici *et al* (1985) described the treatment of 16 patients with 150 mg vitamin B_6 for 4 months and 8 patients with 300 mg B_6 for 4 months. Measurement of distal motor latency indicated a clinical improvement. No details of neurological assessment were given and the duration of the study limits its value for assessing adverse effects after long-term treatment. Ellis *et al* (1979) reported detailed results on one patient out of a group of 22 patients with carpal tunnel syndrome who had been treated with pyridoxine (120 mg/day). Treatment with either 2 mg or 100 mg per day resulted in clinical improvement in symptoms ($P<0.01$ and $P<0.001$ respectively), which is consistent with the authors proposal that this condition is related to pyridoxine deficiency. A subsequent paper, Ellis (1987) described the successful treatment of 35 selected cases

of carpal tunnel syndrome with pyridoxine (100-200 mg per day), which took 12 weeks to improve or relieve the signs and symptoms.

No clinically significant side effects were reported in a group of ten 6-year old patients with autism who were given a combination of pyridoxine (639 mg) and magnesium (216 mg) daily for a period of 10 weeks using a double blind, placebo-controlled study design (Findling *et al*, 1997).

Additional data have been published from studies in patients with known metabolic abnormalities or inborn errors of metabolism. These are given briefly below for completeness, but their interpretation in relation to the safety of vitamin B_6 for normal subjects is unclear.

Bernstein and Lobitz (1988) reported no deterioration of peripheral nerve function in a group of 16 patients with painful diabetic neuropathy who were treated with pyridoxine 150 mg/day for a period of up to 6 months, but only 4 subjects completed the study for the full 6 months.

Mitwalli *et al* (1984) reported the absence of neurological effects in a group of 22 patients with hyperoxaluria who developed kidney stones, and who received doses of 250-500 mg of pyridoxine per day for 8 months to 6 years (average 2.3 years). Nerve conduction studies in a sub-group of 7 of these patients revealed no abnormalities. This paper is frequently stated as supporting the safety of intermediate doses of pyridoxine but it is limited by the study size; also hyperoxaluria can be a result of pyridoxine deficiency (Nath *et al*, 1990), so that a metabolic abnormality related to pyridoxine cannot be excluded.

Patients with homocysteinuria may be given high dose pyridoxine treatment for many years from birth. A brief report by Mpofu *et al* (1991) described a group of 17 subjects who were given 200-500 mg of pyridoxine per day for between 7 and 24 years. Because the patients were treated from ages 2 weeks to 14 years, the doses ranged from 10-90 mg/kg/day during the first 10 years of life. The treatment was associated with very high concentrations of pyridoxine and pyridoxal phosphate in the plasma. Nerve conduction was within the normal range in each of the 4 nerves studied in each patient. Although this study appears to support the safety of doses of 200-500 mg of pyridoxine per day, such patients typically show a range of mental, ocular, skeletal and cardiovascular disease if untreated. The influence of the condition on the response to potentially neurotoxic doses of pyridoxine is not known, but the absence of effects at such high doses (comparable to those of Berger *et al*, 1992) raises questions over the usefulness of such data in relation to the general population.

4.3. Establishment of a no-observed adverse effect level (NOAEL)

The available dose-response data in humans are difficult to analyse because many of the publications relate to case reports and true incidence data are not available. The studies of Schaumburg *et al* (1983) showed the potential severity of the hazard but there was little information on dose-response. Parry and Bredesen (1985) reported a case series of 16 patients with sensory central-peripheral distal axonopathy, who had taken from 0.2 to 5 g/day for prolonged periods. Berger *et al* (1992) reported adverse effects in 4 out of 5 healthy volunteers who were given 1 g or 3 g/day in a controlled study. Berger and Schaumburg (1984) described a case of reversible pyridoxine-induced, sensory ataxia in a woman who had taken 200 mg/day for 2 years followed by 500 mg/day for 1 year. A similar case was reported by Waterston and Gilligan (1987) in a woman who had taken 1000 mg/day for 1 year.

The data described above, and summarised in Table 2, are the basis for the generally accepted conclusion that 500 mg of pyridoxine daily represents a potentially toxic dose for adults.

The data for doses between 100 mg/day and 500 mg/day are less clear, largely because they relate to case reports or observations in groups of patients, that were not subject to a proper double-blind, placebo-controlled evaluation. The case series described by Parry and Bredesen (1985) included 3 patients who had taken <1 g/day, all of whom had taken the high dose supplements for more than 1 year; one subject had taken a maximum of 200 mg/day for at least 3 years. Brush (1988) reported a low incidence of possible pyridoxine related side-effects (5 subjects reported tingling and/or numbness) in a cohort of 336 subjects who had taken 200 mg per day (see above); however the duration of treatment and details of other medication for these subjects were not given. Similar effects were not reported in a previous group, but the majority had been followed for less than 12 months (Brush, 1988).

As discussed above, many of the observational clinical studies which report no adverse effects of pyridoxine are limited in relation to duration of intake, size of study group, lack of adequate assessment

of adverse effects and/or lack of an appropriate protocol (double-blind, placebo-controlled) (see Wyatt *et al*, 1999). Many of these criticisms can also be applied to the study of Dalton and Dalton (1987) with the important exception of the duration of exposure. This study divided a group of 172 women into those who reported altered sensations in their limbs or skin, or muscle weakness, or pain (n = 103), and those who did not. Comparison between the two sub-groups was used as the basis for the analysis, which found no difference in pyridoxine intake, but a significantly greater duration of intake in those with symptoms (2.9 years) compared with those without (1.6 years). Although this study is open to criticism, the finding in relation to duration is not inconsistent with other data at higher doses and in animals. The authors did not give a separate statistical analysis in relation to the difference in duration of intake for those who reported likely pyridoxine-related symptoms such as paraesthesia (n = 59), hyperaesthesia (n = 33) and numbness (n = 21) compared to the symptom-free group. The lower end of the dose-response for pyridoxine-related neurological effects has not been defined clearly, especially for long-term intake. The various studies show clear effects at 500 mg/day or more, a low incidence of effects at 200 mg/day in one study (if taken for up to 2 years) and the possibility of effects at about 100 mg/day (if consumed for about 3 years). In consequence a clear NOAEL has not been established and an intake of 100 mg/day cannot be excluded as a possible effect level for long-term intake.

4.3.1. Previous reviews and evaluations

There have been a number of reviews published on the establishment of a safe upper limit for pyridoxine. Bender (1989) reviewed the risks and benefits of B_6 therapy and concluded that doses of "50 mg/day" and above must be considered to be potentially hazardous. Although Bendich and Cohen (1990) in their review of published data concluded that total amounts of 100 g taken over periods of 20 months (which can be estimated as equivalent to approximately 170 mg/day) are not associated with neuropathy, the database they presented included 2 cases in which neuropathy was reported at doses of around 100 mg/day for approximately 14 months. An earlier SCF evaluation (1993) concluded that "intakes greater than 500 mg/day are associated with neurological damage and intakes of more than 50 mg/day are potentially harmful in adults". An upper level of 10 mg per day was suggested by the UK Committee on Toxicity in 1997, which was based largely on the data from studies in dogs, divided by a safety factor, and supported by the data from the study of Dalton and Dalton (1987) and other available human data.

The Food and Nutrition Board of the Institute of Medicine (FNB, 1998) in the USA recently set an upper level of 100 mg/day for adults. That report did not use the study of Dalton and Dalton (1987) to establish the NOAEL because they considered that the weaknesses of the study and the inconsistency of the results with the weight of evidence pertaining to the safety of higher doses of pyridoxine ruled out the use of these data to base an upper level. The report highlighted a number of methodological weaknesses in the study, but in reality many of these apply also to the other studies available on pyridoxine. The FNB report identified a NOAEL of 200 mg/day based on two studies (Bernstein and Lobitz, 1988; Del Tredici *et al*, 1985). The FNB report (FNB, 1998) supported the NOAEL of 200 mg/day with additional studies that they stated were not as carefully executed or reported as the study by Bernstein and Lobitz (1988), but which reported no neuropathy in hundreds of individuals given pyridoxine doses of 100 to 500 mg/day (Brush *et al*, 1988; Ellis *et al*, 1979; Mitwalli *et al*, 1984; Tolis *et al*, 1977). Careful scrutiny of the papers used by the FNB to establish a NOAEL of 200 mg/day shows that the studies that were the principal basis were of too short duration to be useful; Bernstein and Lobitz (1988) reported data for only 16 patients, at doses of 150 mg/day and the duration of intake was only up to 6 months (with only 5 subjects studied after 5 months), while Del Tredici *et al* (1985) studied only 24 patients for 4 months. The large number of subjects studied in the other publications used to support the NOAEL of 200 mg/day was largely due to the work of Brush and colleagues (which was not unequivocally without possible adverse effects – see above), whereas Ellis *et al* (1979) reported on only 22 subjects [Ellis (1987) discusses data for 35 cases], Mitwalli *et al* (1984) gave data for only 7 in detail (but these had been treated for 2.8 years) and Tolis *et al* (1977) only 9 patients. Against this general background of inadequate data, especially with respect to the duration of treatment, it is not reasonable to dismiss the study by Dalton and Dalton (1987).

These previous analyses did not consider adequately the possibility of an inverse relationship between duration of intake and the lowest dose producing adverse effects. Also, these reviews did not consider intakes during potentially critical periods of development.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The toxicity of concern with pyridoxine is neurotoxicity, which has been demonstrated clearly in experimental animals and humans. Other effects reported in animals, which occur at high doses (see earlier), have not been investigated in humans, because the main effect in animals is on spermatogenesis and the main use of high doses in humans is for premenstrual tension.

The deficiencies and uncertainties in the available database make the identification of a clear no-effect intake very difficult. Daily doses of about 500 mg are necessary to produce severe neurological effects. In contrast, some of the subjects who reported minor neurological symptoms in the study of Dalton and Dalton (1987) were taking only 50 mg of pyridoxine per day. The dose-response data in humans, which are the basis for determining the upper level, are derived largely for women with premenstrual syndrome. Pyridoxine neuropathy develops very slowly in humans even at high doses; intake for 12 months or longer is necessary to produce neurotoxicity at doses of 2 g per day or less (see Table 2). Severe effects have been reported in a number of case reports that involved daily intakes about 500 mg, with one case report after 100-200 mg for 36 months (Table 2). The second cohort described in the paper by Brush (1988) is consistent with mild effects at 200 mg/day in a small number of treated patients. The data in the study of Dalton and Dalton (1987), and their validity are critical to establishing the upper level. The study of Dalton and Dalton (1987) is difficult to interpret with respect to both the incidence and also the dose-response relationship; however, the duration difference and the reversibility data indicate that the effects cannot be dismissed. Based on the apparent inverse relationship between dosage and duration of intake, a significant difference in duration of intake (average 2.9 years), but not dosage in women with “neurological effects” while taking low doses is exactly the relationship that would be predicted.

In summary therefore the data indicate that severe toxicity can be produced at doses of 500 mg/day or more, and that minor neurological symptoms may be apparent at doses of 100 mg/day or more if consumed for long periods. Neurotoxicity has not been reported at doses of 100 mg/day when consumed for a period of up to a few months but such data are not relevant to assessment of neurotoxicity, because of the slow development of symptoms at high doses and the inverse relationship between dosage and the onset of symptoms.

An upper level has been calculated by dividing the average intakes in the study of Dalton and Dalton (1987) of approximately 100 mg per day (the mean intake was 117 mg/day and the median was <100 mg/day) by a factor of 2, because the intake corresponds to a possible effect level for long-term intake, and by a second factor of 2 to allow for deficiencies in the database. A larger uncertainty factor is considered not to be necessary, because the data of Dalton and Dalton (1987) were for a sub-group with high plasma concentrations, and because the resulting upper level of 25 mg per day has not been associated with adverse effects in any of the large number of published studies. This value is below the lowest doses associated with minor neurological effects following long-term intake, and is 10- or 20-fold lower than doses associated with more severe adverse effects. In addition an intake of 25 mg/day is below the doses producing subtle and minor effects when taken for only 10 days (Molimard *et al*, 1980).
There are no subgroups that are known to be unusually susceptible to the adverse effects of vitamin B_6. There are no reports of adverse neurological effects on infants born to mothers with high intakes of vitamin B_6, or of neurological effects in lactating women, although controlled clinical studies are lacking. Therefore the UL of 25 mg per day should be considered to apply also to pregnant and lactating women. However, there are no adequate animal developmental neurotoxicity data that address these stages of development, and this is identified as a database deficiency (see recommendations).

The upper level intakes for children are based on body weight differences compared to adults:

Age (years)	Tolerable Upper Intake Level (UL) (mg per day)
1-3	5
4-6	7
7-10	10
11-14	15
15-17	20
Adults	25

6. CHARACTERISATION OF RISK

There is a wide margin between the UL of 25 mg per day and intakes from food sources only (see Table 1) and there are no safety concerns in relation to vitamin B_6 intake from food sources. The combined intake that would occur from the foods and from supplements is generally below the UL. However, recent data on vitamin B_6 intake from foods and supplements in Ireland indicate that, while the 95th percentile intake of 18-64 year old women is 8 mg/day, the intakes of 2.5% of this population group exceed the UL of 25 mg (range of intake of 30-62 mg/d) due to supplement use (IUNA, 2000). There are supplements available in some countries that contain amounts per tablet/capsule that are considerably higher than the upper level. The UL does not apply to individuals taking vitamin B_6 under medical supervision.

7. RECOMMENDATIONS

Neurotoxicity has been reported only after prolonged periods of treatment at high doses. The vitamin itself is rapidly eliminated and there is no molecular mechanism to explain the delay between exposure and the development of adverse effects. Information on the mechanism may allow a better understanding of the inter-relationships of the dosage, the duration of intake and the severity of effect.

Pyridoxine deficiency has a significant effect on neuronal development (Kirksey *et al*, 1990), but there are no data on the neuronal toxicity of excessive pyridoxine during development of the nervous system. A major deficit in the database for this vitamin is the absence of information from adequate developmental neurotoxicity studies. Such research would clarify if the dose-response relationship of the developing nervous system is comparable to that indicated by studies in adults. Information on the neurobehavioural development of the offspring of women who become pregnant while taking high-doses of vitamin B_6 for pre-menstrual syndrome, or who were intentionally given high doses of B_6 during pregnancy (see Ellis, 1987), may provide data relevant to this issue.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN B_{12}

(EXPRESSED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Vitamin B_{12} is the generic name for a specific group of cobalt-containing corrinoids with biological activity in humans. This group of biologically active corrinoids is also described as cobalamins. Cyanocobalamin is the commercialy available form used in food supplements and food fortification. In foods, hydroxo-, methyl- and 5'-deoxyadenosyl-cobalamins are the main cobalamins present. Sulphitocobalamin, with a sulphite ligand chelated to the central cobalt atom in the corrin ring, may occur is some processed foods.

Vitamin B_{12} functions primarily as a coenzyme in intermediary metabolism. Only two vitamin B_{12} dependent reactions have been identified thus far for humans: 1) the methionine synthase reaction with methylcobalamin, and 2) the methylmalonyl CoA mutase reaction with 5-deoxyadenosylcobalamin as the active coenzyme, respectively.

A dietary vitamin B_{12} deficiency can occur in strict vegetarians or after gastrectomy, and other diseases affecting cobalamin absorption. About two-thirds of patients with vitamin B_{12} deficiency have pernicious anemia (PA), an autoimmune disorder associated with gastric atrophy and absence of Intrinsic Factor (IF) which results in vitamin B_{12} malabsorption. The key symptom in vitamin B_{12} deficiency is macrocytic megaloblastic anemia. These haematological abnormalities are indistinguishable from those seen in folate deficiency, because of the interrelated function of both vitamins (Herbert, 1986). Another key symptom of vitamin B_{12} deficiency are neurological complications, such as paraesthesia, leg weakness, memory loss, etc, due to progressive lesions in the lateral and posterior columns of the spinal cord (subacute combined degeneration of the spinal cord). Neurological symptoms occur in about 75-90% of all individuals with (untreated) vitamin B_{12} deficiency, and appear generally at a later stage. In about 25% of all cases neurological symptoms are the only symptoms, i.e. without haematological abnormalities (for review see Bower & Wald, 1995; Lindenbaum *et al*, 1988).

2. NUTRITIONAL BACKGROUND

Vitamin B_{12} plays a specific role in amino acid metabolism, i.e. in methylation reactions, together with folate, in the methionine synthase reaction, and in the rearrangement of methylmalonyl CoA into succinyl CoA (for review see Herbert, 1984; Ellenbogen & Cooper, 1991).

The average dietary requirement for vitamin B_{12}, as established by the Scientific Committee for Food (Nutrient and Energy Intakes for the European Community, Reports of the SCF, 31th Series, 1993) is 1.0 µg/day, with a population reference intake (PRI) for adults of 1.4 µg/day. This is approximately the amount needed to maintain an adequate vitamin B_{12} body pool (about 2.5 mg), and to compensate for daily losses (about 0.1% of the total body pool). Dietary vitamin B_{12} only comes from animal sources, mainly from dairy products, fish and (red) meat. Daily intakes between 2 and 6 µg have been reported for omnivores. Individuals consuming large amounts of liver and some types of fish (sardines) may have high intakes; in contrast, individuals avoiding animal products need supplemental sources of vitamin B_{12}.

The prevalence of marginal cobalamin deficiency in elderly, characterized by low serum cobalamin and increased plasma methylmalonic acid (MMA) levels, has been estimated at about 25% (van Asselt, 1998). This is likely due to a decreased bioavailability of vitamin B_{12} from dietary sources, but not from synthetic vitamin B_{12}. In the US the use of vitamin B_{12} supplements is encouraged for elderly because of their compromised absorption (FNB DRI Report, IOM, 1998).

Other groups at risk for a marginal intake of vitamin B_{12} are those avoiding animal products, such as vegans and individuals on a macrobiotic diet, and subjects with an undiagnosed pernicious anemia.

Detailed intake data on vitamin B_{12} in EU countries are scarce. Recently, average intakes of 4.9 and 3.9 µg/day were reported for adult men and women, respectively, from a representative household survey in The Netherlands, using a two-day dietary record method (Blokdijk *et al*, 2000). The mean vitamin B_{12} intake in Dutch elderly subjects was about 5 µg/day, with a range of 0.5-16.9 µg/day from dietary intake, and up to 32 µg/day for the total intake (including supplements) (van Asselt *et al*, 1998).

Data from Ireland (IUNA, 2000) indicate a mean vitamin B_{12} intake from all sources (food + supplements) of 5.4 and 4.1 µg/day for men and women, respectively. The upper 97.5th percentiles were 15.0 and 15.1 µg/day, respectively. Mean intakes from food sources only were 5.2 and 3.6 µg/day, for males and females, with upper 97.5th percentiles of 13.1 and 11.8 µg/day, respectively.

Data from the United Kingdom (HMSO, 1990) indicate a mean vitamin B_{12} intake from all sources (food + supplements) of 7.3 and 5.4 µg/day for men and women, respectively. The upper 97.5th percentiles were 23.0 and 18.2 µg/day, respectively. Mean intakes from food sources only were 7.2 and 5.2 µg/day, for males and females, with upper 97.5th percentiles of 22.9 and 17.8 µg/day, respectively.

Data from the Boston Nutritional Status Survey on vitamin B_{12} supplement use among elderly show that the median (percentile 50) intake from supplements is 5 µg/day for males and 6 µg/day for females (total intake from diet + supplement: 9.7 and 9.0µg/day, respectively); the percentile 95 intake from supplements was 77 µg/day in men and 100 µg/day in women, and the corresponding values for total intake were 83 µg/day in men and 106 µg/day in women, respectively. Data from NHANES III (USA) give a highest mean intake from diet + supplements for males (31-50 years) of 17 µg/day, the percentile 95 intake in pregnant females was 37 µg/day (data taken from FNB DRI Report, IOM, 1998).

Dietary vitamin B_{12} is absorbed through a receptor mediated mechanism in the ileum. Food-bound vitamin B_{12} has first to be liberated through peptic digestion and gastric acid secretion in the stomach. The 'free' vitamin B_{12} becomes then bound to haptocorrins (or R-proteins) secreted by the salivary glands and the gastric mucosa. In the small intestine the R-binders are degraded by pancreatic protease action and the cobalamins are subsequently bound to the Intrinsic Factor (IF), a glycoprotein secreted by the parietal cells of the stomach. Uptake in the ileum is specific for the IF-cobalamin complex. Fractional absorption decreases as the oral dose is increased. Ileal receptors are saturated with dosages between approximately 1.5 and 2.5 µg of vitamin B_{12} per meal. At intakes around 1 µg about 50% is absorbed, at dosages around 25 µg only 5% is absorbed. Very small amounts (ca 1%) can be absorbed by passive diffusion, in the absence of IF.

In blood the cobalamins are transported by specific binding proteins, called transcobalamins. Although normally about 80% of the plasma cobalamins (mainly methyl-, adenosyl- and hydroxocobalamin) are bound by the glycoproteins TC I and TC III, the other 20% is bound to TC II, which is the essential B_{12} carrier in the delivery of the vitamin to the non-hepatic, metabolically active tissues such as the bone marrow and the brain.

After parenteral administration of hydroxycobalamin a rapid decline in plasma levels has been observed in the first 7 hours, followed by a slower decline with a half-life elimination of 21-29 h (Loew, 1988).

Vitamin B_{12} is an exceptional B-vitamin as it can be stored in significant amounts, especially in the liver and the kidney. The average concentration in human liver is between 0.5-1 µg/g; the total body pool size is estimated between 2-3 mg (Grasbeck *et al*, 1958; Adams *et al*, 1972). The main excretion is through the bile, but there is a considerable reabsorption of these biliary cobalamin losses in the ileum (enterohepatic circulation). Average daily losses *via* the stool are estimated at ca 0.5 µg. In PA patients these losses are higher, estimated at about 0.2% of the total body pool size, because of a lack of (IF mediated) reabsorption. Urinary excretion is minimal, and increased only if the plasma binding capacity is exceeded, e.g. following parenteral or intravenous administration. Total daily losses are estimated at about 0.1% of the total body pool (for review see Scott, 1997; Ellenbogen & Cooper, 1991).

3. HAZARD IDENTIFICATION

No adverse effects have been associated with excess vitamin B_{12} intake from food or supplements in healthy individuals. Vitamin B_{12} has a history of safe long-term use as a therapeutic agent given in high dosages per os, or *via* intramuscular injections, for treatment of disorders associated with impaired

vitamin B_{12} absorption, such as in gastrectomy and malabsorpion. In vitamin B_{12} replacement therapy oral or intramuscular dosages between 1-5 mg vitamin B_{12} are used, with no supportive evidence of adverse effects. The usual treatment in PA patients is 1 mg administered intramuscularly once every 1 to 3 months, but oral dosages of 300-1000 µg daily could also provide adequate treatment (Berlin *et al*, 1968; Hathcock & Troendle, 1991). At these dosage rates the cobalt and cyanide contributions are toxicologically insignificant (see Hathcock & Troendle, 1991).

Mangiarotti *et al* (1986) studied the effect of massive supplementation with vitamin B_{12} in a group of dialysis patients. One group of 106 patients received a multivitamin preparation containing 2.5 mg vitamin B_{12} plus 0.7 mg folic acid, 12 mg niacin and 150 mg vitamin C at the end of each dialysis period during 3 years. Serum vitamin B_{12} levels at the end of the treatment period were 4 times greater than normal, but no adverse effects were reported.

High dosages have also been used in other experimental studies, mostly short term, such as for the treatment of sleep-waking rhythm disorders, in which study vitamin B_{12} (no form specified) was given in dosages between 1.5 and 3 mg/day for 8 weeks (n = 13 cases) , with no adverse effects recorded (Maeda *et al*, 1992).

High dose vitamin B_{12} (1 mg cyanocobalamin intramuscular weekly for 1 months, followed by monthly injections for a minimum of 6 months), has also been used to improve cognitive functions in geriatric patients (Martin *et al*, 1992). Cobalamin therapy resulted in cognitive recovery in some patients, and no adverse effects were reported.

In some studies intravenous (i.v.) dosing has been associated with dermal abnormalities, e.g. acne formation in some cases. Ten cases were reported by Puissant *et al* (1967) after series of up to 12 injections with 5 mg of hydroxocobalamin, but not with cyanocobalamin. The authors suggest that degradation products, formed from the less stable hydroxocobalamin, might have been responsible for the acne formation, rather than the intact compound (but no further data provided).

One case of acneiform eruption, described as acne rosacea, was reported for a 53 yr old women who used vitamin supplements containing 100 mg vitamin B_6 and 100 µg vitamin B_{12} and 10,000 IU vitamin A and an unknown amount of zinc. Upon discontinuation of the supplement a 'dramatic' improvement was observed. The author ascribe the acne to the vitamins B_6/B_{12} without further testing (Sheretz, 1991).

Hydroxocobalamin is used as a cyanide antidote and has also a history of safe and effective use. For this purpose intravenous dosages up to 5 g are given (Forsyth *et al*, 1993).

Foulds *et al* (1969) described 4 PA patients presenting with tobacco amblyopia (optic neuropathy) who were treated parenterally with 0.25-1 mg cyanocobalamin per month. This treatment restored the haematological, but not the visual abnormalities. When the treatment was changed to hydroxocobalamin the visual impairment also improved. Cyanide, derived from tobacco smoke, has been implicated in the pathogenesis of tobacco amblyopia, and the positive effect of hydroxocobalamin is likely explained by cyanide detoxification.

One case has been reported of a 32 year old man handling animal feed who developed contact dermatitis to vitamin B_{12} and developed skin plaques (Rodrigues *et al*, 1994). However, this anecdotal evidence without follow up is not considered relevant in deriving an UL.

3.1. Carcinogenicity

A tumour promoting effect of vitamin B_{12} has been reported in one study in rats. Rats kept on a methionine deficient diet supplemented with 5 µg/100 g vitamin B_{12} (trademark: Rubramin) and treated with the carcinogen *p*-dimethylaminobenzene (DAB) had a higher incidence of hepatomas compared to the group without supplemental vitamin B_{12}. A control group receiving the supplemented diet without DAB showed no hepatic tumours (Day *et al*, 1950).

Kalnev (1977) studied the effect of methylcobalamin and cyanocobalamin on the growth of Walker's carcinosarcoma and on the longevity of rats with implanted Zajdela ascites hepatoma cells, and reported reduced survival of rats upon treatment with both compounds.

4. DOSE RESPONSE. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL

No systematic toxicological studies have been reported for vitamin B_{12}. There are no reports attributing carcinogenic or mutagenic or teratogenic potential to cyanocobalamin (see Ellenbogen & Cooper, 1991). In one study a tumour promoting effect was reported in a rat model, but this study is not considered relevant for safety assessment in humans.

There are also no adverse effects known for vitamin B_{12} from foods, or from supplements in amounts far in excess of needs. Some studies suggested acne formation after high parenteral doses of hydroxocobalamin, but not with cyanocobalamin, or after a combination of vitamins A, B_6 and B_{12} given orally.

Oral and parenteral supplementation with dosages between 1-5 mg every fortnight or month have been given for long periods, up to at least 5 years, to patients with compromised vitamin B_{12} absorption, without any identified adverse effects. It should be noted, however, that these studies were not designed to find adverse effects.

Therefore there are no clearly defined adverse effects produced by vitamin B_{12} that can be used to define a LOAEL or NOAEL, which can be used as a basis for deriving an UL.

5. CHARACTERISATION OF RISK

Average intakes of vitamin B_{12} are about 2-6 µg/day from food; intakes up to 32 µg/day have been reported for the total intake (including supplements) in elderly Dutch subjects (van Asselt *et al*, 1998). For the UK (HMSO, 1990) upper intake levels (97.5th percentile) from food sources only were reported to be 22.9 and 17.8 µg/day, for males and females, respectively. Upper intake levels from all sources were hardly higher, i.e. 23.0 and 18.2, respectively.

Data from the USA (see Section 2) show 95th percentile intakes from food and supplements of 83 µg/day in elderly men, 106 µg/day in elderly women, and 37 µg/day in pregnant women. Although it is not possible to derive an UL, there is no evidence that the current levels of intake from foods and supplements represent a health risk.

In addition, adverse effects have not been reported in the treatment of patients with compromised B_{12} absorption who received dosages up 1000 µg/day orally for prolonged periods; however, there was no systematic assessment of adverse effects in these patients.

Supplements available on the market usually contain dosages between 1-5 µg, but higher dose supplements with 50 µg or more are available.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF FOLATE

(EXPRESSED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Folate is the generic name for a number of compounds having a similar activity as folic acid (pteroylglutamic acid, PGA), i.e. being involved in single carbon (C_1-) transfer reactions. Folic acid (PGA) is a synthetic folate compound used in food supplements and in food fortification because of its stability, and becomes biologically active after reduction. Natural (dietary) folates are mostly reduced folates, i.e. derivatives of tetrahydrofolate (THF), such as 5-methyl-THF (5-MTHF), 5-formyl-THF and 5,10-methylene-THF, and exist mainly as pteroylpolyglutamates, with up to nine additional glutamate molecules attached to the pteridine ring.

2. NUTRITIONAL BACKGROUND

Folates play an important role in the transfer of C_1-groups (i.e. methyl-, methylene- and formyl-groups), maintaining the methylation balance, such as in the biosynthesis of DNA bases and in amino acid metabolism.

Green vegetables and certain (citrus) fruits are important dietary sources of folates. The population reference intake set by the SCF of the EU is 200 µg/day for adults, and 400 µg/day in pregnancy.

Food folates, mainly present as polyglutamates, have to be hydrolysed by a (brush border associated) deconjugase enzyme in the gut before absorption can occur. Folate absorption from natural food is generally lower than synthetic forms (e.g. folic acid) contained in supplements, due to matrix effects and the presence of inhibitors of the conjugase enzyme in some foods. Folic acid (PGA) enters the folate cycle after reduction by a (dihydro-)folate reductase. This enzyme is present in the intestinal mucosal cell, but also in other tissues, such as liver and kidney. Reduction of PGA may be a slow process in some subjects and at higher intake levels (> ca 260 µg) PGA may appear unchanged in the circulation (i.e. in the postprandial state after supplement use (Kelly *et al*, 1997). Under normal conditions 5-MTHF (as monoglutamate) is the only form present in plasma, mainly protein-bound. Tissue uptake is carrier-mediated and/or through folate binding proteins. In tissues folates are retained as polyglutamates and the folate coenzymes can be interconverted in numerous (de-)methylation reactions, such as in DNA synthesis (formation of thymidilate from deoxyuridine), amino acid interconversions, such as the remethylation of homocysteine to methionine. In this latter methionine synthase (MS) reaction vitamin B_{12} is also involved as a cofactor. About 50% of the folate body store, estimated to be 13-28 mg, is considered to be present in the liver (for review see Report of the Standing Committee on the scientific evaluation of dietary reference intakes (DRIs) and its panel on folate and other B-vitamins and choline. Food and Nutrition Board, Institute of Medicine, 1998).

In European countries the average folate intake in adults was found to be remarkably similar, i.e. around 300 µg/day in adult males, and 250 µg in adult women (De Bree *et al*, 1997). This is about the recommended intake level, but lower than recommended for pregnant women and women with a pregnancy wish. For these groups an intake >400 µg/day is considered protective against neural tube defect (NTD). More than 90% of women in the childbearing age range have dietary folate intakes below this optimal level. In The Netherlands and the UK women with a pregnancy wish are advised to take daily a folic acid supplement of 400 µg between 4 weeks before up to 8 weeks after conception. In some European countries, such as the UK, cereals and breads are fortified with folic acid, contributing 25-100 µg per serving.

Additional data on mean and high (P-97,5) intakes, reported for the various EU member states are summarised below.

Table 1. Folate intake in EU countries (µg/day)

Country intake	Population	Mean intake	High intake
Austria[1]	M + F (20-60 y) (n = 2488)	398	1795 (P-97.5)
Germany[2]	M (26-50 y) F (26-50 y)	255 210	- -
Ireland[3]	M (n = 662) (18-64 y) F (n = 717) (18-64 y)	332 260	662 (P-97.5) 638 (P-97.5)
Italy[4]	M + F (n = 2734)	287	550 (P-97.5)
The Netherlands[5]	M + F (n = 5958)	251	412 (P-97.5)

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3. HAZARD IDENTIFICATION

Megaloblastic anemia is the ultimate consequence of an inadequate folate intake. More recently, an increased plasma (total) homocysteine level, an independent risk factor for vascular disease, has also been associated with low folate intakes, respectively with lower folate plasma levels (Selhub *et al*, 1993; Morrison *et al*, 1996). A low folate level or intake is also a risk factor for NTD risk in women (Daly, 1995, 1997).

No adverse effects have been associated with the consumption of excess folate from foods (Butterworth & Tamura, 1989). Adverse effects are exclusively reported from use of the synthetic compound folic acid (PGA). Synthetic 5-MTHF and 5-formyl-THF (as a racemic mixture of the 6S,6R-compound) are also commercially available, but as far known, these compounds are not being used in food supplements or in food fortification, but only for therapeutic use, such as for treatment of neuropsychiatric patients, and/or in 'rescue therapy' of cancer patients treated with antitumour (i.e. antifolate) agents. The natural form of the reduced folates is thought be mainly the (6S) diastereoisomer, which has a greater biological activity than the (6R) isomer.

Both for PGA and the synthetic reduced folate compounds, no systematic toxicological evaluation has been reported and/or is available. However, adverse effects have been reported for folic acid. Based upon these studies the following safety issues have to be considered:
- modification of vitamin B_{12} deficiency (pernicious anemia) symptoms due to folic acid supplementation:
1) masking of haematological symptoms,
2) exacerbation of neurological symptoms;
- epileptogenic and neurotoxic effects of folic acid;
- decreased efficacy of folate antagonists used in chemotherapy;
- potential adverse effects of folate supplementation on zinc absorption and status;
- carcinogenicity;
- assumed hypersensitivity for folate.

These items have been extensively reviewed (see Butterworth & Tamura, 1989; Campbell, 1996; Dickinson, 1995). The scientific data on folic acid toxicity were also thoroughly reviewed by the Food and Drug Administration (FDA, 1993) when considering folic acid fortification of cereal grain products for prevention of neural tube defects (NTD).

The available evidence with respect to the various safety issues is shortly summarised and discussed below.

3.1. Modification of vitamin B_{12} deficiency symptoms

Folate and vitamin B_{12} are interrelated vitamins, both involved in the remethylation of homocystein to methionine. The vitamin B_{12} deficiency ultimately results in a partly or secondary folate deficiency as folate becomes 'trapped' as 5-methyl tetrahydrofolate (5-MTHF) and therefore no longer available for

the formation of the 5,10-methylene-THF coenzyme which is involved in the formation of the DNA base thymidine from deoxyuridine. In either a folate- or vitamin B_{12}-deficiency megaloblastic changes occur in the bone marrow and other replicating cells due to an impaired DNA synthesis, because of a lacking thymidine production. Supplementation of a vitamin B_{12}-deficient subject with folic acid, but not with 5-MTHF, the predominant folate occurring in natural foods, can result in the 'renewed availability' of 5,10-methylene-THF, and the subsequent repair in DNA synthesis and remission of the haematological abnormalities. The neurological complications of a vitamin B_{12} deficiency do not respond to folate or folic acid supplementation.

3.1.1. Incidence of vitamin B12 (cobalamin) deficiency

About two-thirds of cases of vitamin B_{12} deficiency are due to pernicious anemia (PA), i.e., an autoimmune disorder characterised by decreased amounts of intrinsic factor (IF) resulting in cobalamin malabsorption. Figures on the prevalence of PA in W-Europe vary between 1.2/1000 in the UK and 1.98/1000 in Scandinavian countries, the incidence in these latter countries was estimated at 0.167 per 1000 person years (data taken from Bower & Wald, 1995). Among Caucasians, PA is mostly found in elderly people, but in African-Americans cases were also reported under 40 years of age.

Other causes of hypocobalaminemia, i.e., low serum cobalamin levels without specific anemic abnormalities, were found to be associated with dementia, AIDS, and malignant diseases. Recent data show a high prevalence (ca 25%) of marginal cobalamin deficiency in elderly, characterised by low serum cobalamin and increased plasma methylmalonic acid (MMA) levels, but not, or hardly associated with haematological abnormalities (van Asselt, 1998).

3.1.2. Modification of vitamin B12 deficiency symptoms: masking of haematological symptoms

In the earlier days when vitamin B12 was not yet identified as a separate vitamin, individuals with macrocytosis and other haematological abnormalities were treated with folic acid (>5 mg/day). With these higher dosages complete haematological remission has been observed in most (>60%) of PA patients. A suboptimal improvement has been reported for dosages between 1-5 mg/day. Also with lower (intramuscular) dosages <1 mg a haematological response has been reported, but this is considered very rare (for review see Butterworth and Tamura, 1989; Bower and Wald, 1995; and the FDA subcommittee report on folic acid, 1993).

It should be noted that haematological abnormalities do not occur in all PA patients. In a subgroup of patients only neurological symptoms are observed (see below). Campbell (1996) stated in his review that 11-33% of patients with neurologic abnormalities due to vitamin B12 deficiency has normal routine haematological tests. Lindenbaum *et al* (1988) evaluated all records of patients with low serum cobalamin levels that were seen in their centre (Columbia Presbyterian Medical Center, NY, US) between 1968-1985, and concluded that neuropsychiatric disorders due to cobalamin deficiency occur frequently in the absence of anemia or macrocytosis. In 28.4% of their cases with clinical cobalamin deficiency (n = 141) haematocrit and MCV were normal (without any treatment).

3.1.3. Masking of vitamin B12 deficiency: exacerbation of neurological symptoms

In many, but not all untreated PA patients neurological abnormalities may develop due to subacute combined degeneration of the spinal cord. Contrary to the effect of folic acid supplementation on the haematological symptoms, the neurological abnormalities in vitamin B12-deficient patients are not cured by folic acid.

In a report on 10 cases of pernicious anemia (PA) patients treated with 5-25 mg folic acid, one patient experienced neurological symptoms after 8 days, and 2 patients after 4 and 9 months of treatment, respectively (Wagley, 1948). In another study, neurological symptoms remained stable or improved in 4/70 patients treated with folic acid for a period of 6-12 months, but deteriorated in 3 subjects (Bethel and Sturgis, 1948). In a study from Schwartz *et al* (1950), cited by Dickinson (1995), 98 PA patients treated with folic acid were followed for a period up to 3.5 years, 4 patients relapsed neurologically within 1 year, and 19 in the next year. These studies show that neurological symptoms, especially posterolateral spinal cord disease and peripheral neuritis, may occur in cobalamin deficient patients treated with high doses of folic acid to maintain an haematological remission, but not necessarily. Reports are available on patients treated with large doses of folic acid (10-100 mg daily) for many years without the development of neurological complications. However, in some studies it has been claimed that folic acid therapy in patients with vitamin B12 deficiency might aggravate, or even induce neurological lesions.

However, after reviewing the available literature, Dickinson (1995) concluded that there is no convincing evidence for such an effect. A folic acid-induced aggravation or induction of neurological symptoms would be difficult to demonstrate, as the progression of such symptoms in untreated subjects is already highly variable between patients, e.g. between a few months up to 5 years in development of paraesthesiae. Also studies in fruit bats with nitrous oxide-induced vitamin B_{12} deficiency, showing exacerbation of neurological signs after folic acid administration, are not completely convincing because of methodological flaws (van der Westhuyzen *et al*, 1982; see also comment in Dickinson, 1995).

These fruit bats were given large oral daily doses of folic acid (1.54 mg/kg; equivalent to ca 100 mg/day in humans), or daily intramuscular injections of formyl-THF (1.15 mg/kg). The fruit bats given the oral folic acid reached the same stage of neurological impairment ("flight reduced to hops"), but this occurred slightly, but not significantly, earlier.

In another study with experimentally (diet) induced vitamin B_{12} deficiency in rhesus monkeys, three of the nine monkeys received 5 mg/week of supplemental folic acid intramuscularly, followed by 5 mg in the drinking water (5 days/week) (Agamanolis *et al*, 1976). Five animals developed visual impairment and optic atrophy, including the 3 monkeys that received supplemental folic acid. Apparently, the optical nerve lesions occurred earlier (by 10-11 months) in the folic acid-treated animals. It should be noted that the visual lesions observed in these monkeys are only rarely noted in human disease. Spastic paralysis of hind legs and tail was found in 3 animals, including 2 animals receiving folic acid. Other lesions in cranial and peripheral nerves and in the white matter of the spinal cord were observed in some animals, but were apparently not affected by supplemental folic acid.

Vegetarians are at risk to develop a vitamin B_{12} deficiency, while their folate intake is generally high. It has been reported that in this group neurological symptoms due to B_{12} deficiency, such as myelin damage, occur with only minor haematological damage (Herbert, 1994). It was even reported that vegetarians with greater myelin damage had generally higher red cell folate levels. However, there is generally no clear correlation between haematological and neurological features of vitamin B_{12} deficiency.

3.2. Neurotoxic effects of folic acid

Animal studies have shown that folic acid can be a neurotoxin, and can cause convulsions in laboratory animals (e.g. Hommes and Obbens, 1972; Spector, 1972). This evidence is in part based upon *in vitro* tissue and cell culture studies, and/or using very high dose levels (i.v. dosages 60-90 mg). There is however no clear evidence for a folic acid-induced neurotoxicity in humans. Some cases of neurological deterioration have been reported following ingestion of folic acid tablets (3 mg), or folic acid containing multivitamin supplements, but the presence of an (undiagnosed) vitamin B_{12} deficiency cannot be ruled out in these cases (see Dickinson, 1995). In one study with epileptic patients electroencelographic changes were noted after administration of 7.2 mg of folic acid, and seizures after 14.4 and 19.2 mg. However, in other studies no such changes or effects were observed after i.v. dosage of 75 mg (see Campbell, 1996). These studies are therefore inconclusive.

Hunter *et al* (1970) reported disturbing toxic effects, i.e. sleep disturbances and mental changes, after treatment of healthy volunteers with 15 mg folic acid for 1 month. This study was however not placebo controlled, and the results were not confirmed in another double blind, randomised study (Hellstrom *et al*, 1971).

Concern has been expressed that folic acid might exacerbate seizures in persons with uncontrolled, or drug controlled epilepsy. However, no such effects were found in several controlled studies with dosages between 15-20 mg/day. Supplementation studies (15 mg/day for 45 days) with Parkinson disease patients did not show an effect on the incidence of neurological defects. Also after i.v. dosing with dosages up to 150 mg no adverse effects have been reported (for review see Butterworth & Tamura, 1989; Campbell, 1996). As already mentioned, some anticonvulsant drugs may reduce serum folate levels. However, as far as data are available, there seems apparently no increased risk for patients with epilepsy, or interference with anticonvulsant medication, at higher folate intakes.

3.3. Potential adverse effects on zinc absorption and status

Dietary zinc deficiency and a relative shortage of maternal zinc has been associated with NTD in human (Milunsky *et al*, 1992). It has been suggested (Quinn *et al*, 1990) that in the presence of a zinc deficiency the administration of high-dose folate increases the teratogenicity of such a deficiency. The enzyme gamma-glutamyl hydrolase is zinc-dependent and converts polyglutamates to monoglutamates, which

is an important step in the absorption of folate. Therefore, the availability of folate is dependent on the glutamyl hydrolase activity, which is regulated by the concentration of zinc (Canton *et al*, 1990).

Some earlier studies indicated competitive interactions between folic acid and zinc, however, results are conflicting. In reviews on this item from Butterworth and Tamura (1989) and from Zimmerman and Shane (1993) it is concluded that there is as yet no convincing evidence for negative effects of folate supplements on serum or red cell zinc contents (in a study in which women were dosed with 10 mg/day for 6 months), nor for negative effects of folic acid supplementation on zinc status in pregnant women. Contradictory results most likely result from methodological problems in assessment of zinc status/bioavailability.

3.4. Carcinogenicity

Folic acid has been associated with an increased incidence of oropharynx, hypopharynx and all cancers (Selby *et al*, 1989), but, as indicated by the authors of this epidemiological study, these cancers are largely smoking- and/or alcohol-related and this finding likely related to these confounding factors. In other (observational) studies an inverse relation was found between folate intake and/or status and colorectal cancer (e.g. Giovanucci *et al*, 1993; White *et al*, 1997), and with cervical cancer (Butterworth, 1993). Treatment of smokers with 10 mg folic acid plus 500 µg hydroxocobalamin for 4 months resulted in a reduction in atypical bronchial squamous metaplasia (Heimburger *et al*, 1988).

3.5. Decreased efficacy of folate antagonists

Folate antagonists such as methotrexate are used in the treatment of various cancers, e.g. leukemia, and also in rheumatoid arthritis, bronchial asthma and psoriasis. There are also a number of other drugs that interfere with folate metabolism, such as pyrimethamine, phenytoin, colchicine, etc. The FDA discussed the issue of potential effects of increased folate intake on the efficacy of antifolate therapy and concluded that there are relatively little data (Food Labelling: Health Claims and Label Statements; Folate and Neural Tube Defects, 1993). The American College of Rheumatology has stated that a dose of 1 mg/day of folic acid does not appear to inhibit the efficacy of low-dose methotrexate therapy in rheumatoid arthritis. High dose folic acid is also used to reduce methotrexate toxicity (see Campbell, 1996). So, there is currently little scientific data on potential adverse effects of high folate intakes on antifolate medication.

3.6. Assumed hypersensitivity

A limited number of case reports have been published on hypersensitivity reactions to oral and parenteral folic acid, but it cannot be excluded that these reactions were due to other components in the formulations. So, hypersensitivity may occur, but is most likely very rare (see Campbell, 1996).

4. DOSE-RESPONSE ASSESSMENT

From the available data it can be concluded that (synthetic) folic acid can cause adverse effects, while no adverse effects have been reported with the consumption of excess folate from foods. Animal data and *in vitro* tissue and cell culture studies indicate that neurotoxic and epileptogenic effects of folic acid can occur at high dose levels (60-90 mg i.v.). However, there is no clear evidence for a folic acid-induced neurotoxicity in humans. In one study with vervet monkeys a dose of 25.6 mg folic acid per day was given to 3 males for 99 days without any obvious toxic side effect (Venter *et al*, 1993). However, it was not indicated what was examined in this study. The animal data available cannot be used to derive a LOAEL or NOAEL.

The most serious adverse effect known in humans is modification of vitamin B_{12} neurological sequela in PA (pernicious anemia) patients as a result of folic acid supplementation, such as masking of the haematological signs and the potential of progression of neurological symptoms. Although the evidence for an exacerbation of the neurological symptoms in humans is equivocal because of the variability in severity and appearance of these symptoms in PA patients, there is some evidence for such a progression in monkeys.
Masking of the haematological signs in PA patients occurs with high frequencies and consistently with daily intakes of 5 mg; however, insufficient data are available for evaluation of dose levels between 1-5 mg.

This masking effect was considered the most serious adverse effect of folic acid by the Folic Acid Sub-committee of the FDA (in 1993) and used as the basis to derive a safe upper uptake limit (UL). Because of the uncertainty of potential adverse effects in the dose range between 1-5 mg, and because of the

fact that at higher intake levels of folic acid unmetabolised (oxidised) folic acid appears in the blood, exposing body tissues to a form of the vitamin not encountered before, the UL was set at 1 mg per day total folate (dietary folate plus folic acid). Although it was agreed that the safety data are all based upon trials with folic acid, and thus might warrant an UL for folic acid, rather than total folate, it was stated that in all trials folic acid was given on a variable background (dietary) intake of folate, and therefore, it could not be concluded that folic acid nor folate are responsible for the masking effect. In an update in 1996, based upon comments received on the proposal in 1993 for the UL level of 1 mg total folate, this conclusion remained unchanged (FDA, 1996).

More recently, the US Committee evaluating the new dietary reference intakes and the subcommittee on Upper Reference Levels of Nutrients (FNB DRI Report, 1998) concluded that progression of the neurological symptoms due to folic acid supplementation should be considered as the most serious adverse effect, and not the masking effect. Masking of the haematological signs in PA patients was considered a diagnostic problem that could be circumvented by using more specific tests (i.e. measuring serum MMA and/or Hcys) to identify cases of undiagnosed B_{12} deficiency. This Committee set a lowest-observed-adverse-effect level (LOAEL) of 5 mg folic acid and used an uncertainty factor of 5 because no NOAEL could be derived, resulting in an UL of 1 mg of folic acid.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Although no systematic toxicological studies of folic acid or other folates are available, an upper safe level can be set for (synthetic) folic acid (PGA) on the basis of findings in PA patients treated with high doses of folic acid. There is no evidence for risk associated with high intakes of natural, reduced folates, and thus no data to set an UL for natural folate. Although there is no conclusive evidence in humans, the Committee concludes that the risk of progression of the neurological symptoms in vitamin B_{12}-deficient patients as a result of folic acid supplementation cannot be excluded and should be considered the most serious adverse effect. In nearly all studies showing neurological relapse, dose levels >5 mg folic acid per day have been applied and data on the effect of dose levels between 1-5 mg is limited to a few cases.

In analogy with the US DRI Committee, it is concluded that the best available estimate for a lowest-observed-adverse-effect level (LOAEL), obtained from a sensitive group, for folic acid is 5 mg, and as dosages up to 1 mg of folic acid are unlikely to cause masking of the haematological signs in PA patients, the UL is set at 1 mg of folic acid. No data are available to suggest that other life-stage groups have increased susceptibility to adverse effects of high folic acid intake. Therefore, the UL is also applicable for pregnant or lactating women. It seems prudent, however, to adjust the ULs for children on the basis of bodyweight.

UL for children and adolescents

Age (year)	UL (µg)
1 - 3	200
4 - 6	300
7 - 10	400
11 -14	600
15 - 17	800

6. CHARACTERISATION OF RISK

Average folate intake in Europe is around 300 µg/day in adult men, and 250 µg in adult women (De Bree *et al*, 1997). Dietary folate consists only of reduced folates, and contains no folic acid, unless added. High intake levels (97.5th percentile) for folate from dietary sources around 500 µg/day have been reported, the higher data reported for Austria (see Section 2), are likely from all sources, including supplements.

Data from the 2nd Dutch National Food Consumption Survey on supplement use indicated that the mean folic acid intake from supplements among users is 100 µg, with a 97.5th percentile and maximum intake of 400 and 800 µg, respectively (Ronda *et al*, 1996).

Data from the Boston Nutritional Status Survey on folic acid supplement use among elderly males and females show that the median (P-50) intake from supplements is 400 µg/day; the P-95 was 2400 (M) and 1000 (F) µg/day, respectively (data taken from FNB DRI Report, IOM, 1998).

Regular supplements available on the market usually contain 400-500 µg folic acid. Women with a pregnancy wish are advised to use a daily supplement containing 400-500 µg folic acid between 4 weeks before and up to 8 weeks after conception to reduce NTD (neural tube defect) risk. In some European countries, such as the UK, cereals and breads are fortified with folic acid, contributing 25-100 µg per serving.

Subjects at risk for too high folic acid supplementation are those with an (undiagnosed) vitamin B_{12} deficiency, such as in pernicious anemia (PA) patients and in other conditions associated with cobalamin malabsorption. Figures on the prevalence of PA in W-Europe vary between 1.2/1000 in the UK and 1.98. Among Caucasians, PA is mostly found in elderly people, but in African-Americans individuals it was also reported under 40 years of age. Other cases of hypocobalaminemia, i.e. the occurrence of a low serum cobalamin level without specific anemic abnormalities, were found to be associated with dementia, AIDS, and with malignant diseases. Recent data show a high prevalence (ca 25%) of marginal cobalamin deficiency in elderly, characterised by low serum cobalamin and increased plasma methylmalonic acid (MMA) levels, but not, or hardly associated with haematological abnormalities (van Asselt, 1998).

Other groups at risk for a marginal intake of vitamin B_{12} are groups avoiding animal products, such as vegans and macrobiotics.

7. RECOMMENDATIONS FOR FURTHER RESEARCH

The extent to which a high dietary folate intake affects the symptomatology of a vitamin B_{12} deficiency (i.e. the occurrence of neurological *vs*. haematological signs) remains to be investigated. As elderly are especially at risk for a marginal vitamin B_{12} status, not necessarily related to PA, the potential risks of folic acid supplementation in this group needs further investigation.

The safety and efficacy of synthetic reduced folates, i.e. 5-MTHF, as an alternative for folic acid (PGA), also needs further study.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF MANGANESE

(EXPRESSED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Manganese can exist in a number of oxidation states, of which Mn(II) is the predominant form in biological systems. Food is the most important source of manganese exposure for the general population. The concentrations in foodstuffs vary considerably, but are mostly below 5 mg/kg. Grain, rice, and nuts, however, may have manganese levels exceeding 10 mg/kg or even 30 mg/kg in some cases. High concentrations have been found in tea. A cup of tea can contain 0.4 to 1.3 mg manganese (WHO, 1981; WHO, 1996). The dietary intake of adults has been estimated to range from 0.9 to 7 (Schlettwein-Gsell and Mommsen-Straub, 1973), 2 to 9 (WHO, 1981) and 1.2 to 9.4 mg Mn/day (Ellen *et al*, 1990). A Total Diet Study showed that the estimated average intake of manganese in the UK population in 1994 was 4.9 mg/day, including 2.3 mg/day from beverages (MAFF, 1997). The intake can be higher for vegetarians because higher levels of manganese occur in food of plant origin. The consumption of tea may contribute substantially.

The daily intake of manganese from the ambient air is lower. The annual average has been estimated to be less than 2 µg/day. In areas associated with ferromanganese or silicomanganese industries the daily exposure may rise to 10 µg, and 24-h peak values may exceed 200 µg (WHO, 1981). For workers in industries using manganese, the major route of exposure might be inhalation from air rather than ingestion of food.

2. NUTRITIONAL BACKGROUND

Manganese has been shown to be essential for various species. It is a component of arginase, pyruvate carboxylase and superoxide dismutase and plays a role as co-factor of certain enzyme systems. Accordingly, manganese-deficient animals exhibit adverse effects, e.g. impaired growth, skeletal abnormalities, reproductive deficits, ataxia of the newborn, and defects in lipid and carbohydrate metabolism. In contrast, evidence of manganese deficiencies in man is poor. A specific deficiency syndrome has not been described in humans (SCF 1993; Freeland-Graves, 1994; WHO, 1996).

Currently, there is no formal Recommended Dietary Allowance (RDA) for manganese. However, an estimated safe and adequate dietary intake (ESADDI) of 2-5 mg/day for adults was established by the US National Research Council (Freeland-Graves, 1994), and the Scientific Committee for Food of the EU estimated 1-10 mg/day as an acceptable range of intake (SCF, 1993).

3. HAZARD IDENTIFICATION

About 3-8% of orally ingested manganese is absorbed in the gastrointestinal tract, but absorption may be greater for young animals and infants. The absorption of manganese is inversely related to the level of iron and calcium in the diet. Highest tissue concentrations of manganese are found in the liver, kidney, pancreas, and adrenals. Preferentially, it is retained in certain regions of the brain in young animals and infants. Manganese is almost entirely excreted in the faeces. In humans, elimination is biphasic, with half-lives of 13 and 34 days (WHO, 1996).

3.1. Toxic effects in laboratory animals

The acute toxicity of manganese is relatively low. The oral LD50 of manganese chloride is reported to be in the range of 275-450, 250-275, and 400-810 mg Mn/kg body weight (bw) in mice, rats, and guinea pigs, respectively (WHO-IPCS, 1981).

The ingestion of diets containing manganese (II) sulphate monohydrate in 13-week feeding studies at doses ranging from about 110 to 2000 mg/kg bw/day in F344/N rats and 330 to 7400 mg/kg bw/day in B6C3F1 mice, equivalent to 36 to 650 mg Mn/kg bw/day or 107 to 2400 mg Mn/kg bw/day, respectively, was associated with lower body weight gains, lower absolute and relative liver weights, and haematological changes, partly in all exposed groups. In addition, epithelial hyperplasia and hyperkeratosis of the forestomach occurred in the highest exposed male mice (NTP, 1993).

Special studies in rodents have been performed to elucidate neurochemical effects of manganese exposure. These studies demonstrated changes of neurotransmitter levels in the brains of rats given 1 mg $MnCl_2$.$4H_20$/ml in drinking water, equivalent to 39 mg Mn/kg bw/day (Lai *et al*, 1981, 1982; Leung *et al*, 1981; Chandra *et al*, 1981). In addition, some behavioural effects were found in rats and mice at the same dose (Chandra *et al*, 1979; Ali *et al*, 1981). Unfortunately, only one drinking water concentration was tested in these studies. After administration of diets containing 2g Mn/kg equivalent to about 200 mg Mn/kg bw/day in the form of several manganese compounds to mice for 100 days (Komura and Sakamoto, 1991) and 12 months (Komura and Sakamoto, 1992), retarded growth, changes of biogenic amines in the brain and changes in the motor activity were observed. Another study showed effects of manganese on the biogenic amine metabolism in the regions of the rat brain following administration of 0.54 mg $MnCl_2$.$5H_20$/ml in drinking water. The average intake was 4.5 mg Mn/day equivalent to about 20 mg Mn/kg bw/day (Subhash and Padmashree, 1991). Similarly, earlier studies revealed neurochemical alterations in the brain of neonatal male rats orally exposed to 10 and 20 mg Mn/kg bw/day (Deskin *et al*, 1980) and changes in motor-activity of male Sprague-Dawley rats (hyperactivity in the first month and hypoactivity from months 7 to 8) at concentrations of 0,1 and 5 mg Mn/ml drinking water (Bonilla, 1984).

The lowest dose affecting the central nervous system was found in a study with growing male rats, in which 50 µg $MnCl_2$.$4H_20$/rat, initially equivalent to about 0,28 mg Mn/kg bw, were given by stomach tube daily for 15 to 60 days and reported to increase significantly the monoamine oxidase in the brain and to cause neuronal degeneration in the cerebral and cerebellar cortex (Chandra and Shukla, 1978). A similar dose of 0.357 mg Mn/kg bw/day was reported to decrease significantly the learning ability of female rats following intragastric administration of $MnCl_2$.$4H_2O$ for a period of 15 and 30 days (Öner and Sentürk, 1995).

A study with four male rhesus monkeys who were given orally 25 mg $MnCl_2$.$4H_20$ daily for 18 months, corresponding to 6,9 mg Mn/kg bw/day, revealed muscular weakness, rigidity of the lower limbs and marked degeneration with de-pigmentation of neurons in the region of substantia nigra (Gupta *et al*, 1980). The same animals had increased testis weights with interstitial oedema and degeneration of seminiferous tubules (Murthy *et al*, 1980).

3.2. Genotoxicity and related effects

The results of genetic toxicity tests with manganese are dependent on the particular assay and the protocol used.

Manganese sulphate was not mutagenic in *Salmonella typhimurium* strains TA97, TA98, TA100, TA1535 and TA1537 with and without exogenous metabolic activation (S9) (Mortelmans *et al*, 1986), although it was reported to be mutagenic in strain TA97 by Pagano and Zeiger (1992). Manganese chloride was not mutagenic in *S. typhimurium* strains TA98, TA100 and TA1535, but it was mutagenic in TA1537 (Wong, 1988). Both manganese sulphate and manganese chloride were positive on tester strain TA102 only without S9 mix. (De Méo *et al*, 1991). Manganese sulphate was found positive in a gene conversion/reverse mutation assay in *Saccharomyces cerevisiae* strain D7 without exogenous metabolic activation (Singh, 1984).

Manganese chloride was found positive in the mouse lymphoma assay ($tk^{\pm}$) without S9 (Oberly *et al*, 1982). It was also reported to be able to induce DNA damage in cultured human lymphocytes using the single-cell gel assay technique (comet assay) without S9, but not with S9 (De Méo *et al*, 1991). Manganese sulphate was reported to be able to induce sister chromatid exchanges (SCEs) with and without S9, and chromosomal aberrations only in the absence of S9 in cultured Chinese hamster ovary (CHO) cells (Galloway *et al*, 1987). Manganese chloride was not clastogenic in cultured FM3A cells in the absence of S9 (Umeda & Nishimura, 1979); in contrast it was found able to induce chromosomal aberrations in the root tips of *Vicia faba* (Glass, 1956). Manganese sulphate was found able to induce SCEs and chromosomal aberrations in CHO cells without S9, and only SCEs in the presence of S9 (NTP, 1993). Magnesium chloride was reported to be able to induce cell transformation in Syrian

hamster cells (Casto *et al*, 1979). Manganese chloride was unable to induce somatic mutations in *Drosophila melanogaster* (Rasmuson, 1985); manganese sulphate did not induce sex-linked recessive lethal mutations in *D. melanogaster* (Valencia *et al*, 1985). Oral doses of manganese chloride did not cause chromosomal aberrations in the bone marrow or spermatogonia of rats (Dikshith and Chandra, 1978); oral doses of manganese sulphate induced micronuclei and chromosomal aberrations in bone marrow cells and sperm-head abnormalities in mice treated for three weeks (Joardar and Sharma, 1990). In view of the known affinity of Mn^{2+} for chromosomal components, the authors suggested that the effects were mediated by these ions.

No induction of heritable translocations in mice or dominant lethal mutations in rats were observed after administration of manganese sulphate in the diet for 7 weeks (mice), or by gavage once a day for 1 to 15 days (rats) (NTP, 1993).
It seems probable that the positive results reported in several short term tests are not due to intrinsic, direct genotoxicity of manganese, but to indirect mechanisms, as it occurs for other elements. The genotoxicity of manganese compounds seems to be mediated by the bivalent ion Mn^{2+} at relatively high and cytotoxic concentrations.

Based on the presently available data no overall conclusion can be made on the possible genotoxic hazard to humans.

3.3. Carcinogenic potential

Under the conditions of a 2-year feed study with F344/N rats, there was no evidence of carcinogenic activity of manganese (II) sulphate monohydrate in rats receiving 60, 200, or 615 mg/kg bw/day (males) or 70, 230, or 715 mg/kg bw/day (females), equivalent to 19.5, 65 or 200 mg Mn/kg bw/day and 23, 75 or 232 mg Mn/kg bw/day, respectively. There was, however, equivocal evidence of carcinogenic activity in a 2-year feed study with $B6C3F_1$ mice receiving 160, 540, or 1800 mg/kg bw/day (males) or 200, 700, or 2250 mg/kg bw/day (females), equivalent to 52, 176 or 586 mg Mn/kg bw/day or 65, 228 or 732 mg Mn/kg bw/day, respectively, based on marginally increased incidences of thyroid gland follicular cell adenoma (high-dose animals) and significantly increased incidences of follicular cell hyperplasia. In addition, increased severity of nephropathy in male rats, focal squamous hyperplasia of the forestomach in male and female mice, and ulcers and inflammation of the forestomach in male mice were observed in the highest dose groups of these studies (NTP, 1993).

3.4. Reproductive toxicity

Teratogenicity studies with manganese (II) sulphate monohydrate conducted in rats, mice, hamsters and rabbits revealed no clearly discernible effects on nidation or on maternal or fetal survival. The number of abnormalities did not differ from the control animals (NTP, 1973).

Several studies in rats and mice indicate that the ingestion of manganese can delay reproductive maturation in male animals. Male rats administered an oral dose of 13 mg manganese/kg bw/day for 100-224 days had reduced testosterone levels. Delayed growth of the testes was observed in young rats ingesting 140 mg manganese/kg bw/day for 90 days. These effects do not appear to be severe enough to affect sperm morphology or male reproductive function. In rabbits, chronic parenteral administration of manganese produced marked degenerative changes in the seminiferous tubules, resulting in infertility (WHO, 1996).

3.5. Toxic effects in humans

In workers chronically exposed to manganese dusts and fumes, neurological effects of inhaled manganese have been well documented. The syndrome known as “manganism” is characterised by weakness, anorexia, muscle pain, apathy, slow speech without inflection, emotionless “mask-like” facial expression, and slow clumsy movement of the limbs. In general, these effects are irreversible. The minimal exposure level producing neurological effects is not certain but is probably in the range of 0,1-1 mg/m^3 (WHO, 1996).

A study in Japan described an epidemic outbreak of an encephalitis-like disease in a six members family and ten of their neighbours having similar symptoms. It was caused by an intoxication due to manganese dissolved accidentally in drinking water. Two different chemical analysis of the well waters consumed by all patients showed a concentration of manganese close to 14 mg/l. The source of the manganese was 400 dry-cell batteries buried near a drinking-water well. Sixteen cases of poisoning were reported,

with symptoms including lethargy, increased muscle tone, tremor, and mental disturbances. Two of the severe cases died and one of the moderate cases committed suicide from melancholy. The most severe instances were seen in elderly people, with only minor effects in children. Zinc was the other metal analysed quantitatively at a concentration close to 17 mg/l. However, the clinical observations in this study were typical for subacute manganese poisoning (Kawamura *et al*, 1941).

An epidemiological study in Greece investigated the possible correlation between manganese exposure from water and neurological effects in elderly residents. The levels of manganese were 3,6-14,6 µg/litre in the control area and 82-253 µg/litre and 1800-2300 µg/litre in the test areas. The authors concluded that progressive increases in manganese concentration in drinking-water are associated with progressively higher prevalences of neurological signs of chronic manganese poisoning and manganese concentration in the hair of older persons. However, no data were given on exposure from other sources such as food and dust, and little information was provided on nutritional status and other possible confounding variables (Kondakis *et al*, 1989).

In an area with sewage irrigation, where the manganese content of drinking water was high (0.241-0.346 mg/l) compared to a control area (0.03-0.04 mg/l), the neurobehaviour of pupils aged 11-13, measured by scores of a number of tests, was impaired (He *et al*, 1994). The available abstract of this study does not discuss that also the exposure to other chemicals might have been responsible for the children's neurobehavioral changes.

In cohorts from rural dwellings located in northern Germany exposed to manganese in well water of either 0.3-2.16 or less than 0.05 mg/l, differences in neurological examinations including the assessment of possible Parkinsonism signs could not be detected (Vieregge *et al*, 1995).

In addition to these studies, there are other reports indicating that the intake of manganese by the oral route may be of concern (Velazquez and Du, 1994). Some investigators have reported an association between the elevated hair levels of manganese and learning disabilities in children (Pihl and Parkes, 1977; Barlow and Kapel, 1979; Collipp *et al*, 1983). Gottschalk *et al* (1991) found elevated levels of manganese in jail inmates convicted of violent felonies. Banta and Markesbury (1977) raised the possibility that symptoms of classic manganese poisoning in a 59-year-old male were caused by the patient's consumption of large doses of vitamins and minerals for 4 to 5 years.

In an area of Japan, a manganese concentration of 0,75 mg/litre in the drinking-water supply had no apparent adverse effects on the health of consumers (Suzuki, 1970).

According to a footnote without further details, no signs of toxicity were noticed in patients given 30 mg manganese citrate (9 mg manganese) per day in a mildly alcoholic tonic for many months (Schroeder *et al*, 1966).

A number of sub-populations has been reported to be more susceptible to manganese neurotoxicity than the general population. One group that has received special attention is the very young, because neonates retain a much higher percentage of ingested manganese, presumably as consequence of increased absorption. Other groups of potential concern are elderly people, individuals with iron-deficiency anaemia and people with liver disease (ATSDR, 1997).

4. DOSE RESPONSE ASSESSMENT

The available data clearly show that manganese can cause adverse effects, both in humans and experimental animals. The most important target is the central nervous system. There is clear evidence that exposure to relatively high concentrations of manganese by inhalation results in profound neurotoxic effects in humans.

There are also human studies reporting effects of manganese contained in drinking water. Assuming a consumption of 2 litres of drinking water/day, the cohorts showing the reported effects were exposed to at least 28 mg Mn/day (Kawamura *et al*, 1941), 0.16-0.5 and 3.6-4.4 mg Mn/day (Kondakis *et al*, 1989) and 0.48-0.69 mg Mn/day (He *et al*, 1994), plus the contribution from food. In another study, 0.6-4.3 mg Mn/day from drinking water plus contribution from food showed no effects (Vieregge *et al*, 1995). However, the limitations of these studies including the uncertainty of the contribution from food make firm conclusions difficult.

Similarly, the dose-response relationship of adverse effects in experimental animals has not been

clarified sufficiently. Although the animal data are more extensive, no-observed-adverse-effect levels (NOAELs) for the critical effects cannot be derived. The lowest-adverse-effect-levels (LOAELs) following oral administration observed so far are 0.28 mg/kg bw/day in growing male rats, still producing biochemical and neurological changes in the brain (Chandra and Shukla, 1978), and 0.36 mg/kg bw/day in adult female rats, decreasing their learning ability (Öner and Sentürk, 1995). In rhesus monkeys, 6.9 mg/kg bw/day, given for 18 months to four male animals, caused muscular weakness, rigidity of the lower limbs and marked neuronal degeneration with depigmentation in the region of the substantia nigra (Gupta *et al*, 1980) as well as testicular changes (Murthy *et al*, 1980).

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Exposure to manganese by inhalation is neurotoxic. Oral intake of manganese despite its poor absorption in the gastrointestinal tract has also been shown to cause neurotoxic effects. The limitations of the human data and the non-availability of NOAELs for critical endpoints from animal studies produce a considerable degree of uncertainty. Therefore, an upper level cannot be set.

6. CHARACTERISATION OF RISK

The margin between oral effect levels in humans as well as experimental animals and the estimated intake from food is very low. Given the findings on neurotoxicity and the potential higher susceptibility of some subgroups in the general population, oral exposure to manganese beyond the normally present in food and beverages could represent a risk of adverse health effects without evidence of any health benefit.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF SELENIUM

(EXPRESSED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. NUTRITIONAL BACKGROUND

1.1. Selenium forms in foods

Foods contain a number of different selenium forms. In animal foods, there are specific selenium proteins where selenium is incorporated *via* selenide as selenocysteine, while selenomethionine, and possibly also selenocysteine to some extent, are non-specifically incorporated as analogues to methionine and cysteine in foods both of animal and plant origin. Selenomethionine, as well as the inorganic forms selenite and selenate, are the most common forms in food supplements and fodder additives. Although extensively used for research purposes, it is uncertain if the inorganic forms occur in foods. In addition to these forms a number of uncharacterised forms exist, e.g. in fish (Åkesson and Srikumar, 1994), but their contribution to total dietary selenium is unknown.

Selenium forms used in supplements are inorganic selenite and selenate and organic selenium in the form of selenomethionine, selenocystine and selenium enriched yeast. The forms of selenium found in yeast vary according to production process and the selenomethionine has been suggested to comprise 20 to 50% of the selenium and that some is bound as selenotrisulphides (SCF, 1999).

It should be noted that selenium compounds other than those nutritionally relevant, i.e. those present in food and with a tradition of use as supplements to meet nutritional requirements, are outside the scope of this document. The toxicity and biological properties of such selenium compounds (there are numerous synthetic ones) can be quite different from the nutritionally relevant selenium compounds.

1.2. Selenium intake and selenium status in European countries

The amount of selenium available in the soil for plant growth and corresponding variations in the intake of selenium by humans varies considerably among regions and countries (Gissel-Nielsen *et al*, 1984; Frøslie, 1993). The mean intakes of non-vegetarian adults in different studies are Belgium 28-61 µg/day, Denmark 41-57 µg/day, Finland 100-110 µg/day, France 29-43 µg/day, United Kingdom 63 µg/day, The Netherlands 40-54 µg/day, Norway 28-89 µg/day, Spain 79 µg/day, and Sweden 24-35 µg/day (Alexander and Meltzer, 1995; van Dokkum, 1995; Johansson *et al* 1997).

1.3. Metabolism of selenium

The available data indicate that selenium-containing aminoacids and probably other selenium forms, such as selenite and selenate, can be converted to selenide in mammals (Young *et al*, 1982). Selenide is a central metabolic form of selenium, which is utilised for the formation of selenocysteine, incorporated into specific selenoproteins, and in case of high exposure, into excretory products such as dimethyl selenide (which is exhaled) and trimethylselenonium ions (which are excreted into urine). Selenomethionine and selenocysteine formed by transsulfuration of selenomethionine can be non-specifically incorporated into protein as analogues to methionine and cysteine. Other forms of protein-bound selenium may also occur (Sunde, 1990; Alexander and Meltzer, 1995; Johansson *et al*, 1997).

1.4. Bioavailability of different forms of selenium

Most forms of selenium salts and organic bound selenium, i.e. selenomethionine and selenocysteine, are easily absorbed from the gastrointestinal tract. Only a few studies on the bioavailability of selenium have been performed in humans (Mutanen, 1986; Neve, 1994). Selenium in blood or serum is most

effectively raised by selenium-rich wheat or yeast selenium (the latter may vary in quality), probably because of non-specific incorporation of selenomethionine into proteins. Inorganic selenium as selenate and selenite can be incorporated specifically into selenium proteins via selenide as selenocysteine and increase seleno-enzyme activity until saturation (Levander *et al*, 1983 (Alfthan *et al*, 1991). A few studies have also compared selenium bioavailability from different foods. Van der Torre *et al* (1991) found that supplementation with selenium-rich forms of bread and meat gave similar increases in circulating selenium levels. Christensen *et al* (1983), using a triple stable-isotope method, found that the absorption of selenium from selenite was 36% and that from intrinsically labelled poultry meat was 71%. Selenium consumed from fish had no apparent effect on the amount of selenium incorporated into functional selenoproteins and a low effect on general level of selenium in plasma (Meltzer *et al*, 1993, Åkesson and Srikumar, 1994; Svensson *et al*, 1992; Huang *et al*, 1995). Given different bioavailabilities and differences in non-specific incorporation of selenium compounds from different sources such as cereals, meat, fish and organic and inorganic supplements, the selenium concentration in whole blood will relate differently to the total intake of selenium (Alexander and Meltzer, 1995).

The total body pool of selenium has been estimated to be 5-15 mg in adults. Kinetic studies indicate that blood plasma contains at least four components with half-lives between 1 and 250 hours (Patterson *et al*, 1989).

1.5. Functional forms of selenium – selenoproteins

At least eleven selenoproteins containing the amino acid selenocysteine have been identified in mammals: cellular glutathione peroxidase (cGSHPx), extracellular glutathione peroxidase (eGSHPx), phospholipid hydroperoxide glutathione peroxidase (phGSHPx), gastrointestinal glutathione peroxidase (giGSHPx), iodothyronine deiodinase types I, II and III, prostatic epithelial selenoprotein (PES), selenoprotein P (SeP), selenoprotein W, thioredoxin reductase (Alexander and Meltzer, 1995; Johansson *et al*, 1997).

1.6. Daily requirements

The amount of dietary selenium (as DL-selenomethionine) required to saturate the selenium need of extracellular GSHPx was used as one of the approaches to define a Dietary Reference Intake for Selenium in the USA in 2000 (55 µg/day for adult men and women) (NAS; 2000). A so-called Population Reference Intake of 55 µg selenium per day for adults, but also other levels of intakes based on other criteria, were established by the Scientific Committee for Food of the European Commission (1993).

A joint FAO/IAEA/WHO Expert Consultation (WHO, 1996) gave several modes for the calculation of requirements of the individual and populations. For a 65 kg reference man the average normative requirement of individuals for selenium was estimated to be 26 µg/day, and from this value the lower limit of the need of population mean intakes was estimated to be 40 µg/day. The corresponding values for a 55 kg reference woman were 21 and 30 µg selenium/day, respectively. The latter value was estimated to increase to 39 µg/day throughout pregnancy and to attain the values of 42, 46 and 52 µg selenium/day at 0-3, 3-6 and 6-12 months of lactation, respectively. The Nordic Nutrition Recommendations (1996) have set a recommended intake of 50 µg/day for men, an average requirement of 35 µg/day and a lower limit of needed intake of 20 µg/day, the corresponding values for women being 40, 30 and 20 µg/day, respectively.

The approach to define a biochemical index for the saturation of the functional selenium requirement using a limited number of selenoproteins has given variable results (Nève, 1991). The estimations are also complicated by the fact that different forms of dietary selenium (organic *vs*. inorganic) give variable responses in different measures of selenium status (Alfthan *et al*, 1991) and the physiological relevance of the 'saturation of selenium dependent enzymes approach' can be questioned (Johansson *et al*, 1997).

1.7. Selenium deficiency and selenium in disease states

The most obvious example of a relationship between selenium status and disease is the cardiomyopathy, Keshan disease, that occurs in selenium-deficient areas of China (Xia *et al*, 1994). Prophylactic treatment with selenium supplementation dramatically decreased disease incidence. The disease is not a clear-cut selenium deficiency syndrome since it is not obligatory at very low selenium status and moreover several other factors affect its incidence.

A suspected selenium deficiency syndrome has also been demonstrated in a few patients treated with parenteral nutrition without added selenium (see Rannem *et al*, 1996). Muscular pain and muscular and

cardiac dysfunction has been demonstrated in some patients, but no uniform symptomatology has been described.

In several epidemiological studies the incidence of different diseases, such as cancer and cardiovascular disease, has been related to selenium status. In addition, selenium has been related to immune function, viral infection, reproduction and mood (WHO, 1987; Flohé, 1989; Knekt *et al*, 1990; Virtamo and Huttunen, 1991; Willett *et al*, 1991; Kok *et al*, 1991; Clarke *et al*, 1996, Rayman, 2000).

2. HAZARD IDENTIFICATION AND CHARACTERISATION

2.1. Adverse and toxic effects

2.1.1. Mechanisms of toxicity

The molecular mechanisms of selenium toxicity remain unclear. Several mechanisms have been suggested: redox cycling of auto-oxidisable selenium metabolites, glutathione depletion, protein synthesis inhibition, depletion of S-adenosyl-methionine (cofactor for selenide methylation), general replacement of sulphur and reactions with critical sulphydryl groups of proteins and cofactors (Anundi *et al*, 1984; Hoffman, 1977; Martin, 1973; Stadtman, 1974; Vernie *et al*, 1974). No unifying hypothesis is possible and it is likely that several mechanisms may operate and vary among different selenium compounds. Growth reduction in experimental animals is apparently caused by selective selenium accumulation and toxicity to growth hormone producing cells in the anterior pituitary gland (Thorlacius-Ussing, 1990).

2.1.2. Acute toxicity

Selenite, selenate and selenomethionine are among the most acutely toxic selenium compounds (Högberg and Alexander, 1986). Intake of 250 mg selenium as a single dose or multiple doses of 27-31 mg resulted in acute toxicity with nausea, vomiting, nail changes, dryness of hair, hair loss, tenderness and swelling of fingertips, fatigue, irritability and garlicky breath (Jensen *et al*, 1984; WHO, 1987).

In Sweden, several cases of toxicity in children occur each year due to accidental overconsumption of selenium tablets. Acute symptoms such as vomiting have been observed, but so far no serious cases of toxicity have been recorded (Johansson *et al*, 1997).

2.2. Chronic toxicity

2.2.1. Animal toxicity data

Animals show growth reduction, liver changes, anaemia, pancreatic enlargement and some domestic animals also exhibit neurotoxicity following selenium exposure above 0.03-0.4 mg/kg bw (Alexander and Meltzer, 1995).

2.2.1.1. Cancer studies

Several early studies showed tumours following selenium exposure (Nelson *et al*, 1943; Volgarev and Tscherkes, 1967; Innes *et al*, 1969; Schroeder and Mitchener, 1971a; Schroeder and Mitchener, 1972; Schrauzer and Ishmael, 1974; IARC, 1975; US EPA, 1980). These studies have been evaluated on several occasions and, in general, the data have been considered inconclusive due to many problems with the studies (Diplock, 1984). Nelson gave low-protein diets supplemented with seleniferous wheat or 10 mg selenium salts/kg bw. A number of rats died before 18 months, but none with tumours. In surviving rats hepatic tumours were found in animals with liver cirrhosis. It has also been questioned whether identified tumours were actually regeneration nodules. The study of Volgarev and Tscherkes (1967) lacked adequate controls. Also the study by Schroeder and Mitchener (1971) lacked adequate controls, and the colony suffered from severe infections.

Synthetic selenium compounds that have shown effects indicative of carcinogenicity are as follows. Selenium diethyldithiocarbamate given to mice (10 mg/kg by gavage daily for three weeks) was found to increase the incidence of hepatomas, lymphomas and pulmonary tumours (Innes *et al*, 1969). Seifter *et al* (1946) gave 0.05% of bis-amino-phenyl selenium dihydroxide in the diet to rats and found an increased incidence of adenomatous hepatic hyperplasia and thyroid adenomas. Selenium sulphide in large oral doses (3 and 15 mg/kg bw/day to rats and 20 and 100 mg/kg bw/day to mice) was found to be carcinogenic to rats and mice (NCI, 1980a). In a separate study in mice, selenium sulphide was applied to the skin and there was no increased incidence of tumours attributable to selenium treatment (NCI, 1980b); under most conditions the systemic uptake of topically selenium sulphide might be

insignificant (Cummins and Kimura, 1971). Carcinogenicity of selenium compounds seems primarily to be associated with the nature of the compound than with the element itself. The selenium compounds mentioned above are not used as sources of selenium in food, nor as nutrients.

2.2.1.2. Reproductive effects

It is well established that several selenium compounds such as selenate, selenite, selenocysteine and in particular selenomethionine are teratogens in avian species and in fish (Franke *et al*, 1936; Moxon and Rhian, 1943; Halverson *et al*, 1965; Palmer *et al*, 1973; Dostal *et al*, 1979; Birge *et al*, 1983; Heinz *et al*, 1987; Woock *et al*, 1987; Hoffman *et al*, 1988; Pyron and Beitinger, 1989). Both inorganic and organic forms of selenium cross the placenta in humans and experimental animals (Willhite *et al*, 1990). Terata have also been produced in sheep (Holmberg and Ferm, 1969) and pigs (Wahlström and Olson, 1959). Effects of selenium compounds on reproduction and offspring in rodents have usually been associated with overt maternal poisoning and nutritional deprivation (Schroeder and Mitchener, 1971b; Berschneider *et al*, 1977; Nobunaga *et al*, 1979; Ferm *et al*, 1990). Recent studies on macaques fed selenomethionine (25, 150 and 300 µg/kg bw/day) during organogenesis showed no signs of terata (Tarantal *et al*, 1991). A dose-dependent maternal toxicity was observed in this study. Whereas no signs of treatment related toxicity in the dams were observed at the dose of 25 µg/kg bw/day (NOAEL), maternal toxicity as indicated by poor appetite and emesis was observed in the mid- and high-dose groups. No treatment-related changes in the teeth, skin or nails were found. No indication of teratogenicity of selenium has been shown in humans even in the areas of high selenium intake in China (Yang *et al*, 1989b).

2.2.1.3. Genotoxic effects

A moderate genotoxic activity of selenium compounds (i.e., selenite, selenate, selenide, selenocysteine and selenosulphide) has been found in several *in vitro* systems (Löfroth and Ames, 1978; Ray and Altenburg, 1978; Noda *et al*, 1979; Whiting *et al*, 1980; Ray, 1984; Tennant *et al*, 1987; Kramer and Ames, 1988). There is one *in vivo* study showing chromosomal aberrations and increased SCE in hamster bone marrow cells after selenite treatment (Norppa *et al*, 1980). This occurred only at doses of 3, 4, and 6 mg Se/kg bw i.p. that were associated with severe systemic toxicity, including lethality, some hours after dosing. The numbers of aberrations in these groups were 13-55%, compared to 0.9-1% in the controls. Doses of 0.3, 0.6, 1 and 2 mg Se/kg bw did not cause any clastogenic effects. A non-pregnant macaque dosed with 600 µg selenomethionine for 15 days (lethal dose) showed in comparison with the control animal a sevenfold increase in bone marrow micronuclei (Choy *et al*, 1989). In pregnant macaques receiving 0, 150 or 300 µg selenomethionine/kg bw and showing signs of selenosis, foetal bone marrow smears did not show any increase in the number of micronuclei (Choy *et al*, 1993).

In vitro studies indicate that the mutagenic effects of selenium salts are associated with production of reactive oxygen radicals and that glutathione promotes these reactions (Kramer and Ames, 1988). It is well known that auto-oxidisable selenium metabolites, such as hydrogen selenide, can undergo redox cycling producing oxygen radicals and cause DNA strand breaks (Anundi *et al*, 1984; Nuttall and Allen, 1984; Garberg *et al*, 1988). Detoxification of selenide by methylation is saturable depending on the supply of methyl donors. *In vivo*, only toxic amounts were shown to be active, keeping in mind the central role of hydrogen selenide in the metabolism of most selenium compound it is likely that overproduction of this and other auto-oxidisable selenium metabolites could promote the formation of DNA reactive oxygen radicals. It is possible that glutathione might play a central role as well. It follows, given such a mechanism, that expression of selenium dependent genotoxic activity is likely to be concentration- and threshold-dependent, but this remains to be shown (Högberg and Alexander, 1986).

2.2.2. Human toxicity data

2.2.2.1. Exposure to supplements

Two individuals took selenium-containing yeast at doses of 200 and 400 µg selenium daily for 18 months. Together with dietary intake they received about 350 and 600 µg/day. Marginal haematological changes and a borderline increase in serum ALAT (alanine amino transferase) were seen (Schrauzer and White, 1978).

A small group of patients with rheumatoid arthritis receiving 250 µg Se as organic selenium in addition to selenium from food for 6 months had decreased levels of somatomedin C in serum in comparison with a group receiving placebo (Thorlacius-Ussing *et al*, 1988). A similar effect was not observed when graded doses of 100, 200 and 300 µg selenium as selenium wheat was given to healthy, Norwegian

volunteers for a six week period (Meltzer *et al,* 1993), nor was the effect observed in North Americans with a natural selenium intake range of 68-724 µg/day (Salbe *et al*, 1993).

In a study by van Dokkum *et al* (1992) two groups of 6 male volunteers were given 8 slices of bread per day for six weeks. The bread was made with selenium-rich and -poor wheat. In the treatment group the bread provided 200 µg selenium/day per subject. In a study by Longnecker *et al* (1993), groups of 4 healthy male volunteers were fed bread containing 32.4, 206 or 388 µg selenium/day. Prior to the study the intake was 80 µg/day. In both studies no adverse effects were reported, although such information was not specifically sought.

In a supplementation study where 400 µg/day of selenium as selenite or selenomethionine (total dose 450-500 µg/day) were given for 3 months to 32 healthy women, half of them experienced symptoms of depression and extreme tiredness during the month following the termination of the study (Meltzer, 1995).

In a randomised, double blind, placebo-controlled study, the effect of selenium supplementation on prevention of skin cancer was investigated (Clark *et al*, 1996). A total of 1312 patients (mean age 63, range 18-80) with a history of basal cell or squamous cell carcinoma were treated with 200 µg selenium/day in the form of high-selenium brewer's yeast tablet (Nutrition 21, La Jolla, Calif.) or placebo for up to ten years (mean 4.5 years). The percentage of males in the control and treatment groups was 75.6 and 73.8, respectively. Mean plasma selenium concentration at the start of the study was 114 µg/l (1.44 µmol/l), which was in the lower end of the range of normal plasma levels reported in the US (in most European countries, however, the mean serum levels are lower (Alexander and Meltzer, 1995)). Plasma selenium levels remained constant throughout the study in the placebo group, while plasma selenium rose to 190 µg/l (2.4 µmol/l) in the treatment group within 6-9 months from the beginning. The safety endpoints investigated included known signs of frank selenosis (see below), including garlic breath, pathological nail changes and brittle hair. Patients were assessed every 6 months and the authors observed no dermatological or other signs of selenium toxicity. A total of 35 patients upset, 21 in the selenium group and 14 in the control group, complained about adverse effects, mostly gastrointestinal, which resulted in withdrawal from the study.

Although it is difficult to assess the intake based on serum values, as these might vary according to the source of selenium, an estimate can be that a mean intake of approximately 90 µg selenium/day would correspond to a serum value of 114 µg/l (1.44 µmol/l) (Alexander and Meltzer, 1995). Hence, the total intake after supplementation would be approximately 290 µg selenium/day.

2.2.2.2. Long term exposure, epidemiological studies

Health effects of high dietary intakes of selenium have been investigated in selenium-rich areas of South Dakota, USA (Smith and Westfall, 1937). The most common symptoms were gastrointestinal disturbances, icteroid discoloration of the skin, and decayed and bad teeth. It is difficult to evaluate the exposure levels and validity of these findings (WHO, 1987). Children living in a seleniferous area in Venezuela have been compared to children living in Caracas (Jaffe, 1976). The level of selenium in blood averaged 813 µg/l (10.3 µmol/l) in the seleniferous area, and in one child reached 1,800 µg/l (22.8 µmol/l). Using the Chinese data on blood/intake relationships (Yang *et al*, 1989a), a level of 813 µg/l (10.3 µmol/l) corresponds to a daily intake of about 10 µg Se/kg bw. It was found that pathological nail changes, loss of hair and dermatitis were more common in the seleniferous area. However, whether these differences were due to selenium toxicity was not entirely clear, as the groups differed in several other nutritional aspects.

Clinical symptoms associated with selenium poisoning such as those described above are usually referred to as selenosis. A more detailed description of symptoms is given below.

In China, endemic selenium intoxications due to high selenium in soil have been studied by Yang and colleagues (Yang *et al*, 1983). Morbidity was 49% among 248 inhabitants of five villages with a daily intake of about 5,000 µg selenium. The main symptoms were brittle hair with intact follicles, new hair with no pigment, and thickened nails as well as brittle nails with spots and longitudinal streaks on the surface. In more severe cases fluid effused from around the nail bed. Another common finding was lesions of the skin, mainly on the backs of hands and feet, the outer side of the legs, the forearms, and the neck. Affected skin became red and swollen, followed by the appearance of blisters and the occurrence of eruptions. Symptoms of neurological disturbances were observed in 18 of the 22 inhabitants of one heavily affected village alone. Patients complained of peripheral anaesthesia, acroparaesthesia, pain, and hyperreflexia. At a later stage numbness, convulsions, paralysis, and motor

disturbances developed. The daily intake among those with clinical signs of selenosis was estimated to range from 3,200 to 6,690 µg, with an average of 4,990 µg selenium. The mean blood level was 3,200 µg/l (40.5 µmol/l), and the mean urine level was 2,680 µg/l (33.9 µmol/l). Livestock were also affected in these areas. The residents recovered as soon as the diets were changed. In high selenium areas without occurrence of selenosis the daily intake of selenium was calculated to range from 240 to 1510 µg, with a mean intake of 750 µg. The corresponding blood levels were 440 (350-580) µg/l (5.6 (4.4-7.3) µmol/l) (mean and SD). The chemical forms of selenium were determined in Chinese rice and maize and the major form was selenomethionine (Beilstein *et al*, 1991).

In a follow up to their earlier work, Yang *et al* (1989a, 1989b) studied a population of about 400 individuals which was evaluated for clinical and biochemical signs of selenium toxicity. A detailed study of selenium intake *via* various food items as well as measurements of selenium in tissues, i.e., whole blood, urine, hair and finger- and toe-nails, allowed more accurate estimation of the dose-response relationships observed for selenium toxicity.

The average daily intakes based on lifetime exposures were 70, 195 and 1438 µg, and 62, 198 and 1288 µg for adult males and females, respectively, in the low-, medium- and high-selenium areas. Clinical signs of selenosis (i.e., hair or nail loss, nail abnormalities, mottled teeth, skin lesions and changes in peripheral nerves) were examined among 349 adult residents and were observed among subjects in the high selenium area. Subjects with clinical signs of selenosis were classified as ++ or + (mainly finger-nail disease/changes alone and with severe hair loss/skin changes). No clinical signs were observed among those with a blood selenium concentration below 1000 µg/l (12.7 µmol/l) (intake according to regression equation, figure 1 Yang *et al* 1989a: 853 µg Se/day). The prevalences of subjects with selenosis ++ varied between 3-7% in the groups with blood concentrations 1000-1250, 1250-1500 and 1500-2000 µg/l (12.7-15.8, 15.8-19.0, 19.0-25.3 µmol/l). The prevalences of subjects with selenosis + varied from 10-35% in the same groups. No dose response relationships were seen. The prevalence of subjects with selenosis + was 45% in subjects with a blood concentration above 2000 µg/l (25.3 µmol/l). (All prevalence figures were taken from figure 4 of Yang *et al*, 1989b.) Blood selenium concentrations in five subjects with long persistent clinical signs ranged from 1054 µg/l to 1854 µg/l (13.3 to 23.5 µmol/l) with a mean of 1346 µg/l (17.0 µmol/l), corresponding to a daily intake of 1260 µg Se (range: 913-1907 µg Se) (intake calculated from the regression equation). Prolonged bleeding time was observed clinically upon blood collection in the high selenium areas. Prolonged prothrombin time (>14 sec.) was observed among 1 of 20 subjects with a blood selenium of 200 to 990 µg/l (2.53-12.5 µmol/l) and among 45% of those subjects with a blood selenium above 1000 µg/l (12.7 µmol/l) (corresponding to an intake of about 850 µg). The mean prothrombin time only increased marginally, but ranges were not given in the publication. A strong reduction in the plasma-Se/red cell-Se was seen at blood concentrations exceeding 900 µg Se/l (11.4 µmol/l), corresponding to an intake of 750 µg/day. A decreased concentration of glutathione in blood was observed at dietary intakes exceeding 850 µg/day. The prevalence of mottled enamel teeth of school children of 7-14 years of age were 0, 49 and 95% in groups with low (130 ± 20 µg/l (1.65 ± 0.25 µmol/l)), medium (370 ± 320 µg/l (4.68 ± 4.05 µmol/l)) and high (1570 ± 440 µg/l (19.9 ± 5.57 µmol/l)) blood selenium concentrations, respectively.

The five patients showing overt signs of selenosis were followed up in a later study (Yang and Zhou, 1994). The symptoms disappeared after a change in the diet. Their blood levels decreased from 1346 ± 366 to 968 ± 115 µg Se/l (17.0 ± 4.63 to 12.3 ± 1.46 µmol/l), corresponding to an intake of 1270 ± 450 to 819 ± 126 µg Se/day (calculated from the regression equation) (819 µg corresponds to about 15 µg/kg bw, according to the authors). The latter mean value had a lower 95% confidence limit of 567 µg per day. The range was 654 to 952 µg Se/day.

In a study (Longnecker *et al*, 1991), 142 subjects from geographical areas with high dietary selenium intakes in USA were followed over a 2-year period with respect to adverse health effects. The daily dietary intake was assessed by several 48 h duplicate-plate food collections for selenium determination from one person in the household and by diet questionnaires. The subjects were followed for one year, completed health questionnaires, and underwent physical examinations and clinical chemistry tests. Selenium in whole blood, serum, urine and toenails were also determined.

The average selenium intake was 239 µg/day, varying from 68 to 724 µg/day; half of them had an intake above 200 µg/day and 12 individuals above 400 µg/day. There was no variation in the prothrombin time with selenium intake. However, an association of selenium intake with alanine aminotransferase (ALAT) in serum was observed. The values were within the reference range and considered clinically insignificant. Increased prevalence of lethargy was also seen with increased selenium values. Nail abnormalities were not related to selenium intake, neither were any other symptoms or physical findings. In contrast

to a study from Denmark (Thorlacius-Ussing *et al*, 1988) (see above), no relationship between plasma somatomedin C and any of the selenium indices was observed in this study (Longnecker *et al*, 1991; Salbe *et al*, 1993).

Brätter and Negreti de Brätter (1996) studied the influence of high dietary selenium intake on the thyroid hormone levels in serum of 125 lactating mothers 20-24 days post partum from three regions with different selenium intake in Venezuela. The serum concentration of FT_3 (free, unbound T_3) (T_3 is formed from T_4 in the liver) was lower in the group having the highest mean intake, 552 (range: 250-980) μg/day, but all values were found to be within the reference range. The two other regions had mean intakes of 274 (range 170-500) and 205 (range: 90-350) μg Se/day, respectively. None of the investigated individuals had signs of selenosis.

3. DOSE RESPONSE ASSESSMENT

Acute and chronic selenium toxicity have been demonstrated in a wide variety of animals and human. Soluble selenium salts and selenomethionine show approximately similar toxicity and there is no substantial variation between animal species. Soluble selenium salts (in cases of supplementation and selenium from drinking water) may be acutely more toxic than organic bound selenium from food. On the other hand, organic forms may be more toxic during long-term consumption due to incorporation into proteins rather than excretion.

Except for some selenium compounds not used in food, i.e. selenium sulphide, selenium diethyldithiocarbamate, bis-amino-phenyl selenium dihydroxide, experimental data do not indicate that inorganic selenium salts or organic selenium compounds relevant in food and nutrition are carcinogenic. Adequate human data do not exist.

Genotoxicity has been seen in a number of *in vitro* systems and also *in vivo* at toxic doses. It is likely, however, that these effects may be related to the generation of reactive oxygen radicals, being dose dependent and showing a threshold *in vivo* and not occurring at nutritionally adequate intakes.

There is no evidence for teratogenicity neither in humans nor in macaques fed selenomethionine.

Except for the early studies of the population in seleniferous areas in USA (Smith and Westfall, 1937), the more recent Chinese studies of endemic selenium toxicity in humans (Yang *et al*, 1983; Yang *et al*, 1989a; Yang *et al*, 1989b; Yang and Zhou, 1994) and the 1991 American study (Longnecker *et al*, 1991), there are only anecdotal reports on chronic selenium toxicity in humans.

Based on the Chinese studies (Yang *et al*, 1989a; Yang *et al*, 1989b; Yang and Zhou, 1994), the minimum daily dietary intake sufficient to cause symptoms of selenosis (i.e., hair or nail loss, nail abnormalities, mottled teeth, skin lesions and changes in peripheral nerves) is about 1200 μg Se (range: 913-1907 μg Se). The LOAEL for clinical symptoms of selenosis is about 900-1000 μg Se/day. No clinical signs of selenosis were recorded in individuals with blood selenium below 1000 μg/l, corresponding to an intake of about 850 μg/day, which could be taken as a NOAEL for clinical selenosis. Symptoms were also observed in a man taking 913 μg Se/day as selenite (Yang *et al*, 1983). In the follow up study (Yang and Zhou, 1994) of 5 patients (from the study of 349 individuals) recovered from selenosis when their mean intake was reduced to 819 μg Se/day, the 95% lower confidence limit of the mean intake was 567 μg/day).

Symptoms from the liver, which is also affected in animal studies, manifested in increased prothrombin time due to impaired synthesis of coagulation factors in the liver, became statistically significant at dietary intakes at and above 850 μg Se/day (Yang *et al*, 1989b). In the American study (Longnecker *et al*, 1991) no signs of toxicity were seen in a population consuming on average 239 μg Se/day from food. The liver enzyme ALAT in serum, although within the reference range, showed a correlation with selenium intake (68 to 724 μg/day), but this was not considered to be clinically significant. No effect on prothrombin time was seen in the latter study. However, the American population studied covered a lower range of selenium intake than the Chinese study and, considering mean body weights, it is also likely that the intake per kg bw was greater in the Chinese study, in comparison with the American one.

Taken together, increased prothrombin time (in the Chinese study) and slight ALAT increase (in the American study) are both signs of subclinical/biochemical liver effects. The clinical relevance is uncertain.

4. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Taking all this information into account, the Committee decided to derive the UL using the NOAEL of 850 μg/day for clinical selenosis in the study on 349 subjects of Yang *et al* (1989b). In support of this NOAEL is the follow up study of Yang and Zhou (1994) of the 5 individuals who recovered from selenosis when their intake had been reduced to a mean of 819 μg Se/day. The NOAEL used was derived from a study on a large number of subjects and is expected to include sensitive individuals. It was decided to use an uncertainty factor of 3 to allow for the remaining uncertainties of the studies used in deriving an upper level.

An UL of 300 μg Se/day was derived for adults. This value covers selenium intake from all sources of food, including supplements.

The supplementation study by Clark *et al* (1996), who did not observe any signs of selenosis in the supplemented group (selenium enriched yeast), having an estimated mean total intake of about 300 μg selenium/day, the American study (Longnecker *et al*, 1991) and the study of lactating women from Venezuela (Brätter and Negreti de Brätter, 1996) further support this UL.

No data are available to suggest that other life-stage groups have increased susceptibilityto adverse effects of high selenium intake. Therefore the UL of 300 `μg per day should be considered to apply also to pregnant and lactating women.

There are no reports of adverse effects on infants born from mothers with high intakes of selenium or adverse effects on lactating women with dietary selenium intakes below the UL for adults. Therefore, the UL for pregnant and lactating women is the same as for non pregnant and non-lactating women.

There are no data to support a derivation of an UL for children. The data on mottled enamel do not allow a NOAEL to be set. On the other hand, there are no reports indicating that children are more susceptible to adverse effects from selenium. Hence, it seems appropriate to extrapolate the UL from adults to children on a body weight basis. The reference weights derived by the Scientific Committee on Food (SCF, 1993) are used as a basis for the calculations.

Age (years)	UL (μg selenium/day)
1-3	60
4-6	90
7-10	130
11-14	200
15-17	250

Some selenium compounds were reviewed in the context of "Substances for nutritional purposes which have been proposed for use in the manufacture of foods for particular nutritional purposes" by the SCF (SCF, 1999). The Committee found sodium selenate, sodium selenite and sodium hydrogen selenite acceptable for use in food for particular nutritional uses, but did not find other forms of selenium acceptable on the basis of current data. Therefore, the UL of this report relate only to the selenium compounds found acceptable and, in addition, to selenium naturally present in food.

5. CHARACTERISATION OF RISK

Based on the information on selenium toxicity, there are areas in the world where there is a human intake of selenium with no or only very small safety margins to levels where toxicity may occur. However, in most European countries the mean intake levels are much lower, in the lower range of 30-90 μg Se/day, except for Norway, that has a somewhat higher mean intake (60 μg Se/day) due to import of wheat rich in selenium. Finland has an intake of 100-110 μg Se/day because of selenium fertilisation. The margin between the present mean intake, excluding supplements, in the European population and an UL (adult) of 300 μg Se/day would be between 2.7 to 10. The 97.5 percentile intake was 81 and 90 μg Se/day in Italy and The Netherlands, respectively, giving a margin to the UL of about 2.7.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF MOLYBDENUM

(EXPRESSED ON 19 OCTOBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Molybdenum (Mo) is widely distributed in nature, the crustal abundance being 1.5 mg Mo/kg. Molybdenite (MoS_2) is the major source for industrial production of molybdenum compounds. It is used in the manufacture of high strength steel, in electrical equipment, in catalysts and molybdenum pigments. Mo compounds are also used in agriculture for direct seed treatment or in fertiliser formulations (Patty's, 1981; WHO, 1996b).

Mo exists in several valency states, e.g. $Mo^{II}O$, $Mo^{IV}S_2$, $Mo^{VI}O_3$, and as the stable salts $(NH_4)_2Mo^{VI}O_4$ (ammonium molybdate), $(NH_4)_6Mo^{VI}_7O_{24}.4H_2O$ (ammonium molybdate tetrahydrate) and $Na_2Mo^{VI}O_4.2H_2O$ (sodium molybdate dihydrate). These latter salts are used in food preparations for special medical purposes (IDACE, 1995).

Mo is ubiquitous in food and water as soluble molybdates. Mo-containing enzymes are found in many plants and animal organisms. In plants and lower organisms these enzymes are involved in the bacterial fixation of N_2, in the conversion of NO_3 to NH_3, in protein synthesis and in some redox reactions. In human and animal tissues the enzymes xanthine dehydrogenase (XD)/oxidase (XO), aldehyde oxidase (AO) and sulfite oxidase (SO) require molybdopterin as cofactor and part of the enzyme molecule. In molybdopterin Mo is bound by two S atoms to the pterin. The redox potential of Mo^V/Mo^{VI} is appropriate for the electron exchange with flavinmononucleotides. Mo is therefore an essential component of flavin- and Fe-containing enzymes (WHO, 1996a).

This evaluation covers those forms of Mo which are found naturally in food and water, as well as soluble molybdates added to foods.

2. NUTRITIONAL BACKGROUND

2.1. Levels in food

Good food sources of Mo are sorghum, leafy vegetables (levels depending on soil content, those grown on neutral or alkaline soil are rich in Mo, those grown on leached acid soil are Mo deficient (WHO, 1996), legumes (beans), grains (cereals, wheat germ), organ meats (liver, kidney), milk and eggs (Rajagopalan, 1987; SCF, 1993). Some 40% of Mo in cereals is lost on milling (Rajagopalan, 1987; SCF, 1993). Fruits, root vegetables, and muscle meat are poor sources (SCF, 1993). High concentrations have been found in shellfish. Soft tissue of fish contain about 1 mg Mo/kg, vascular plants 0.03-5 mg Mo/kg (Patty's, 1981). Mo levels in drinking water range from 0-68 µg/L, but usually do not exceed 10 µg/L (WHO, 1996b).

2.2. Intake estimates

Estimates of daily intake vary widely regionally depending on the soil type. For adults, the representative range of mean estimates of Mo intakes in different countries is 80-250 µg/day and analysis of representative total diets from 11 countries yields an average adult intake of approximately 100 µg/day (WHO, 1996). Intakes for breast fed infants (aged 0-3 m) vary, typically, from 0.1-0.5 µg/kg bw/day, children (from weaning to 3 years) from 5-7 µg/kg bw/day, and adolescents/adults from 1.5-2.5 µg/kg bw/day (WHO, 1996a). In mining areas with contaminated drinking water (levels up to 400 µg/L) intakes from food plus 2 L water can reach about 1000 µg Mo/day.

Estimates of Mo intake in USA range from 44-460 µg/day, in The Netherlands from 48-96 µg/day, in Sweden from 44-260 µg/day, in the UK 50-400 µg/day (mean 128 µg/day), in Germany 60-500 µg/day, and in Finland 120-150 µg/day (SCF, 1993; SCF, 1998). Other data quote for the USA 120-240 µg/day, of which cereals supply 30-40%, legumes 5-20% and to which potable water contributes up to 20 µg/L (Rajagopalan, 1987).

2.3. Nutritional requirements

There are no reliable estimates of human requirements for Mo and no recommended intake has been established by the EC other than that current intakes appear to be adequate and safe (SCF, 1993). The US Food and Nutrition Board (FNB, 1989) set a provisional estimated range of safe and adequate daily dietary intakes for Mo of 75-250 µg for adults and older children, based on average reported intakes. The range for other age groups was derived through extrapolation on the basis of body weight, i.e. 15-30 µg for infants 0-0.5 yr, 20-40 µg for infants 0.5-1 yr, and 25-50, 30-75 and 50-150 µg for children aged 1-3, 4-6 and 7-10 yr, respectively (FNB, 1989). WHO (1996a) has estimated (tentatively) that adult human basal requirement for Mo could be approximately 25 µg/day, corresponding to approximately 0.4 µg/kg bw.

Attention must be paid to the known antagonism between Mo, Cu and sulphate noted particularly in animals.

2.4. Molybdenum deficiency

Mo deficiency in humans is unknown under normal dietary conditions. Intakes of 25-50 µg Mo/day were reported to cause no clinical signs of Mo-deficiency but were associated with biochemical changes suggestive of functional deficiency of XO activity, e.g. a doubling of xanthine excretion, a 20% decrease in uric acid excretion after purine load. Decrease in AO activity was noted because of nicotinamide metabolism abnormalities.

A human syndrome suggestive of Mo deficiency occurs in prolonged total parenteral feeding in association with intolerance of cysteine and methionine, manifested by irritability, tachycardia, tachypnoea, nightblindness, encephalopathies and coma (Abumrad *et al*, 1984; SCF, 1993; SCF, 1998). Biochemical indicators are low tissue SO and XO, raised plasma methionine, reduced plasma uric acid, high excretion of thiosulphate, xanthine and hypoxanthine, low excretion of inorganic sulphate. Treatment requires reduction of protein intake especially S-containing aminoacids. Clinical symptoms were totally eliminated by administration of 300 µg ammonium molybdate/day (equivalent to 147 µg Mo/day). A similar symptom complex is seen in the short-bowel syndrome and after ileal resection for Crohn's disease with faecal loss of 350-530 µg Mo/day, requiring 500 µg Mo parenterally/day for correction (IDACE, 1995; WHO, 1996a; Vyskocil and Viau, 1999).

Human Mo deficiency is seen also in a rare autosomal recessive syndrome in infants, where there is a defective hepatic synthesis of Mo-pterin cofactor. This disease is associated with abnormal faeces, feeding difficulties, neurological and developmental abnormalities, mental retardation, encephalopathy and ectopy of the lens. The urinary levels of sulphite, thiosulphate and S-sulpho-L-cysteine are increased and urinary sulphate levels decreased. Death occurs by age 3. This condition is not ameliorated by dietary Mo supplementation because it is the result of a defective gene (SCF, 1993).

Low intakes of Mo have been claimed to be associated with oesophageal cancer in the Transkei and in Henan (China), where low serum, hair and urine Mo levels had been found (WHO, 1996a). Keshan disease (myocardial defects associated with Se deficiency) may be linked to low cereal and drinking water levels of Mo, as the incidence was reduced by using Mo fertilisers. However, high Mo levels in rice, wheat and soya combined with high tissue and hair levels were found in some Keshan endemic areas.

Goats kept on 24 µg Mo/kg dry matter in their feed developed Mo deficiency symptoms characterised by reduced conception rate, increased abortion rate, increased mortality of dams and offspring (WHO, 1996a).

2.5. Functions of molybdenum-containing enzymes

Xanthine dehydrogenase (XD) converts tissue purines, pyrimidines, pteridins and pyridins by oxidative hydroxylation to uric acid as an irreversible process. Its normal action is that of a dehydrogenase, but when reacting with O_2, during proteolysis, freezing/thawing or in the presence of reactive -SH reagents it changes into Xanthine oxidase (XO), which produces free radicals of oxygen known to be involved in

tissue damage following physical injury, reperfusion, injury by toxins or Mo excess. Avian XD is stable, hence birds excrete uric acid. Allopurinol oxidises metabolically to alloxanthine, which inhibits XD.

Reduced XD activity is associated with xanthinuria, low urinary uric acid, high blood xanthine levels, high urinary and blood hypoxanthine levels, renal calculi and depositions in muscles with myopathy. Low Mo intake reduces tissue XD activity, however the intake variations from normal diet are insufficient to exert an effect on XD activity, which can cause overt clinical changes. Similarly, a change in the plasma ratio [xanthine + hypoxanthine]/[uric acid] is too unspecific for diagnosing Mo deficiency. Low XD activity can also be due to low protein intake or hepatoma, while high XD activity can be due to high protein intake, low vitamin E status, administration of interferon or administration of agents stimulating interferon release. It is not known whether high Mo intake stimulates tissue XD activity (Rajagopalan, 1987; WHO, 1996a).

Aldehyde oxidase is structurally and chemically similar to XO, has a similar tissue distribution and shares some substrates, e.g.: aldehydes, substituted pyridines, pyrimidines, quinolines and purine derivatives. Its principal metabolic role is unknown (Rajagopalan, 1987).

Sulphite oxidase (SO) is a haem-containing molybdoprotein located in the intermembraneous space of mitochondria. SO converts sulphite to sulphate. Sulphite derives metabolically from S-amino acids, e.g. cysteine, methionine. SO occurs in the liver of man and other species (WHO, 1996a).

2.6. Kinetics and metabolism in laboratory animals

The rate of gastrointestinal absorption of Mo depends on its chemical nature and the animal species. Ingested Mo^{VI} but not Mo^{IV} is readily absorbed from the duodenum and proximal jejunum. Water-soluble molybdates, thiomolybdates and oxothiomolybdates and Mo in herbage and green vegetables are absorbed to 75-97% by laboratory animals and ruminants. Insoluble MoS_2 is not absorbed, Mo^{IV} compounds are not readily absorbed. Intestinal absorption is inhibited by high intraluminal sulphate concentrations, probably because of competition for the common carrier. Silicates also inhibit the absorption of dietary molybdates.

Absorbed Mo rapidly appears in the blood loosely attached to the erythrocytes, specifically bound to α2-macroglobulins (IDACE, 1995). In rodents it is distributed mainly to the liver, converted to molybdate and 36-90% of the total dose is excreted in the urine, less than 1% in the bile and only some in the faeces (IDACE, 1995). In rabbits and guinea pigs Mo is deposited in the tissues within 4 hours after initial high blood and bile levels and eliminated within 72 hours by the kidneys. In horses, cattle and sheep faecal elimination is about half the urinary elimination because of limited absorption. Some bone storage was noted (Patty's, 1981). Mo crosses the placenta. Sulphate reduces the utilisation of Mo by some tissues and increases the urinary Mo excretion (Patty's, 1981; WHO, 1996a). Mo is reabsorbed by the renal tubules but this reabsorption is reduced by S-containing and by acid proteins. The reabsorbed Mo deposits in liver, lung, bone and skin. It is responsible for F storage and aids retention of F in the bone of old rats as well as decreasing caries in rats (Patty's, 1981; Casarett, 1975). Small amounts of Mo increase antibody formation, e.g. agglutinins (Patty's, 1981).

^{99}Mo injected into dogs was concentrated in liver, kidney, pancreas, pituitary, thyroid and adrenals but none appeared in brain, white marrow or fat (Patty's, 1981). The biological half-life varies from a few hours to several days in small laboratory animals and is related to the Cu and S metabolism.

2.7. Kinetics and metabolism in humans

Water-soluble Mo compounds and Mo in herbage and green vegetables are absorbed by man from 40-50% (WHO, 1996a). The absorption rate from drinking water may be the same as from food. Twenty five percent of absorbed Mo appears rapidly in the blood loosely attached to the erythrocytes, specifically bound to α2-macroglobulins (IDACE, 1995), normal blood levels being 2-6 µg/L whole blood or 0.55 µg/L serum. In man, the highest levels appear in kidney, liver and bone, raised levels appear also in adrenals, fat and omentum. There is no bioaccumulation, tissue levels rapidly returning to normal once exposure stops. Increased exposure at the work place or through drinking water is balanced by increased urinary excretion.

16-27% of i.v. administered ^{99}Mo to man was excreted in 5 days in the urine. Faecal excretion over 10 days was 1-7%. Mo was rapidly cleared from the blood within 24 hours (Patty's, 1981).

Data on the Mo status of normal tissues are unreliable. Quoted blood and serum levels vary by 4 orders of magnitude. Serum levels of Mo rise in liver functional defects, hepatitis, hepatic tumours and after certain drugs. Raised blood levels are seen in uraemia, rheumatic disorders and CVS disease. Human liver contains 1.3-2.9 mg Mo/kg dry matter, kidney 1.6 mg/kg dry matter, lung 0.15 mg/kg dry matter, brain and muscle 0.14 mg/kg dry matter, hair 0.07-0.16 mg/kg (WHO, 1996a).

2.8. Relationship between molybdenum, copper and sulphate

The relationship between Mo, Cu and sulphate is complex and varies with the species considered (Mills and Fell, 1960; Arthur, 1965; Huber *et al*, 1971; Casarett and Doull, 1975; Nishioka, 1975; Patty's, 1981).

3. HAZARD IDENTIFICATION

The insoluble compounds MoS_2, MoO_2, and Mo metal are less toxic than the more soluble molybdates. The oral LD_{50} for molybdates in rats lies between 101-330 mg Mo/kg bw (Patty's, 1981). The lethal repeated oral dose for mouse, guinea pig and rabbit lies between 60-330 mg Mo/kg bw (Mills and Davis, 1987). No syndrome of industrial toxicity due to handling of molybdenum compounds is known in humans, but the chronic inhalation of 4 mg Mo/m^3 as MoO_3 for 4 years was associated with pneumoconiosis (Casarett and Doull, 1975; Vyskocil and Viau, 1999). Signs of human Mo toxicity are diarrhoea, anaemia, immaturity of erythrocytes, uricaemia. Thiomolybdate at levels of 5 mg Mo/kg bw causes in experimental animals diarrhoea, anaemia and skeletal lesions (Mills and Davis, 1987).

Na_2MoO_4 was a primary skin irritant for 24 hrs after application, but the skin lesions had cleared within 72 hrs. A 20% solution caused conjunctival redness but no corneal irritation. There was no sensitisation (Patty's, 1981). Mo released from surgical metal implants can induce a delayed type of hypersensitivity with PUO and ANA-ve systemic lupus erythematosus (positive lymphocyte transformation test) (Federmann *et al*, 1994).

Chronic small doses of molybdate have been reported to inactivate the glutaminases of brain and liver causing a decrease in ammonia release (Patty's, 1981). Small amounts of molybdate have similarly been reported to impair the intestinal utilisation of carotenes and to reduce vitamin A status (Patty's, 1981).

The evidence for anticariogenicity in man is contradictory and inconclusive. Mo accumulates in teeth and dental enamel. In a study on the cytopathogenicity of Mo against distinct cell types Mo^{5+} was tested *in vitro* against L-929 murine fibroblasts and primary human gingival fibroblasts because of release from dental alloys. Mo^{5+} had only a low potency and a low NOEL, as measured by its effect on DNA pattern and cell ultrastructure, which latter showed necrosis but not apoptosis. It had no effect on human mast cells. The dose range tested was 0.0033-1.0 mmol/L (Schedle *et al*, 1995).

Rodent cardiomyocyte cultures require Mo and Se for survival, growth and normal electrophysiological function (WHO, 1996a).

3.1. Molybdenosis

In animals, molybdenosis can occur in cattle, sheep and horses by pollution of pasture with fly ash, indicating ready bioavailability of Mo (Ladefoged and Sturup, 1995). Intoxication (known as teart in cattle and sheep) occurs also on feeding forage growing on shales, mineralised granites and some peats, containing 20-100 mg/kg Mo. The symptoms are loss of appetite, listlessness, diarrhoea, poor growth, anaemia with low Hb and RBC and in ruminants are probably due to secondary Cu deficiency. Cattle, rabbit and chicks also develop fatty degeneration of liver and kidneys. The signs are osteogenic defects with skeletal and joint deformities, spontaneous sub-epithelial fractures, mandibular exostoses, reduced AP activity and reduced proteoglycan content of cartilage. In ruminants it is always associated with "conditioned" Cu-deficiency. Typically, anaemia, cardiac hypertrophy, and achromotrichia from defective melanin synthesis are found. In other species, inhibition of phosphoadenosine-phosphosulphate synthesis, oestrus disturbance, testicular degeneration were noted. Mo^{vi} is more toxic to rats and guinea pigs. $(NH_4)_2MoO_4$ in hepatotoxic doses reduced succinic dehydrogenase and cytochrome-c oxidase in rats. Other changes reported were depletion of tissue nicotinamide nucleotides, hyperaminoaciduria, reduction in erythrocyte life span and hypothyroidism, accompanied by low plasma thyroxine and inhibition of thyroid hormone secretion (Patty's, 1981).

In humans high Mo intakes occur with industrial exposure or through food. It is associated with raised XD activity, uricaemia, uricosuria and a higher incidence of gout (IDACE, 1995). In areas with high

geological Mo levels the human XO level is increased (IDACE, 1995). Biochemical changes noted were hypoalbuminaemia, a rise in α-globulins, and raised serum bilirubin as sign of hepatotoxicity. It may be associated with oesophageal cancer.

3.2. Toxic effects in animals

Groups of 4 Holtzman rats were fed daily for 6 weeks diets containing 75 mg and 300 mg Mo/kg feed (equivalent to doses of 3.75 mg Mo/kg bw or 15 mg Mo/kg bw). Growth was significantly inhibited and Cu and Mo concentrations in the liver increased, but the addition of sulphate reduced these effects. The femorotibial joints were enlarged and the epiphyses of femur and tibia were thickened. The LOAEL was 3.75 mg Mo/kg bw for both bodyweight loss and bone deformities (Miller *et al*, 1956; Vyskocil and Viau, 1999).

In an 8 week study in male rats doses of 40 or 80 mg Mo/kg bw/day were given by gavage and the nephrotoxicity investigated. The NOAEL was 40 mg Mo/kg bw/day based on bodyweight loss and nephrotoxicity. The nephrotoxicity was moderate (Bompart *et al*, 1990; Vyskocil and Viau, 1999).

Weanling Long-Evans rats received in their diet 50 or 80 mg Mo/kg bw over 5-8 weeks. Diarrhoea and reduced weight gain were noted. Hepatic Cu levels increased (Suttle, 1980).

Rabbits were exposed for 6 months to oral doses of 0.025, 0.5, 5, 50 mg Mo/kg bw/day. Body weight loss and histological changes in liver and kidney were noted at doses of 5 mg/kg bw/day and above, the NOAEL being 0.5 mg/kg bw/day. The weakness of the study was the uncertainty of the analytical method (Asmangulyan, 1965; Vyskocil and Viau, 1999).

Rabbits were exposed for 4 months to oral doses of 40, 500, 1000, 2000, 4000 mg Mo/kg feed (equivalent to 1.8, 23, 46, 92, 184 mg Mo/kg bw/day for a 1.3 kg rabbit consuming 60 g feed/day). The NOAEL was 23 mg/kg bw/day based on bodyweight loss, skeletal abnormalities and anaemia (Arrington and Davis, 1953; Vyskocil and Viau, 1999).

Male and female guinea pigs were treated for 8 weeks with doses of molybdenum in their diet rising by increments of 1000 mg to 8000 mg Mo/kg feed (1000 mg/kg feed corresponds to 75 mg Mo/kg bw/day for a guinea pig weighing 400 g and consuming 30 g feed/day). The LOAEL was 75 mg Mo/kg bw/day based on loss of Cu, growth depression and achromotrichia. Guinea pigs appear to be a less sensitive species to large doses of molybdenum (Arthur, 1965; Vyskocil and Viau, 1999).

3.2.1. Carcinogenicity

There are no relevant studies in animals or man. Molybdates are not on the MAK list, EPA list or ACGIH list (Vyskocil and Viau, 1999). Intraperitoneal administration to strain A mice of MoO_3 significantly increased the incidence of lung adenomas (Stoner *et al*, 1976). Mo has been found to prevent oesophageal, forestomach and mammary cancer induced by N-nitroso compounds in laboratory animals (Luo *et al*, 1983; Wei *et al*, 1985).

3.2.2. Genotoxicity

$(NH_4)_6Mo_7O_{24}$ was mutagenic in two of three *Escherichia coli* strains. $MoCl_5$ was negative and $(NH_4)_6Mo_7O_{24}$ positive in the *Bacillus subtilis rec*-assay using strains H17 (repair-competent) and strain M45 (repair-deficient) (Nishioka, 1975). Ammonium and sodium molybdate were neither mutagenic nor recombinogenic in *Saccharomyces cereviseae* reverse mutation and gene conversion assays (Singh, 1983).

3.2.3. Cytotoxicity

In a study on the cytopathogenicity of Mo against distinct cell types (Schedle *et al*, 1995), Mo^{5+} (dose range from 0.0033 to 1.0 mmol/L) was tested *in vitro* against L-929 murine fibroblasts and primary human gingival fibroblasts because of release from dental alloys. Mo^{5+} had only a low potency for inhibition of ^{3}H-thymidine incorporation relative to other metal cations. The cytopathogenic effect on both the DNA pattern and the ultrastructure of the cells revealed signs of necrosis but no signs of apoptosis. Mo^{5+} had no effect on human mast cells.

3.2.4. Reproduction and teratogenicity

Four pregnant Cheviot ewes were given in their feed an extra 50 mg Mo/day as ammonium molybdate. Three of the four newborn lambs showed ataxia with histological evidence of cortical degeneration, demyelination of the cortex and spinal cord (Mills and Fell, 1960).

Two male Holstein calves received daily orally by capsules either 4.1 or 7.8 mg Mo/kg bw. Gradual disappearance of spermatogenic and interstitial testicular tissue was noted. The LOAEL was 4.1 mg Mo/kg bw (Thomas and Moss, 1951).

Five pairs of mice (Charles River CD) were given a daily single dose of 10 mg Mo/L (1.5 mg Mo/kg bw) as molybdate in their drinking water for 6 months or about 3 generations. As water consumption was not measured, the calculated daily intake is based on a 20 g mouse consuming 3 ml water/day. Excess pup deaths (15/238) in the F_1 generation and 7/242 pup deaths plus 5 dead litters and 1 maternal deaths in the F_2 generation and infertility were noted. This would correspond to a LOAEL of 1.5 mg Mo/kg bw/day (Schroeder and Mitchener, 1971; Vyskocil and Viau, 1999).

In a 13 week study Long-Evans rats were given in the diet doses of 20, 80, 140, 700 mg Mo/kg feed (calculated to represent approximately 2, 8, 14, 70 mg Mo/kg bw/day for a 100 g rat consuming 10 g feed/day) and either 5 or 20 mg Cu/kg bw additionally. Growth depression was observed at the lowest dose in males, and male fertility was depressed at 14 mg/kg bw/day as shown by fewer litters and degeneration of seminiferous tubules. There was less milk production by females on high dose Mo as pups gained less weight. The LOAEL for growth depression for males was therefore 2 mg/kg bw/day and the NOAEL for infertility of males was 2 mg/kg bw/day. For females the NOAEL for growth depression was 2 mg/kg bw/day (Jeter and Davis, 1954; Vyskocil and Viau, 1999).

In a 9 weeks study in SD rats on the effects of Mo supplementation on oestrus activity, fertility and foetal development, 5 groups, each of 21 female weaning rats, were given for 6 weeks a basic diet containing 0.025 mg Mo/kg diet as well as 6.3 mg Cu/kg diet, and additionally in their drinking water doses of 0, 5, 10, 50 and 100 mg Mo/L as sodium molybdate ($Na_2MoO_4.2H_2O$) for 3 weeks until the 21st day of gestation. Six animals in each group were sacrificed after 6 weeks to determine the oestrus cycle length. The remaining 15 animals in each group were mated with untreated males and allowed to continue gestation for 21 days. The average mean weekly supplementary Mo intakes were 0.0, 0.64, 1.12, 5.81 and 11.56 mg Mo/rat (equivalent to 0, 0.91, 1.6, 8.3 and 16.7 mg Mo/kg bw/day assuming an average rat weight of 100 g). There was no effect on fertility, food and water consumption. Oestrus cycle was prolonged from 1.6 mg/kg bw/day and higher supplementation. Gestational weight, litter size and foetal weights were less than controls for the groups fed 1.6 mg/kg bw/day and higher doses. Histopathology showed delayed histological development of foetal structures, delayed oesophageal development, delayed transfer of foetal haematopoeisis from liver to bone marrow, and delayed myelination of the spinal cord at doses of ≥1.6 mg/kg bw/day. Foetal resorption increased at doses of 1.6 mg/kg bw/day and higher. SO and XDH/XO activity increased with Mo supplementation but less in pregnant animals at dose levels of 1.6 mg/kg bw/day and above. The NOAEL was 0.9 mg Mo/kg bw/day. The study was well designed. (Fungwe *et al*, 1990; Vyskocil and Viau, 1999).

3.3. Toxic effects in humans

There are no well-designed chronic studies in man which can be used for risk assessment.

In an area in Armenia, where the population is exposed to a high dietary intake of Mo for geophysical reasons from soil levels of 77 mg Mo/kg and 39 mg Cu/kg, aching joints and gout-like symptoms have been reported. The daily intakes of Mo and Cu, calculated from analysis of levels in different foods, were 10-15 mg Mo/day (equivalent to 0.14-0.21 mg Mo/kg bw/day for a 70 kg adult) and 5-10 mg Cu/day, compared to intakes of 1-2 mg Mo and 10-15 mg Cu in a control area. Biochemical investigations showed abnormally high serum uric acid levels in humans and livestock (81 mg/L in humans with symptoms). Tissue XO activity was also high. Individuals with symptoms had hyperuricosuria and a raised Mo blood level (310 µg/L). Serum molybdate and XO levels were positively correlated with serum uric acid levels. Serum uric acid levels increased with residence time from 37.5 mg/L after 1 year to 68 mg/L after 5 years. Weaknesses of this study were the low blood Cu level of 1130 µg/L in affected persons *vs*. 1830 µg Cu/L in controls (possibly contaminated samples) and the ratio of 5 controls to 52 exposed cases. The US NRC concluded that the involvement of Mo was speculative (Kovalskiy *et al*, 1961; Vyskocil and Viau, 1999).

In another study on 25 workers exposed for an average of 30 years in a MoS_2 roasting factory to time-weighted average air concentrations of 9.5 mg Mo/m^3 the serum uric acid and ceruloplasmin levels were raised. After 4 years exposure there was a greater incidence of aching joints and headaches than in controls. The minimum daily dose of Mo as dust was calculated as 10.2 mg. The weakness of the study was the high turnover of workers, which made epidemiological assessment impossible (Walravens *et al*, 1979; Vyskocil and Viau, 1999).

In another study on 4 volunteers three different oral doses of Mo were administered. Urinary uric acid excretion was found to be unchanged up to doses of 22 µg/kg bw/day (Deosthale and Gopalan, 1974; Vyskocil and Viau, 1999).

Serum uric acid levels were compared in individuals of 2 cities with high and low Mo levels in their drinking water. The adequately determined Mo intake of the exposed individuals was ≥7 µg/kg bw/day, yet serum uric acid levels were lower than in the controls. Only two Mo levels were compared (Chappel *et al*, 1979; Vyskocil and Viau, 1999).

In a study on 4 young men fed dietary doses of Mo varying from 22-1490 µg/day for 24 days ^{100}Mo was fed 5 times, ^{97}Mo was infused 3 times and ^{94}Mo was used for assessing the total Mo content of urine and faeces by isotope dilution. Absorption was found to be 88-93% efficient, especially the larger the dose. Urinary excretion was proportional to the dietary load and slow at low doses. Mo retention appeared to be regulated by urinary excretion. No adverse effects were noted with doses up to 1500 µg/day for 24 days (Turnlund *et al*, 1995).

In 60 patients on long-term haemodialysis the relationship of serum Mo levels to serum ß2-microglobulin and C-parathyroid hormone levels and to the incidence of arthritis was investigated. Haemodialysis reduced the Mo serum level from 2.7 to 1.4 µg/dl (normal 0.02-0.13 µg Mo/dl). Serum Mo levels correlated with ß2-MG and C-PTH levels and serum Ca^{2+}. In 9 patients with arthritis the serum Mo levels averaged 12.8 µg/dl, suggesting that Mo accumulation contributes to arthritis (Hosokawa and Yoshida, 1994).

4. DOSE RESPONSE ASSESSMENT

There are no adequate human data for establishing a UL. Growth depression occurs in rats at 2-8 mg Mo/kg bw/day (Jeter and Davis, 1954; Miller *et al*, 1956) and skeletal changes at 7.5 mg Mo/kg bw/day (Miller *et al*, 1956). Reproductive and developmental changes were found in rats at 1.6-2 mg Mo/kg bw/day (Jeter and Davis, 1954; Fungwe *et al*, 1990). In mice infertility and early pup deaths were noted at 1.5 mg Mo/kg bw/day (Schroeder and Mitchener, 1971). In rabbits skeletal changes and nephrotoxicity were found at 5 mg Mo/kg bw/day (Asmangulyan, 1965), while skeletal changes, bodyweight loss and anaemia were seen at 25-46 mg Mo/kg bw/day (Arrington and Davis, 1953; McCarter *et al*, 1962). Reduced growth occurred in guinea pigs at 75 mg Mo/kg bw/day (Arthur, 1965). Adverse spermatogenic effects were seen in calves at 4 mg Mo/kg bw/day (Suttle and Field, 1969). Thiomolybdate intoxication can occur in experimental animals at intakes of 5 mg Mo/kg bw (Mills and Davis, 1987).

From these studies the critical effects of molybdenum in the rat and mouse appear to be effects on reproduction, particularly foetal development. The pivotal animal study is the 9 weeks study in the rat showing a NOAEL of 0.9 mg Mo/kg bw/day (Fungwe *et al*, 1990).

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

A tolerable upper intake level (UL) can be established using the 9-week study in the rat (Fungwe *et al*, 1990). This study in rats is pivotal because of its satisfactory design, the use of an adequate number of test animals, demonstration of a clear dose-response relationship and clear toxicological endpoints. The NOAEL of this study was 0.9 mg/kg bw/day for reproductive toxicity. An uncertainty factor of 100 is used. This comprises a factor of 10 for protecting sensitive human sub-populations with inadequate Cu intake or with deficient Cu metabolism in view of the species differences in antagonism between Mo and Cu, and another factor of 10 to cover the lack of knowledge about reproductive effects of Mo in humans and incomplete data on the toxicokinetics in man. Because the exposure in this 9-week rat study is sufficient to cover the relevant period of foetal development, a further uncertainty factor is unnecessary. This provides a UL of approximately 0.01 mg/kg bw/day, equivalent to 0.6 mg/person/day for adults, which also covers pregnant and lactating women.

A further consideration is required in relation to ULs for children, since an adverse effect on growth of young animals was seen in another study in rats (Jeter and Davis, 1954; Vyskocil and Viau, 1999), with a LOAEL of 2 mg/kg bw/day. This indicates that the UL for children should be derived by extrapolating from the adult UL on a body weight basis using the reference body weights for Europe published by the Scientific Committee for Food (SCF, 1993).

Age (years)	UL (mg/day)
1-3	0.1
4-6	0.2
7-10	0.25
11-14	0.4
15-17	0.5

6. CHARACTERISATION OF RISK

The UL is six times the mean estimated intake of 100 µg Mo/day for adults in 11 different countries (WHO, 1996a) and exceeds the upper range of intakes for The Netherlands (96 µg/day), Sweden (260 µg/day), the UK (400 µg/day), Germany (500 µg/day), and Finland (150 µg/day) (SCF, 1993; SCF, 1998). However, in mining areas with contaminated water supplies, drinking water levels may reach up to 400 µg Mo/L. In these circumstances, the daily potential intakes from food and 2 L drinking water could reach 1000 µg Mo/person/day.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN B2

(EXPRESSED ON 22 NOVEMBER 2000)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Riboflavin (vitamin B2) is chemically specified as a 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine. The free vitamin is a weak base normally isolated or synthesised as a yellowish-orange amorphous solid. Riboflavin is widely distributed in foodstuffs and all plant and animal cells contain it, but there are very few rich sources. Only yeast and liver contain more than 2 mg/100g. Other good sources are milk, white of egg, fish roe, kidney and leafy vegetables (Elmadfa and Leitzmann, 1998).

Riboflavin is a precursor of certain essential coenzymes such as flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD). In these coenzyme forms riboflavin functions as a catalyst for redox reactions including flavoprotein-catalyzed dehydrogenations that are either pyridine nucleotide dependent or independent reactions with sulphur-containing compounds, hydroxylations, oxidative carboxylations, dioxygenations and the reduction of oxygen to hydrogen peroxide. Flavo-coenzymes are also involved in the biosynthesis of niacin-containing coenzymes from tryptophan via FAD-dependent kynurenine hydroxylase, the FMN dependent conversion of the 5´-phosphates of vitamin B6 to pyridoxal 5´-phosphate and the FAD-dependent dehydrogenation of 5,10-methylene-tetrahydrofolate to the 5´-methyl product, with the vitamin B_{12}-dependent formation of methionine and sulphur amino metabolism.

2. NUTRITIONAL BACKGROUND

In foodstuffs riboflavin occurs free or combined either as FAD and FMN as a complex with protein. Protein bound riboflavin is hydrolysed in the gastrointestinal tract to free riboflavin, the form absorbed. At physiological concentrations the uptake of riboflavin occurs by an active, saturable transport system. At high levels of intake riboflavin is absorbed by diffusion (McCormick, 1989). The amount absorbed depends on the intake, it is increased by bile salts and when riboflavin is given orally, with food. Absorption rate of free riboflavin is 50-60% for a dose range of 2-25 mg (Elmadfa and Leitzmann, 1998). In plasma, riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins, and mainly found as FAD. Although the significance of this protein binding is not fully understood, the main function is the transport of riboflavin from plasma into the central nervous system (Steier *et al*, 1976; Natraj *et al*, 1988). Phosphorylation and dephosphorylation are features of intracellular metabolism.

In the cellular cytoplasm of most tissues, the small intestine, heart, liver and kidney, riboflavin is converted into the coenzymes FMN with flavokinase and FAD by the apoenzyme FAD-synthetase. In the body tissue riboflavin is predominantly present as the coenzyme FAD, which can be evaluated by determination of the blood FAD level. In healthy adults riboflavin accounts for 60-70% of the excreted urinary flavins (McCormick, 1989). The urinary excretion of riboflavin varies with intake, metabolism, and age. This is an alternative approach for determination of riboflavin status, because it reflects an excess of current intake beyond tissue requirements. A recent study of the pharmacokinetics of riboflavin uptake in human subjects indicated an upper limit of absorption from a single dose of 27 mg. The half-life of absorption was 1.1 h. Stool analysis to estimate riboflavin excretion was not done in this study. It also demonstrated relatively modest changes in plasma riboflavin or flavoenzymes following oral administration (Zempleni *et al*, 1996).

One of the methods commonly used for assessing riboflavin status involves the determination of the erythrocyte glutathione reductase (EGR) activity. The method generally preferred for the estimation of riboflavin status is the stimulation of FAD-dependent EGR *in vitro*, which relies on an associated oxidation of NADPH which can be readily monitored spectrophotometrically (Bates *et al*, 1986).

The second biochemical method used is the detection of riboflavin in the urinary excretion – a normal adult excretes 120 µg or more per 24 h. Less than 40 µg per 24 h (Horwitt, 1950) or 27 µg/g of creatinine (Sauberlich, 1999) is an indicator for riboflavin deficiency.

Red blood cell FAD and FMN (after modest hydrolysis from FAD) have been used as indicators of the cellular concentration of riboflavin in its form of coenzyme, since these forms comprise over 90% of riboflavin.

The highest mean intake of riboflavin from diet and supplements was reported for males aged 31-50 years: 6.9 mg/day. The highest reported intake at the ninety-fifth percentile was 11 mg/day in females over 70 years (Food and Nutrition Board, 1998).

The RDA (Recommended Daily Allowance) for riboflavin varies from 0.5-0.9 mg/day for children, 1.3 mg/day for male adults and 1.0-1.1 mg/day for female adults (Food and Nutrition Board, 1998); 0.8-1.6 mg/day children/male, 0.8-1.3 mg/day children/female, 1.3 mg/day male adults and 1.1 mg/day female adults (SCF, 1993), 0.7-1.6 mg/day children/male, 0.7-1.3 mg/day children/female, 1.2-1.5 mg/day male adults and 1.2 mg/day female adults (D-A-CH Referenzwerte, 2000).

3. HAZARD IDENTIFICATION

3.1. Studies on genotoxicity

Riboflavin and FMN were found not to be mutagenic in the Ames test with *Salmonella typhimurium* (strains TA97A, TA102, TA98, TA100). Both suspensions and plate overlay tests were conducted and assays were done with and without mammalian activation systems (Fujita and Sasaki, 1986; Kale *et al*, 1992).

DNA-damage was found in human cell cultures after multivitamin administration together with light. It was suggested that riboflavin was involved in this photodynamic damage, but only in synergism with other multivitamin components, because riboflavin solely was not able to damage DNA even in a 30 fold higher concentration (Ennever *et al*, 1983).

3.2. Special studies on reproduction

Weaned male and female rats were fed daily doses of 10 mg of riboflavin for 140 days. The animals were mated and normal litters were obtained from the riboflavin and control groups. At three weeks of age the offspring of the first generation were fed with 10 mg/kg bw/day of riboflavin. Daily feeding over periods of 140 days was continued for three generations. No differences in development, growth, maturation and reproduction of treated and control animals were observed. Autopsies at the end of the test period did not show any gross changes (Unna & Greslin, 1942).

Thirteen female rats were fed diets containing 100 ppm of riboflavin/day for two weeks, prior to mating and subsequently during gestation and lactation. Control rats received 4 ppm riboflavin in the diet. There was no difference between groups except an apparent decrease in the viability of the offspring in the high riboflavin group as a result of the loss of one litter (Schumacher *et al*, 1965).

No differences in the number per litter, mortality or weight gain of offspring in young female Wistar rats were found between diets containing 4 or 40 ppm of riboflavin during pregnancy and lactation (Le Clerc, 1974).

3.3. Toxicity studies

Early reports of some toxic effects with riboflavin in laboratory animals were due to the effects of the solvent and not caused by the vitamin. Unna and Greslin (1942) reported a lack of toxicity in rats receiving 10 g/kg orally, or 5 g/kg subcutaneously, and in dogs receiving 2 g/kg orally. The first reliable report of toxicity in animals for riboflavin was an investigation in rats receiving 0.6 g/kg intraperitoneally where the animals became anuric and riboflavin crystals were found in the renal tubes (Unna and Greslin, 1942). The monodiethanolamine salt of FMN was fed to groups of 10 weaned female rats five days per week for 29 weeks at doses of 1, 4, 10 and 40 mg/day (= 5, 20, 50 and 200 mg/Kg bw). No effects were observed at 5 and 20 mg/Kg bw levels. A slight decrease in haemoglobin concentration was observed at 50 mg/Kg bw. At 200 mg/Kg bw two rats died and the surviving eight animals showed slight anaemia and decreased weight gain (Randall, 1950). Groups of four rabbits each received 10 or 100 mg (5 or 50 mg/Kg bw) monodiethanolamine riboflavin by intravenous or intramuscular injection five days per week

for three weeks. One of the rabbits died with evidence of renal damage following seven intravenous injections at 50 mg/kg bw. No toxic effects were noticed after intramuscular injection (Randall, 1951). The administration of 25 mg/kg bw of riboflavin for five months did not cause any toxic effects in dogs (Unna and Greslin, 1942).

Riboflavin (chemically synthesised or produced by fermentation, food grades purity 98%) was examined in a sub-chronic 13-week oral feeding study in three groups of 16 male and 16 female Wistar rats at doses of 20, 50 or 200 mg/kg bw (SCF, 1998). There were no dose related differences regarding feed consumption, feed conversion efficiency and water intake. A 6% (<10%) growth retardation was found in female rats given 200 mg/kg bw/day riboflavin ex fermentation and males and females treated with 50 mg/kg bw/day riboflavin ex synthesis. No dose related changes in haematological parameters, urine analysis or clinical chemistry were noted, except for borderline variations in the haemoglobin concentration, red blood cell and reticulocyte counts in females with 200 mg/kg bw/day riboflavin ex synthesis. Gross and histopathological findings showed no significant treatment related lesions in any test group.

The LD_{50} after an intraperitoneal riboflavin injection was 340 mg/kg for the mouse and 560 mg/kg for the rat (Yoneda, 1984). Death, which occurs after 2-5 days, was from formation of riboflavin crystals in the kidney, leading to anuria and azotemia. Vitamin crystallisation in the kidney occurs when the riboflavin blood level exceeds 20 µg/ml in rats. Urinary levels of 150 µg/ml may be a sign of toxicity (Machlin, 1991).

The low toxicity following oral administration can probably be explained by the limited capacity of the intestinal absorption mechanism (Machlin, 1991).

There are no published data from studies using animal models or in humans that connect riboflavin with genotoxic, carcinogenic, teratogenic or reproductive toxic effects or with toxic effects in humans.

3.4. Mechanistic studies

Evidence of adverse effects associated with the group of flavins is based on *in vitro* studies showing involvement in the formation of active oxygen species and in the axonal degeneration on intense exposure to ultraviolet and visible light (Spector *et al*, 1995, Lucius *et al*, 1998).

3.5. Human studies

The few studies performed involving large doses of riboflavin were not designed to evaluate adverse effects, but identification of hazards as a first step is possible based on the studies done with high dose supplementation and large intakes of riboflavin.

The highest doses orally administered over a longer time period were in two studies by Schoenen *et al* In the first study, Schoenen *et al* (1994) reported no side effects in 49 patients treated for migraine with 400 mg/day of riboflavin taken with meals for at least 3 months. One patient, receiving riboflavin together with aspirin, withdrew from the study due to a gastric upset possibly due to aspirin. No side effects were reported by the other study participants.

In the second study (Schoenen *et al*, 1998), 55 patients with migraine were treated with 400 mg/day of riboflavin (or a placebo) in a random trial of 3 months duration. Minor adverse effects were observed in two cases in the riboflavin group (diarrhoea and polyuria). In the placebo group one case of abdominal cramps was reported.

For the treatment of methaemoglobinaemia due to NADH methaemoglobin reductase deficiency, 120 mg riboflavin/day were administered to the members of a family for a period of 10 months. The intake was reduced to 10-30 mg/day for longer periods; no side effects were observed (Hirano *et al*, 1981).

One 24 year old woman, suffering from chronic fatigue, received 100 mg/day riboflavin for two years without any side effects, and a 14 year old girl with the same disorder received 200 mg/day riboflavin for one year and 100 mg/day for the next 2 years without any side effects (Peluchetti *et al*, 1991).

A 57 year old epileptic woman, treated with barbiturates, was given daily 600 mg of riboflavin as a chronic treatment. There was a small electro-encephalographic abnormality, which was not associated with clinical symptoms and which disappeared 47 days after the treatment was completed (Santanelli *et al*, 1988).

The lack of harmful results from high doses of riboflavin can also be due to its physico-chemical properties – the solubility is limited and, especially, the capacity to absorb riboflavin from the gastrointestinal tract by humans is limited (Stripp, 1965; Zempleni *et al,* 1996).

Stripp (1965) found the single oral administration of 50-500 mg of the sodium salt of FMN without any adverse effect.

One case is described where a woman with multiple myeloma showed an impaired turnover and excretion of dietary riboflavin, causing yellow pigmentation of the skin and hair (Farhangi and Osserman, 1976).

4. DOSE RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The absorption of riboflavin is limited when administered in high doses. Data on adverse effects from high oral riboflavin intake are not sufficient for a risk assessment. Given the lack of any demonstrated functional disorders or adverse structural effects in humans following excessive oral riboflavin intake and considering the limitation of intestinal absorption, the relevance of the mild effects shown in *in vitro* studies to human health *in vivo* is questionable.

Available subchronic data from human studies and on pharmacokinetics studies do not show reported effects on oral toxicity of riboflavin. Apart from a few minor gastrointestinal disorders, which are not clearly related to the riboflavin intake, it is free from serious adverse effects.

Although the studies of Schoenen *et al* (1994, 1998) involved an adequate number of subjects with a daily dose of 400 mg riboflavin, they included only self-reporting of adverse effects and did not include adequate assessment of parameters relevant to the detection of adverse effects (for example biochemical indices of hepatic or renal function). In consequence, these studies were not of sufficient quality and extent to be used for the determination of a Tolerable Upper Intake Level (UL).

The results of a 13 week feeding study in Wistar rats (SCF, 1998) show that a dose of 50 mg riboflavin per kg body weight can be considered as the NOAEL and does not contradict the current JECFA ADI value for riboflavin and riboflavin 5-phosphate of 0-0.5 mg/kg bw (JECFA, 1969). The SCF has not adopted an ADI for riboflavin, but regards its use as food colorant to be acceptable (SCF, 1977).

5. CHARACTERISATION OF RISK

The dietary intake of riboflavin from food was evaluated in the 1990s in different European countries. Mean riboflavin intake in the population from The Netherlands, based on data from the Dutch National Food Consumption Survey (n = 5958, 2-day estimated dietary record) is 1.54 mg/day (97.5 percentile: 2.87 mg/day) (Hulshof *et al*, 1997-1998). Italian data are based on the Italian survey (n = 2734, 7-day weighed record), with a mean intake of riboflavin of 1.6 mg/day (97.5 percentile: 2.7 mg/day) (Turrini, 1994-1996). In the Austrian Study on Nutritional Status (n = 2488, 24-h-recalls), the mean riboflavin intake is 1.49 mg/day (97.5 percentile: 3.29 mg/day) (Elmadfa *et al*, 1998). It should be noted that these studies were not designed to assess specifically the intake of riboflavin from supplements; however, the number of consumers of supplements is probably not sufficient to influence significantly the mean consumption of the total population.

In the UK (EVM, 2000), the mean riboflavin intake from all sources for men and women was 2.3 mg/d and 1.8 mg/d, respectively; riboflavin intake for adults (16-64 years old) from food supplements among supplement consumers only (4% of males, 8% of females) amounted on average to 5.2 mg/day in males and to 3.1 mg/day in females, based on data collected in 1986/87. In Ireland (IUNA, 2000), the mean riboflavin intake from all sources for adults (18-64 years old) was 2.1 mg/day (97.5th percentile: 4.6); mean riboflavin intake from food supplements among supplement consumers only (7% of males, 13% of females) was 2.4 mg/day in males and 3.9 mg/day in females.

No study has reported significant adverse effects in humans of excess riboflavin consumption from food or supplements. This does not mean that there is no potential for adverse effects from high intakes. Although it is not possible, based on the present database, to derive an UL for riboflavin, the limited evidence available from clinical studies indicates that current levels of intake of riboflavin from all sources do not represent a risk to human health.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN B1

(EXPRESSED ON 11 JULY 2001)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Vitamin B_1 or Thiamine, formerly known as Aneurine, is 3-(4-amino-2-methylpyrimidin-5-ylmethyl)-5-(2-hydroxyethyl)-4-methylthiazolium. The free vitamin is a base. It is isolated and synthesized and used in food supplements and in food fortifications as a solid thiazolium salt in the form of thiamine hydrochloride or thiamine mononitrate. The molecular weight of thiamine hydrochloride is 337.29 g per mol. It is soluble in water and stable to heat below pH 5.0 and destroyed rapidly at pH 7.0 or above by boiling. Thiamine forms esters at the hydroxyethyl side chain with various acids. The most important ones are Thiamine monophosphate (TMP), Thiamine pyrophosphate (TPP) and Thiamine triphosphate (TTP) (McCormick, 1988).

2. NUTRITIONAL BACKGROUND AND METABOLISM

Vitamin B_1 was the first vitamin identified in 1926 by Jansen and Donath working on the antiberiberi factor from rice bran extracts. Lack of vitamin B_1 causes the deficiency disease Beriberi already known in Chinese antiquity. Nowadays in the Western world the vitamin B_1 deficiency is mainly found as a consequence of extreme alcoholism and known as Wernicke-Korsakoff syndrome. It is for this reason that thiamine has become the only regularly administered parenteral vitamin supplement in hospital emergency departments.

Vitamin B_1 mainly acts in α-ketoacid decarboxylation (e.g. pyruvate, α-ketoglutarate and branched-chain α-ketoacid acids), in transketolation (e.g. among hexose and pentose phosphates), and possibly in nerve conduction.

Ingested thiamine is well absorbed. It involves two mechanisms; the first is an active rate-limited jejunal uptake mechanism (Thomson *et al*, 1972). When the active transport is saturated, at an intestinal concentration greater than 3 μmol.l^{-1}, there is passive uptake. However, above an oral intake of 5 mg vitamin B_1 absorption rapidly declines (Friedeman *et al*, 1948). In a study of Davis *et al* (1984) with healthy volunteers vitamin B_1 plasma levels rose only marginally (42%) compared to folate and pyridoxine (>1500%), while the vitamin was actively excreted in the urine for up to six hours following an oral test dose of 10 mg. Vitamin B_1 is phosphorylated when it crosses the intestinal epithelium, but enters the blood principally as free vitamin B_1 and diffuses down a concentration gradient in the liver, heart, kidneys, and brain. In the blood vitamin B_1 is distributed between the plasma (10%) and cells (90%). The physiological whole blood concentration of the phosphate ester is 20 to 75 μg.l^{-1}. It is poorly stored and it is eliminated mainly in the urine either unchanged or as several (about 20) metabolites (Ariaey-Nejad *et al*, 1970). Raising the serum level of the vitamin results in active urinary excretion on the basis of the creatinine clearance (mean thiamine/creatinine/ renal clearance ratio of 2.4). After an oral dose of vitamin B_1, peak excretion occurs in about 2 hours and is nearly complete after 4 hours (Levy and Hewitt, 1971), as was already described in the early ninety-forties (Najjar and Holt, 1940; McAlpine and Hills, 1941).

Based on these observations, it is concluded that the plasma concentration of vitamin B_1 is tightly controlled. This is partly explained by Thom (1983), who reported that 20-30% of plasma vitamin B_1 is protein bound, all of which appeared to be as pyrophosphate. All unbound vitamin B_1 is rapidly dephosphorylated to facilitate excretion of an excess of the vitamin.

Vitamin B_1 metabolism is especially sensitive to excess alcohol consumption since the absorption of vitamin B_1 is decreased and its excretion is increased by alcohol. Alcohol also inhibits the activation of vitamin B_1 to its co-enzyme form Thiamine Pyrophosphate ester (TPP) (McCormick, 1988).

A vitamin B_1 kinetic study has been performed by Royer-Morrot *et al* (1992) using pharmacological doses intramuscular or orally. Comparison of 250 mg p.o. every 12 hours versus 500 mg i.m. once a day for 11 days resulted in a steady state plasma vitamin B_1 concentration after 7 and 5.6 days, respectively. The mean elimination half-life value was calculated to be approximately 1.8 days. Biological half-life of the vitamin is probably in the range of 9 to 18 days (Ariaey-Nejad *et al*, 1970). Total vitamin B_1 content of the adult human has been estimated to be approximately 30 mg (McCormick, 1988).

Animal experiments have shown that the rate of vitamin B_1 utilisation depends on the amount of carbohydrate metabolised. Because the principal metabolic role is in energy-yielding metabolism the requirement is related to energy intake. Increased physical activity, pregnancy and lactation increase vitamin B_1 requirements because of greater energy need; but when expressed per MJ the requirement is constant, and this relationship does not vary in such circumstances or with age. Based on this evidence the population recommended intake (PRI) is set at 100 μg per MJ leading to average daily requirements of around 1.0 to 1.2 mg per day (SCF, 1993). For people with energy intakes of less than 8 MJ per day, a minimal vitamin B_1 intake of 0.8 mg per day is suggested.

Vitamin B_1 is found in a large variety of animal and vegetable products but at a relatively low level (<0.5 mg/100 g). Important sources of vitamin B_1 are lean pork, legumes and cereal grains (germ fraction). The Nutriscan EC food and nutrition intake study revealed mean daily intake levels of vitamin B_1 on an EC average of 1.2 mg per day for women, ranging from 1.0 mg per day (NL) to 1.8 mg per day (Portugal) (SCF, 1993). With these figures in this low energy intake group, vitamin B_1 intake is generally considered as adequate in relation to physiological needs.

Biochemical changes in vitamin B_1 status occur well before the appearance of overt signs of deficiency. A number of sensitive tests have been developed to evaluate the vitamin B_1 status mostly based on the activity of enzymes with vitamin B_1 as co-enzyme. Erythrocyte transketolase activity (ETK-activity) and the *in vivo* stimulation of ETK-activity with thiamine phosphate (α-ETK) are reasonable indicators of marginal deficiency (1.20-1.25, 15-24%, respectively) and deficiency (>1.25, ≥25%, respectively) (Schrijver, 1991; Brin, 1980). Furthermore measurement of vitamin B_1 concentration and its phosphorylated esters in blood and urinary excretion under basal conditions is used.

3. HAZARD IDENTIFICATION

3.1. Evidence of adverse effects in humans

Orally ingested vitamin B_1 has a long history of use as an oral supplement without reported adverse effects. Due to its therapeutic action in some frequently observed clinical syndromes, thiamine hydrochoride has been advised and used over a long period of time. There are no reports of adverse effects of oral thiamine, even at dosages of several hundred milligrams a day (SCOGS, 1978; DHEW, 1979; Marks, 1989).

Rare cases of allergic sensitivity are documented mostly in the ninety-forties and have occurred solely in patients who received repeated vitamin B_1 by parenteral route (Tetreault and Beek, 1956). A systematic toxicity study by Wrenn *et al* (1989) on the parenteral use of thiamine at a dose of 100 mg in 989 patients resulted in 0.1% major reactions such as general pruritus. All the reported clinical symptoms suggest an anaphylactic reaction to the vitamin B_1 injection. Symptoms listed include anxiety, pruritus, nausea, respiratory distress, shock and in rare cases death (RDA Committee, 1998). Parenteral doses greater than 400 mg of vitamin B_1 cause nausea, anorexia, lethargy, mild ataxia and a diminution of gut tone (McCormick, 1988). More recent high level studies argue for inherently low toxicity of vitamin B_1 supplementation intravenously and especially orally. Royer-Morrot *et al* (1992) injected 500 mg daily intra-muscularly and reported one case of pruritus disappearing after 6 days of injection. Oral intake of two times 250 mg daily for 11 days did not reveal any adverse effects. Oral doses of 500 mg taken daily for a month did not lead to any adverse effects (Hawk *et al*, 1954).

Of interest is a study about vitamin intake in professional cyclists (Saris *et al*, 1989). This group is well known for their continuous massive intake of vitamins over years. Based on detailed questionnaires and product information, extra intake of vitamin B_1 was calculated to be 30 mg per day orally and 10 mg i.m. Plasma vitamin B_1 values were 237 $nmol.l^{-1}$ (reference value 95-138 $nmol.l^{-1}$) α-ETK was 1.02 (reference value 1.05-1.20). No adverse effects were reported.

A Medline search from 1966 on did not reveal any report on adverse effects after oral intake of vitamin B_1.

3.2. Toxicological data in animals

Lang (1979) reported that in all types of animal tests in his institute with extreme high oral doses of vitamin B_1, they never succeeded to detect any harmful effect. This was also the case under stress-related conditions such as iron deficiency or protein deficiency and using fifty times the normal daily doses of vitamin B_1. Only in one experiment with high vitamin B_1 intake and extreme low intake of protein and other B vitamins, effects were noticed probably caused by the unbalanced diet (Lang, 1979).

Bitsch (1997) reviewed the toxicity of vitamin B_1 given orally, and by intravenous or intraperitoneal injection and concluded that it is extremely safe. The tabulated LD_{50} levels in mice for thiamine hydrochloride were 0.07-0.125 g/kg bw intravenous, 0.317-0.500 g/kg bw intraperitoneal and 3-15 g/kg bw orally. Lang (1979) quoted an oral LD_{50} of vitamin B_1 of 3.0 g/kg bw for mice based on earlier studies of Hecht and Weese (1937). Symptoms by i.v. injections are hypotonia due to vasodilatation, bradycardia and respiratory arrhythmia leading to general neuromuscular inhibition. Death is caused by depression of the respiratory centre (Haley, 1948). The lethal i.v. dose in g/kg bw is for mice 0.125; rats 0.25; rabbits 0.30; and dogs 0.35 (McCormick, 1988). In monkeys up to 0.60 g/kg bw was required to produce toxic symptoms (Gubler, 1991). Similar pharmacological effects in humans are found only with parenterally administered doses hundreds of times larger than that required for optimal nutrition (Campbell *et al*, 1980). Rats have been maintained for three generations on oral doses of 0.08 to 1.0 mg/kg bw vitamin B_1 without any harmful effects (Williams and Spies, 1938). This is about 50 to 100 times the daily requirement. Gubler (1991) concluded that the margin between potential intake and levels of acute toxicity is at least 600 or more.

4. DOSE-RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Due to the lack of systematic oral dose-response intake studies as well as the extreme low toxicity no LOAEL and NOAEL can be established.

5. CHARACTERISATION OF RISK

Since systematic data on adverse effects with oral intake of vitamin B_1 in human are very limited, an exposure assessment as given by the RDA (1998) can be of help. From the NHANES III data in the US, the highest mean intake of vitamin B_1 from food and supplements for any life-stage and gender group reported for males aged 31 through 50 years was 6.7 mg/day, the highest reported intake at 95th percentile was 11.0 mg/day for females aged 51 years and older. In table 1 intake data from a number of EU countries are given.

Table 1. Mean and high percentile vitamin B1 intake (mg/day) from food and supplements in some EU countries

Country	Type of survey	n	Method	Supplements*	Mean	97.5%
Italy[a]	household	2734	7-day weighed	+	1.10	1.90
Netherlands[b]	household	5958	2-day record	-	1.23	2.87
Austria[c]	individual	2488	24 h recall	-	1.36	3.55
Germany[d]	individual (M) individual (F)	4974 5304	7-day record 7-day record	- -	1.40 1.10	2.63 2.11
Ireland[e]	individual (M) individual (F)	662 717	7-day record 7-day record	+ +	2.28 2.13	4.65 6.35
UK[f]	individual (M) individual (F)	1087 1110	7-day weighed 7-day weighed	+ +	2.01 1.26	3.29 3.09

* + data included supplements; - data excluded supplements.
[a] Turrini (INRAN) [b] Hulshof and Kruizinga (1999)
[c] Elmadfa *et al* (1998) [d] Adolf *et al* (1995)
[e] IUNA (2001) [f] Gregory *et al* (1990)

The highest reported mean intake in the EU is 2.28 mg/day with a reported highest intake at 97.5 percentile of 6.35 mg/day. Both are considerably lower than the reported US values.

From the available literature it can be concluded that vitamin B_1 orally ingested has a very low risk of adverse effects. This is related to the fact that with intake levels higher than 5 mg absorption rapidly declines and absorbed vitamin B_1 is actively excreted in the urine. Therefore no adverse effects of orally ingested doses of vitamin B_1 have been reported despite the fact that relatively high doses of vitamin B_1 up to 50-200 mg daily have been used therapeutically over long periods of time (months) as well as the widely available supplements providing intakes up to 50 mg/day without prescription.

Based on parenteral use of vitamin B_1, reports show rare cases of adverse events at levels from 100 to 300 mg i.v. and more frequently at higher doses up to 500 mg i.v. daily.

A Canadian evaluation of micronutrient safety classified vitamin B_1 as a nutrient with no known adverse effects (Program on Food Safety, 1996). The Dutch Nutrition Council reported 500 mg as an upper safe limit (Nutrition Council RDA, 1989) as did the US RDA Committee in 1989. However in the latest report of the US Food and Nutrition Board (1998) no UL could be derived based on the inadequate data. The SCF (1993) mentioned no evidence of toxicity at oral intakes up to 500 mg/day (for 1 month).

Based on the presented evidence, the Committee comes to the conclusion that it is not possible to derive a numerical UL for vitamin B_1. However existing evidence that is available from clinical studies as well as the long history of therapeutic use, indicates that current levels of intake from vitamin B_1 from all sources do not represent a health risk for the general population.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF BIOTIN

(EXPRESSED ON 26 SEPTEMBER OF 2001)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Biotin is a heterocyclic compound, an imidazolidone ring joined to a tetrahydrothiophene ring. The latter possesses a valeric acid side chain. Of eight theoretically possible stereoisomers only D(+)-biotin occurs in nature and binds to and activates four carboxylases found in humans. Its molecular weight is 244.3.

Biotin is soluble in water, insoluble in organic solvents, stable at pH 5 to 8 against air, light and heat. Oxidation of the sulphur atom and shortening of the valeric acid side chain result in loss of vitamin activity.

Biotin cannot be synthesised by mammals, therefore, in humans it must be acquired from exogenous sources. It is still controversial if and how much biotin synthesised by intestinal bacteria can contribute to the body's requirement for biotin.

Quantification of biotin in foods and body fluids for nutritional studies has been done by three basic methods: bioassays, avidin-binding assays or fluorescent derivative assays. As these assays do not have the same specificity the results show major discrepancies and it is important to establish how biotin concentrations were determined when comparing different studies. The avidin-binding assay of biotin and its metabolites after separation by high-performance liquid chromatography is considered as one of the best currently available methods (Mock, 1996; Mock *et al*, 1993; 1995; 1997).

2. NUTRITIONAL BACKGROUND

2.1. Biotin in food

Biotin requirements must be met by dietary uptake. The contribution of bacterial biotin synthesis in the gut has never been quantified.

Biotin occurs in many foods in very variable amounts. The variability of contents reported is due both to natural variation and methodological problems. Rich sources are liver, kidney, egg yolk, some vegetables like soybeans, nuts, spinach, mushrooms and lentils (20 to 100 µg/100 g edible portion). Lean meat, fruit, cereals and bread contain 1 to 20 µg/100 g. Biotin in vegetables, green plants and fruits occurs in water-extractable forms, whereas it occurs in firmly bound complexes in yeast and animal products. There are no reliable data on the bioavailability of biotin from different foods. Biotinidase, present in the intestinal mucosa and in pancreatic juice of mammals can cleave biotin linked to lysine (biocytin) and to oligopeptide. It may play an important role in the uptake of dietary biotin in humans after proteolysis (Wolf, 1995).

Biotin concentrations in human milk vary significantly during any 24-hour period and increase 5- to 30-fold during the progression from colostrum to transitional milk and eventually mature milk (Mock *et al*, 1992a; Salmenperä *et al*, 1985) and are then 20- to 50-fold higher than the plasma concentrations of normal women (Mock *et al*, 1992b). More than 95% of total biotin was found in the skim fraction of milk, of which more than 95% was free as opposed to reversibly or covalently bound biotin. At day 8 postpartum bisnorbiotin and biotinsulfoxide accounted for more than 50% of total biotin (3.9 ng/ml) in the skim fraction. Thereafter only true biotin rises significantly (mean value at day 30 to 40 is 7 ng/ml) (Mock *et al*, 1997a).

2.2. Biotin intake estimates

Dietary biotin intake of children and adults in different populations ranges between 17 and 60 µg per day (Heseker *et al*, 1994; Helbich, 1997; Food and Nutrition Board, 1998; Mensink and Ströbel, 1999; Expert Group on Vitamins and Minerals, UK Food Standards Agency, 2001; IUNA 2001). Data are scarce because in most dietary intake surveys biotin has not been assessed.

The estimated mean dietary biotin intakes in Germany, UK and Ireland between 1986 and 2000 are given in Table 1.

Table 1. Mean and 97.5 percentile biotin intake (µg/day) from food and supplements in three EU countries (adults >16 y)

Country	Type of survey	n	Method	Supplements*	Mean	97.5%
Germany[a]	individual (M/F)	1988	7-day record	-	36.8	86.2
Germany[b]	individual (M) individual (F)	1268 1540	record+interview record+interview	- -	52.9 42.5	53.7 43.1
UK[c] (16-64 yr)	individual (M) individual (M) individual (F) individual (F)	1087 1087 1110 1110	7-day record 7-day record 7-day record 7-day record	- + - +	38.9 39.1 28.3 28.7	69.7 71.4 56.3 58.1
Ireland[d] (18-64 yr)	individual (M) individual (M) individual (F) individual (F)	662 662 717 717	7-day record 7-day record 7-day record 7-day record	- + - +	40.4 42.8 29.8 34.1	74.9 91.8 53.9 103.3

* + data included supplements; - data excluded supplements.
[a] Heseker *et al* (1994) [b] Mensink and Ströbel (1999)
[c] EVM (2001) [d] IUNA (2001)

Dietary supplements containing biotin were taken on a regular basis by 4 to 5% of the German subgroups in the MONICA study 1994/1995. Whereas the median content of supplements was 10 µg biotin, maximal doses up to 5000 µg were reported (Schellhorn *et al*, 1998). Biotin containing supplements were consumed by 1 to 2% of the adult UK population. Biotin intakes from supplements ranged from 0.1 to 130 µg/day (EVM 2001).

In 145 elderly persons (aged 68 to 90 years) mean biotin intakes per day, assessed by seven-days weighed or estimated records were 19.5 µg (range 7.6 to 37) in women and 23.5 µg (range 9.9 to 59) in men (Bailey *et al*, 1997).

2.3. Biotin absorption and metabolism

Biotin is actively transported by different carrier systems.

Biotin absorption in the intestinal brush-border membrane proceeds via a structurally specific, temperature dependent carrier against a concentration gradient. Transport is electroneutral and coupled to sodium. This transport is saturable. At high intakes of biotin in pharmacologic doses simple diffusion predominates. Exit of biotin from the enterocyte across the basolateral membrane is also carrier-mediated, but independent of sodium and electrogenic, and does not accumulate biotin against a concentration gradient.

An electroneutral sodium-dependent transport system for biotin has also been reported for human renal brush border membrane vesicles, resulting in transfer from the lumen of the renal tubule into the blood.

A specific transporter for biotin together with sodium was demonstrated in human lymphocytes. This transport can be stimulated by mitogens apparently as a consequence of an increased number of biotin transporters in proliferating lymphocytes (Zempleni and Mock, 2000a).

Specific transport systems for biotin from the mother to the foetus have been reported, with little evidence of accumulation on the foetal side (Mock, 1996). A sodium dependent multivitamin transporter, which transports pantothenate, lipoate and biotin in an electrogenic process has been identified in

human placenta and is also but to a lesser extent expressed in human kidney, liver, pancreas, heart, brain, lung and skeletal muscle (Wang *et al*, 1999).

Renal clearance of biotin in normal children and adults is 0.4 times the creatinine clearance.

In humans the ratio of free biotin between cerebrospinal fluid and ultrafiltrates of plasma was found to be 0.85 ± 0.50 (Mock, 1989).

From studies with 6 healthy adults given biotin in pharmacologic doses, either orally (512, 2000 or 20,000 µg) or intravenously (4500 µg) it was concluded that oral biotin was completely absorbed. 50% of an oral dose was recovered in urine within 24 hours as biotin plus biotin metabolites. Intravenous administration resulted in a larger percentage excreted as intact biotin. The higher biotin concentration in plasma after intravenous dosage may have exceeded the capacity for renal tubular reabsorption (Zempleni and Mock, 1999).

The elimination half life time from plasma of a single oral biotin dose of 600 µg was calculated to be 110 minutes in 15 healthy volunteers (Bitsch *et al*, 1989).

Acute and short-term (14 days) ingestion of 1200 µg biotin daily by 15 healthy adults increased serum biotin from 60 ng/L (range 34 to 89 ng/L) to a mean of 3,738 ng/L after one day and to 5,521 ng/L after 14 days. Bisnorbiotin (basal level 46 ng/L) and biotin sulfoxide (basal level mean 3.7 ng/L, not detectable in 9 of 15 subjects) increased substantially both after one day (24 fold and 46 fold, respectively compared with pretreatment) and after 14 days (2.5 fold and 2.3 fold compared with day 1). The ratio of bisnorbiotin plus biotin sulfoxide to the total of avidin-binding substances did not change after 14 days. The excretion of biotin, bisnorbiotin and biotin sulfoxide in the urine likewise increased, 324 fold, 85 fold and 114 fold, respectively, providing indirect proof of biotin catabolism in human tissues (Mock and Heird, 1997; Mock and Mock, 1997).

Biotin absorption is reduced by ingestion of raw eggs which contain avidin, a protein resistant to proteases which binds 4 moles biotin per mole of avidin thereby making it unavailable. Prolonged heating at 100°C denatures avidin and sets biotin free.

Biotin is degraded in the human body by β-oxidation of the valeric acid side chain to bisnorbiotin and bisnorbiotinmethylketone and by oxidation of the sulphur in the thiophene ring to biotin-d,l-sulfoxide and biotin sulfone, which are excreted into the urine, and also found in plasma (Mock *et al*, 1993; Zempleni and Mock, 1999). These metabolites are inactive as vitamin. Biotin accounts for only approximately 50% of avidin binding substances in plasma and urine.

Biotin catabolism is increased by anticonvulsant drug treatment, by alcohol consumption and during pregnancy (Mock and Dyken, 1997; Zempleni and Mock, 2000b).

2.4. Functions of biotin

In man biotin is an essential co-factor for four carboxylases which catalyse the incorporation of bicarbonate into a substrate and are involved in gluconeogenesis and provision of intermediates into the citric acid cycle (pyruvate carboxylase, PC, EC 6.4.1.1), fatty acid synthesis (acetyl-CoA carboxylase, ACC, EC 6.4.1.2), leucine catabolism (3-methylcrotonyl-CoA carboxylase, MCC, EC 6.4.1.4.), and propionate catabolism (propionyl-CoA carboxylase, PCC, EC 6.4.1.3). The propionate to be carboxylated has various sources: catabolism of valine, isoleucine, threonine, methionine, the side chain of cholesterol, odd-numbered saturated fatty acids, and metabolism of intestinal bacteria.

Biotin is attached to the carboxylases via an amide bond formed between the carboxyl group of the valeric acid side chain and the epsilon-amino group of a specific lysine in the apocarboxylases by holocarboxylase synthetase (EC 6.3.1.10), driven by hydrolysis of ATP.

In the turnover of cellular protein catalysed by lysosomal proteases the holocarboxylases release biocytin, which is biotin linked to lysine, or biotin bound to oligopeptides. For the cleavage of the amide bond between biotin and lysine a specific hydrolase, biotinidase (EC 3.5.1.12) is needed, present in many tissues. The highest activity is found in serum, liver, kidney and adrenal gland. Serum biotinidase is produced in the liver. Impaired hepatic function is accompanied by decreased biotinidase activity in serum (Grier *et al*, 1989). Biotinidase activity is very low in human brain and cerebrospinal fluid.

Biotinidase is able both to recycle biotin bound to carboxylases and to cleave biotin bound to dietary proteins. Apart from this important function of biotinidase in providing biotin for intermediary metabolism, a function of this enzyme, is the transfer of biotin to nucleophilic acceptor proteins such as histones, thereby affecting gene expression (Hymes and Wolf, 1996) and e.g. embryological development (Bender, 1999; Zempleni and Mock, 2000b). Biotin is essential for cell proliferation. Its proliferative effect in immune cells can become of clinical relevance in biotin deficiency (Zempleni and Mock, 2001).

2.5. Biotin requirement

Biotin requirement cannot be accurately estimated. For infants the amount provided by breastmilk is considered to be adequate. For children and adults either the usual dietary intake or an extrapolation from the intake of exclusively breastfed infants is the basis for setting estimated adequate intakes (FNB, 1998).

The Scientific Committee on Food of the European Commission has defined a biotin reference value for adults of 15 to 100 µg per day (31st Report of the SCF, 1993).

Age-related adequate intakes of biotin have been estimated (Food and Nutrition Board,1998; D-A-CH Referenzwerte, 2000) and are summarised in Table 2.

Table 2. Adequate intakes of biotin (µg/day)

Age	0-4 m	4-12 m	1-4 yr	4-7 yr	7-10 yr	10-13 yr	13-15 yr	>15 yr
D-A-CH (2000)	5	5-10	10-15	10-15	15-20	20-30	25-35	30-60
Age	**0-6 m**	**6-12 m**	**1-3 yr**	**4-8 yr**	**9-13 yr**		**14-18 yr**	**>18 yr**
FNB (1998)	5	6	8	12	20		25	30

2.6. Biotin nutritional status

Mock *et al* (1997) determined serum true biotin concentrations in normal adults to be 60 ± 14.9 ng/L (range 34 to 89 ng/L), bisnorbiotin 46 ± 33 ng/L (range 5 to 145), biotin sulfoxide 3.7 ± 8 ng/L (range 0 to 31). Most (81%) of these avidin-binding substances are free, approximately 12% are covalently bound to plasma proteins and 7% are reversibly bound (Mock and Malik, 1992). The urinary excretion of biotin in normal adults ranges between 18 and 79 nmol/24 h (4.4 µg to 19.3 µg/24 h) plus approximately the same amount of biotin analogues.

The biotin status of an individual cannot be well assessed by determination of the biotin plasma concentrations. This was shown in 10 healthy subjects made biotin deficient by 20 days consumption of egg white containing enough avidin to bind more than 7 times the (normal) dietary biotin intake. The mean serum biotin level did not decrease significantly, and only five subjects had serum biotin concentrations below the lower limit of normal on day 20. In contrast, biotin and bisnorbiotin excretion into the urine decreased significantly from day 3 onwards and was less than the lower limit of normal in 8 of 10 subjects by day 14. The most sensitive indicator was an increase of the amount of 3-hydroxyisovaleric acid excreted per 24 h, which was significant at day 3, and greater than the upper limit of normal (112 ± 38 µmol/24 h) by day 10 in all subjects. Increased 3-hydroxyisovaleric acid excretion is the consequence of decreased activity of 3-methylcrotonyl-CoA carboxylase because of insufficient availability of its prosthetic group biotin (Mock *et al*, 1997). In these experimental subjects no clinical signs of overt biotin deficiency evolved.

There are no systematic studies to show if a deficient biotin status can be recognised by increased excretion of other organic acids as a consequence of impaired activity of propionyl-CoA carboxylase (3-hydroxypropionic acid, methylcitrate), which might also lead to an increase in the proportion of odd-numbered fatty acids in plasma lipids. However, as intestinal bacteria produce an unpredictable amount of propionic acid and as odd-numbered fatty acids are contained in variable amounts in dietary fat it can be expected that these measurements would be unreliable in assessing biotin status (Bender, 1999). Measurement of the activities of carboxylases in blood leukocytes might constitute another potential indicator of biotin status. In children with severe protein-energy malnutrition propionyl-CoA carboxylase activity in blood lymphocytes was significantly reduced in all and pyruvate carboxylase

activity in some compared to normal controls and both increased in response to biotin administration. The plasma biotin concentration in these patients did not correlate with carboxylase activity (Velázquez *et al*, 1995).

2.7. Biotin deficiency

2.7.1. Dietary biotin deficiency

Overt dietary biotin deficiency is rare. It does not occur in breastfed infants. It has been observed in association with total parenteral nutrition without biotin and in chronic feeding of raw egg white and it has been seen in an infant fed an amino acid formula and hypoallergenic rice presumably containing no biotin (Higuchi *et al*, 1996). This latter case especially argues that biotin is not provided by intestinal bacteria in sufficient amounts.

Clinical symptoms of biotin deficiency are alopecia and cutaneous abnormalities such as seborrhoeic dermatitis, periorificial erythema, and fungal infection. In adults effects on the central nervous system are expressed as depression, lethargy, muscular pain, hyperesthesia and paresthesia. Symptoms take months to years to become apparent. Biocytin excretion is normal during symptomatic biotin deficiency.

Biotin-deficient infants become symptomatic within a shorter time (3 to 6 months) and show -in addition to hair loss and skin rash- hypotonia, lethargy and developmental delay.

Subclinical biotin deficiency in patients on anticonvulsant therapy (Mock and Dyken, 1997), in patients undergoing chronic haemodialysis (Yatzidis *et al*, 1984), in alcoholics, patients with gastric disease, or inflammatory bowel disease is a matter of some concern (Mock, 1996; Zempleni and Mock, 2000b). A considerable proportion of pregnant women show increased excretion of 3-hydroxyisovalerate in both early and late gestation with a decrease in urinary biotin excretion (Mock *et al*, 1997b). 3-Hydroxyisovalerate excretion was reduced by 300 µg of biotin per day over two weeks (Zempleni and Mock, 2000b). Subclinical maternal biotin deficiency was shown to be teratogenic in several species (chicken, turkey, mouse, rat, hamster) (Mock, 1996; Zempleni and Mock, 2000b).

2.7.2. Biotinidase and holocarboxylase synthetase deficiencies

Both genetic defects in holocarboxylase synthetase (Burri *et al*, 1981) and biotinidase (Wolf *et al*, 1983) result in multiple carboxylase deficiency with a typical pattern of organic acids in urine (and serum) and a wide spectrum of clinical symptoms, ranging from asymptomatic to neonatal death.

Treatment of biotinidase deficiency requires at least physiologic doses of biotin, but treatment is empirical in most cases with 10 mg of biotin given per day.

Holocarboxylase synthetase deficiency requires higher therapeutic doses of biotin, up to 100 mg/day in individual cases, dependent on the severity of the enzyme defect (Baumgartner and Suormala, 1997).

3. HAZARD IDENTIFICATION

Single or repeated doses of biotin (total dose of 50 and 100 mg/Kg of body weight by subcutaneous injection) given to rats resulted in production of irregularities of the oestrus cycle (Paul *et al*, 1973a and b) and resorption of foetuses and placentae in pregnant rats (Paul *et al*, 1973b) accompanied by decreased uterine weight, reduced glycogen and protein in the uterus and reduced protein in the liver. Estrogen treatment prevented loss of pregnancy and normalised these organ parameters (Paul and Duttagupta, 1975). However, these studies cannot be regarded as conclusive for human dietary biotin uptake because of the route of administration and the extreme dosage selected (corresponding to 10^5 times the adequate human daily intake).

The administration of oral biotin in doses up to 100 mg per day to patients with holocarboxylase synthetase and with biotinidase deficiency did not result in adverse effects, although the metabolic defect may have prevented or masked toxicity. The prenatal administration of 10 mg biotin orally per day during the third trimester of pregnancy in pregnant women at risk of carrying a foetus with holocarboxylase synthetase deficiency have not resulted in apparent adverse effects (Baumgartner and Suormala, 1997; Packman *et al*, 1982; Roth *et al*, 1982). However, systematic studies on biotin effects in healthy humans have not been undertaken.

In a recent publication, however, biotin supplementation of 750 µg per day for 14 days to five healthy adults resulted in significant decreases of mitogen-stimulated proliferation of peripheral blood mononuclear cells in all subjects, and a significantly reduced release of interleukin-1β and interleukin-2 in four of five subjects. This was not due to relative changes in differentiation of individual subsets of peripheral blood mononuclear cells nor to inhibition of cellular pantothenic acid uptake via the multivitamin transporter by high biotin levels. The significance of these findings is not known (Zempleni *et al*, 2001).

4. DOSE RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Due to the lack of systematic oral intake dose-response studies of biotin a quantitative risk assessment can not be carried out and it is not possible to derive a numerical UL for biotin.

5. CHARACTERISATION OF RISK

The risk of human toxicity from the usual dietary intake of biotin and from biotin supplements, such as are described in Table 1, appears to be low according to available data. There are insufficient data to draw any conclusions concerning the safety of very high-level supplements.

Although no numerical UL can be established, existing evidence that is available from observational studies indicates that current levels of intake of biotin from all sources do not represent a health risk for the general population.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF MAGNESIUM

(EXPRESSED ON 26 SEPTEMBER 2001)

FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Magnesium (Mg) belongs to group II of the third period of the Periodic Table of Elements, a group that includes two other physiologically important elements: calcium and zinc. Mg has an atomic weight of 24.312; its atomic number is 12; its valency 2. The earth's crust contains approximately 2% Mg and seawater up to 55 mmol/L. The adult healthy body contains approximately 21-28 g (about 1 mole) of Mg; related to an average body weight of 70 kg, this corresponds to circa 14.3 mmol/kg, or to 0.034% of body weight. It is the fourth most abundant cation in the mammalian body and the second most abundant cation in intracellular fluid. Nevertheless Mg deficiency may occur in plants (chlorosis, low crop yields, forest damage), livestock (grass staggers, or grass tetany in ruminants) and man. Depending on the degree of the deficiency, symptoms are latent, moderate or even life threatening because Mg is a cofactor in hundreds of enzymatic reactions, many of which involve energy metabolism. It also plays an important role in protein and nucleic acid synthesis and has a stabilizing and protecting effect on membranes. Finally, Mg is also considered essential in maintaining Ca, K and Na homeostasis (Aikawa, 1981; Durlach, 1988; Seelig, 1989; Wacker, 1980). As early as in the 19th century magnesia was used as antacid and as an antidote against various poisons, e.g. acids and arsenic, and magnesium sulphate as laxative (Epsom salt).

In food derived from plant and animal sources, Mg is mostly bound or chelated, e.g. to phytic acid, phosphates, chlorophylls or it is included in biological apatites (skeleton). In aqueous solutions, Mg salts (e.g., sulphate, chloride, phosphate, citrate, and carbonate) are mostly dissociated depending on the concentration, pH and temperature. Most Mg salts are hygroscopic and have a bitter taste ("Bittersalz", "Bittererde" in German).

2. NUTRITIONAL BACKGROUND

2.1. Food levels and intake estimates

The Mg content of food varies substantially. It is generally accepted that fats, refined sugars and pure alcohol are more or less free of Mg. Foods containing less than 25 mg Mg/100 g wet weight are: meat and most kinds of fish, fruit, most vegetables and dairy products (Seelig, 1980). Chlorophylls contain maximally 0.2% Mg (Aikawa, 1981). Cacao and bitter chocolate, conches, shrimps, soybeans, butter beans, and beet greens contain over 100 mg Mg/100 g. The Mg content of grain and grain products largely depends on food technology processes: high concentrations (110-180 mg/100 g) are found in whole barley, whole rye or wheat flour or brown rice (Seelig, 1980) but high amounts of phytic acid as well as high levels of dietary fibre probably decrease bioavailability (Schümann *et al*, 1997). In Germany the Mg concentration in drinking water is limited to maximally 50 mg/L but geologically caused higher concentrations up to 120 mg/L are tolerated (Trinkwasser VO). Studies in Germany (Schimatschek *et al*, 2001) revealed median-, 5th and 95th percentile concentrations in tap water of 9.9 (2.2 to 28.4) mg/L, n = 14,330 samples and in mineral water of 33.5 (4 to 101) mg/L, n = 150 samples.

Estimates of intake are usually calculated by using data from food questionnaires together with nutrition tables. However, with respect to Mg, these data are probably by 20 to 30% too high because the concentrations measured in food duplicates were considerably lower than values derived from nutritional tables (Glei and Anke, 1995; Schimatschek *et al*, 1997; Stehle *et al*, 1991; Wörwag *et al*, 1999). With these reservations in mind, the following estimates of intake are presented (in mg Mg per day) in Table 1.

Table 1. Estimates of magnesium intake

Country	Mean	2.5th Percentile	97.5th Percentile
Austria[a]	319	116	628
Germany[b]	327	148	558
males	353	188	618
females	288	134	499
Italy[c]	208	117	350
Netherlands[d]	312	139	558
US[e]			
males	323	177 (5^{th} P)	516 (95^{th} P)
females	228	134 (5^{th} P)	342 (95^{th} P)

[a] Elmadfa *et al* (1999) [b] Heseker *et al* (1992) [c] Turrini *et al* (1996)
[d] Hulshof and Kruizinga (1999) [e] FNB (1997)

2.2. Nutritional requirements

Magnesium kinetics represent an open system consisting of several compartments: the intestinal tract (absorption compartment), blood (central compartment), cells, skeleton, central nervous system (deep compartments) and faeces, urine, sweat and milk during lactation (excretion). Mg balance is positive when the input is greater than the output in urine and faeces. This calculation seems simple at a first glance but becomes highly variable aiming to the following individual factors:

a) At low dietary Mg intakes enteral absorption considerably increases from the normal level of 30-40% up to 80% probably via an active transport system (although this has not yet been proven); this system can, however, be completely defective (so-called "primary Mg deficiency") or insufficient ("poor absorbers"). As in the latter cases Mg uptake depends mostly or exclusively on passive diffusion (10-30%) a Mg deficit will result at intake levels which are sufficient for normal individuals (Durlach, 1988; Schimatschek *et al*, 1997; Seelig, 1980; Wörwag *et al*, 1999).

b) Mg turnover also differs individually, depending for example on age, growth, physical activity, pregnancy-lactation, fluid consumption, stress exposure, drugs and diseases (Classen, 1990). Estimates of requirement have therefore been performed on healthy individuals under strictly standardized essentially steady state conditions (FNB, 1997).

c) Mg losses represent an important variable: Diarrhoea or bowel diseases adversely affect absorption. Under physiological conditions the healthy kidney can reduce daily Mg excretion from 5 mmol to less than 0.5 mmol within a few days of low Mg intake. However, this Mg-sparing mechanism may be disturbed genetically, or affected by diseases associated with polyuria such as diabetes mellitus or by drugs (e.g. most diuretics) or alcohol.

The Food and Nutrition Board of the Institute of Medicine (FNB, 1997) in the USA has established the following EAR (Estimated Average Requirement) and the RDA (Recommended Dietary Allowance) based on data obtained under strictly standardized conditions (metabolic unit; adaptation period of at least 12 days; at least two Mg levels) (Table 2).

Table 2. EAR and RDA for magnesium (FNB, 1997)

	EAR (mg)	RDA (mg)
Men, 19-30 years Women, 19-30 years	330 255	400 310
Men, 31-70 years Women, 31-70 years	350 265	420 320
Pregnancy	+ 35 mg	-

The SCF (31^{st} Series, 1993) determined an Acceptable Range of Intake for Adults of 150-500 mg/day.

2.3. Magnesium deficiency

Mg deficiency always includes secondary electrolyte disturbances (since rats respond in a unique to Mg deficiency these experimental data are not considered here). Extracellularly, hypomagnesaemia is frequently associated with hypocalcaemia (as a consequence of disturbed vitamin D metabolism and disturbed parathyroid hormone activity) and sometimes with hypokalaemia (renin-aldosterone-interactions). Intracellularly, K is decreased and the concentration of Na and Ca is increased (owing to decreased activity of Mg-ATP-dependent ionic pumps and "leaky" membranes). In the CNS, the activity of excitatory amino acids (especially glutamate) is enhanced because Mg is a specific blocker of the glutamate-NMDA receptor. Consequently central-nervous and spastic symptoms predominate in Mg deficiency. The clinical diagnosis should be ascertained by reliable biochemical tests. Although serum/plasma Mg represents only about 0.3% of total body Mg it is today generally accepted that hypomagnesaemia is evidence for a Mg deficit (if pseudohypomagnesaemia owing to hypoalbuminaemia is excluded). Under certain conditions, a Mg deficit may, however, exist despite actual normomagnesaemia; such a diagnosis requires special tests, e.g. retention tests using balance techniques (FNB, 1997; Schümann *et al*, 1997; Spätling *et al*, 2000; Wörwag *et al*, 1999).

Mg deficiency, respectively hypomagnesaemia, generally conditions the body for stress reactions (Classen, 1990; Solymoss *et al*, 1969). Epidemiological studies have revealed significant relations between hypomagnesaemia and increased health risks, for example:

- Low concentration of serum/plasma-Mg and increased cardiac disease (FNB, 1997; Ford, 1999; Gartside and Glueck, 1993; Gottlieb *et al*, 1990; Liao *et al*, 1998; Ma *et al*, 1995; Seelig, 1989; Tjuji *et al*, 1994).

- Low concentration of serum/plasma-Mg and hypertension (Joffres *et al*, 1987; Ma *et al*, 1995; Witteman and Grobbee, 1990) or increased risk of stroke (Ascherio *et al*, 1998).

- Low concentration of serum/plasma-Mg and gestational complications (Seelig, 1980; Spätling *et al*, 1989).

3. BIOLOGICAL CONSIDERATIONS

3.1. Interactions with other electrolytes and drugs

Because Mg has been apostrophized "the natural Ca antagonist", it is frequently claimed that calcium inhibits enteral Mg absorption and *vice versa*. This is not the case under physiological conditions as proven in volunteers Spencer *et al* (1994). On the contrary, as Mg is required for the renal hydroxylation of vitamin D and for the activity of parathyroid hormone, Ca-resistant hypocalcaemia can be compensated by Mg supplements (Schimatschek *et al*, 1997). Similarly, potassium does not inhibit Mg absorption in monogastric mammals, which is in contrast to ruminants and plants.

On the other hand, clinically significant interactions occur between iron and magnesium, Mg-hydroxide or Mg-trisilicate *in vitro* (Disch *et al*, 1994), under experimental (Chadwick *et al*, 1982; Corby *et al*, 1985/86; Hall and Davis, 1969) and also under clinical conditions (Thurnher and Kresbach, 1961; Wallace *et al*, 1998). In a randomized controlled crossover study on 13 healthy adult male subjects, serum-Fe concentrations were determined following the oral administration of 5 mg Fe/kg bw over 12 hours alone, or followed 1 hour later by the oral administration of 4.5 g of $Mg(OH)_2$ per g elemental iron ingested. Mean AUC ± SEM amounted to 144 ± 33 µmol (hr)/L in the controls *versus* 78 ± 23 µmol (hr)/L in the Mg group, $p = 0.03$ by signed rank test. In other words, Fe absorption was inhibited by 46%. Probably a Mg:Fe-precipitate was formed under these conditions, as a water soluble Mg salt did not interfere with Fe-gluconate, neither *in vitro* nor under *in vivo* conditions (Disch *et al*, 1994 and 1996). Interactions with zinc absorption owing to the inhibition of gastric acid by Mg may occur (Sturniolo *et al*, 1991) as well as interactions with certain drugs like tetracycline, penicillin and digoxin (Griffin and D'Arcy, 1981).

Both Mg (see later) and sulphate (Cocchetto and Levy, 1981) may exert an osmotic effect in the intestine resulting in laxation. Thus the osmotic effect of $MgSO_4$ is greater than that of other Mg salts due to the additional osmotic effect of sulphate. High intakes of sulphate ion are required to cause diarrhoea and sulphate, like Mg, is better tolerated when consumed in divided doses. For example, a single dose of 8.0 g sodium sulphate (56 mmol) caused severe diarrhoea in normal adults but when consumed as four equally divided hourly doses providing 2.0 g of sodium sulphate (16.8 mmol) per dose it caused only mild or no diarrhoea (Cocchetto and Levy, 1981). Furthermore, over 4 g sodium sulphate (>30 mmol) was well tolerated in normal adults when consumed in drinking water at a concentration of 1.8 g/l (12.5 mM) throughout the day (Heizer *et al*, 1997).

3.2. Acid-base alterations

The pH of the extracellular fluid is determined by the concentration and chemical properties of the acids and bases dissolved in it. In general, carbonic acid is regulated by pulmonary ventilation. Metabolizable acids -being absorbed from the diet or arising in intermediary metabolism- are regulated by intermediary metabolism. Non-metabolizable acids and bases are absorbed from the diet, they cannot be disposed of by intermediary metabolism or by pulmonary ventilation and hence must be disposed of by renal mechanisms (Shaw, 1989). The principal inorganic bases contributing to the balance are Na, K, Ca and Mg (Sack and Stephensen, 1985) and the principal non-metabolizable acids are hydrochloric acid, phosphoric acid and sulphuric acid. In plasma, [c], i.e. the concentration (mmol/L) of non-metabolizable bases, amounts to:

$[cNa^{+} + cK^{+} + 2 \times cCa^{2+} + 2 \times cMg^{2+}] - [cCl^{-} + 2 \times cSO_4^{2-} + 1.8 \times cP]$
$[140 + 4.5 + (2 \times 2.5) + (2 \times 0.75)] - [102 + (2 \times 0.9) + (1.8 \times 3.4)] = 41$ mmol/L

The anion gap is covered by proteins and organic metabolizable acids. From these data, it can be concluded that the supply of higher amounts of earth alkali and alkali metals tends to alkalinization whereas chloride and o-phosphate favour acidification.

A tendency towards Mg-induced extracellular compensated metabolic alkalosis may be advantageous [e.g., reduction of post-exercise acidosis (Ball and Maughan, 1997); renal Mg conservation (Durlach, 1988); inhibitory effects of Mg plus citrate on Ca oxalate formation (Durlach, 1988)] or it may be harmful [e.g. supporting the development of the tetany syndrome due to decreased concentrations of ionized Mg and Ca (Durlach, 1988; Wacker, 1980), inducing hypokalaemia owing to K-shift into the intracellular space (Urakabe *et al*, 1975); increasing cardiotoxicity of catecholamines (Schimatschek *et al*, 1987)]. Finally the development of the milk-alkali syndrome following ingestion with milk and ice cream has been reported (Yamada *et al*, 1991). It has also been discussed whether high doses of citrates might increase the intestinal absorption of toxic metals like aluminium (Sakhaee *et al*, 1996) or whether alkalinization of the urine favours urinary infections (Sökeland and Sulke, 1992). Alkalinization of the urine affects renal clearance of drugs that are weak acids or bases. Therapy with alkalinizing Mg compound increases e.g. the rate of elimination of salicylates and phenobarbital and decreases the elimination of amphetamine, ephedrine, mecamylamine, pseudoephedrine, and quinidine (Goodman and Gilman, 1990).

A tendency towards compensated extracellular acidoses, e.g. by $MgCl_2$, markedly increases the cardioprotective capacity of Mg salts (Seelig, 1989), as well as antitetany effects (Schimatschek *et al*, 1997), the urinary excretion of weak acids is facilitated and enteral Mg absorption is improved. However high chloride load evokes magnesiuric and calciuric responses and favours Ca-oxalate formation (Classen *et al*, 1995; Houillier *et al*, 1996).

In summary it becomes evident that acid-base metabolism must be evaluated considering the sum of acids and bases; it also becomes evident that with Mg salts one has to consider the respective anion in addition to the cation.

4. HAZARD IDENTIFICATION

Magnesium in foods derived from plant or animal sources has not been demonstrated to induce diarrhoea nor other adverse effects in healthy persons, probably as Mg is bound to matrices and hence is mostly not easily dissociable (see Introduction). On the other hand, easily dissociable magnesium salts (e.g. chloride or sulphate; included are compounds like MgO becoming readily dissociable after the reaction with gastric hydrochloric acid) which are present in water, many supplements and drugs, exert dose-dependent laxative effects. Fine *et al* (1991) analyzed stool samples of 19 normal subjects ranging in age from 23 to 36 years. Mean faecal Mg output amounted to 136 mg/day with a standard deviation (SD) of 73 mg. Using 3 x SD, the upper limit of normal faecal Mg excretion was calculated to amount to 345 mg Mg/day. The mean normal concentration of Mg amounted to 362 mg Mg/L, the standard deviation was 245 mg/L and the upper normal level (mean + 3 SD) was 1,097 mg Mg/L of formed stool. When the volunteers received daily doses of (rounded) 1,200 mg, 2,300 mg or 47,000 mg Mg (as hydroxide) diarrhoea was induced. Fine *et al* concluded that for each 24.3 mg increase in faecal Mg output faecal weight increased by approximately 7.3 g.

One of the first cases of accidental poisoning with Mg sulphate was published by Sang in 1891: a 35 years old woman died 75 minutes after drinking 4 ounces of ordinary Epsom salt (about 120 g) dissolved in a tumbler of hot water. Severe poisoning requiring artificial respiration also occasionally occurred

following the intraduodenal administration of magnesium sulphate during deworming (Thurnher and Kresbach, 1961). According to Stevens and Wolf (1950) seven cases of poisoning with Epsom salt with 5 fatalities were published between 1841 and 1909.

Animal experiments have proven a significant cubic relation between the logarithm of orally administered Mg and the Mg concentrations in plasma and bone (Classen *et al*, 1983). In other words, oral Mg supply has to be considerably increased to increase plasma Mg. Depending on plasma/serum Mg levels the following dose-response relations can be established (Spätling *et al*, 2000; Woods, 1991):

0.76 - 1.10 mmol/L	Reference value
0.80 - 1.10 mmol/L	Optimal concentration
1.10 - 2.50 mmol/L	Therapeutic range (infusion therapy)
2.50 - 3.50 mmol/L	Decreased neuromuscular transmission
3.50 - 7.00 mmol/L	Curare-like effect requiring artificial respiration
10.0 - 12.5 mmol/L	Cardiac arrest

In 6 healthy volunteers about 4% of a 56.5 mmol oral dose of $MgSO_4$ (ca. 1,400 mg of Mg) given in 4 hours was enterally absorbed (Morris *et al*, 1987) without inducing hypermagnesaemia. In the literature, only few cases of toxic hypermagnesaemia (>2.5 mmol/L) have been published, mostly owing to the (ab-)use of Mg as laxatives or antacids in single doses of >100 mmol Mg (ca. 2,500 mg). Woodard *et al* (1990) observed maximal blood levels of 2 mmol Mg/L in 102 patients receiving ca. 380 mmol Mg daily (ca. 9,200 mg of Mg; multiple doses, therapy of drug overdose) and Smilkstein *et al* (1988) observed levels up to 2.5 mmol/L after daily doses of up to 360 mmol $MgSO_4$ (ca. 8,800 mg of Mg). Symptoms were hypotension, nausea and vomiting. Hypoventilation and respiratory depression were reported by Jones *et al* (1986) and by Gren and Woolf (1989) in young women treated with Mg citrate (136 mmol, ca. 3,300 mg of Mg) for salicylate and tricyclic overdose; serum Mg levels were 5.7 and 4.0 mmol/L, respectively. Fung (1995) reported the case of a 69 years old multimorbid woman who took about 990 mmol Mg (ca. 24,000 mg of Mg) daily as an antacid; serum levels increased up to 6.7 mmol/L and caused hypoventilation. The patient recovered. Clark and Brown (1992) identified 12 elderly patients (70 ± 6 yr) among 19,761 hospital admissions with hypermagnesaemia (maximally 3.3 mmol/L); oral daily Mg doses (citrate, hydroxide) ranged between 84 and 256 mmol (2,000 to 6,300 mg of Mg). Hypotension was the most frequent clinical sequelae; 2 patients died due to refractory hypotension to which hypermagnesaemia may have contributed. As bowel disorders were present in most patients it is speculated that active ulcer disease, gastritis, colitis, etc. may enhance Mg absorption. Severely impaired renal function (inulin clearance <10 mL/min) is another risk factor (Aikawa, 1981; Randall *et al*, 1964). High age *per se* is however not a risk factor since Kinnunen and Salokannel (1987) did not observe hypermagnesaemia in 64 geriatric patients (mean age 81 years) receiving daily doses of 28 mmol Mg hydroxide (ca. 680 mg of Mg).

5. DOSE-RESPONSE ASSESSMENT

Easily dissociable magnesium salts, especially the sulphate ("Epsom salt", "Bittersalz") are used as "osmotic" and "saline" laxatives, respectively. Nevertheless mild diarrhoea can be taken as the most sensitive non-desirable effect if Mg supplements are taken for nutritional purposes. However it must be kept in mind that adaptation of the bowel to higher oral Mg intake is known (Nadler *et al*, 1992; Stendig-Lindberg *et al*, 1993; Widman *et al*, 1993), that a mild laxative effect may be desirable ("four patients reported mild diarrhoea in the Mg group, and a similar number felt that their bowel function improved with less constipation"; Gullestadt *et al*, 1991), that mild laxative effects have been frequently observed also in the placebo groups (perhaps caused by taste adjusters, vehicles a.o.) (Sibai *et al*, 1989), that a given daily dose of Mg is better tolerated when it is divided into several portions, and finally that the galenic form (aqueous solution, capsules, tablets, etc.) may play a role. Data from the literature are summarized in Table 3 (next page) including children, pregnant women, tetanic, hypertensive and cardiac patients as well as volunteers. Papers were only considered when the presence or absence of "mild diarrhoea" was stated. Table 3 does not include Mg contained in food derived from plant or animal sources this being considered to be poorly dissociable (e.g. phytates).

As discussed, mild diarrhoea is the most sensitive non-desirable effect of orally administered easily dissociable magnesium salts. From the data presented in Table 1 one can conclude that mild diarrhoea occurs in a small percentage of adult subjects at oral doses of about 360/365 mg Mg per day, hence presenting the LOAEL.

No laxative effects have been observed in adult men and women -also during pregnancy and lactation- at doses up to 250 mg Mg per day. Therefore, this dose is considered as being the no-observed-adverse-effect level (NOAEL).

6. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The NOAEL was derived from studies in which pharmaceutical type of dosage formulation was taken in addition to Mg present in normal foods and beverages. The amounts in food and beverages were not measured or taken into account in the calculation of the NOAEL and therefore the UL for Mg cannot be derived for the intake from all sources. Based on a NOAEL of 250 mg Mg per day and an uncertainty factor of 1.0 an UL of 250 mg Mg per day can be established for readily dissociable magnesium salts (e.g., chloride, sulphate, aspartate, lactate) and compounds like MgO in nutritional supplements, water, or added to food and beverages. This UL does not include Mg normally present in foods and beverages. An uncertainty factor of 1.0 is justified in view of the fact that data are available from many human studies involving a large number of subjects from a spectrum of lifestage groups, including adults, pregnant and lactating women, and children. In addition, the NOAEL is based on a mild, transient laxative effect, without pathological sequelae, which is readily reversible and for which considerable adaptation can develop within days. This UL holds for adults, including pregnant and lactating women, and children from 4 years on.. As no data were available for children from 1 to 3 years, and since it was considered that extrapolation of the UL for older children and adults on the basis of body weight was inappropriate, no UL could be established for this age group.

Table 3. Mild diarrhoea induced by daily oral magnesium supplements

Total Mg Dose* (mg/day)	Diarrhoea (n)	Doses per day	Form	Subjects		Salt	Weeks	Ref.
				Mean age (range) (yr)	Gender			
180	0/130	3	Tablets	5.3-17.4	M, F	Asp. HCL#	3	1
245	0/112	2	Granules	8.1 (4-12)	M, F	Asp.HCL	3	2
245	0/181	2	Granules	4-12	M, F	Asp.HCL	3	3
250	0/31	1	Tablets	58	F	Hydroxide	72	4
360	1/32	3	Granules	37 (18-65)	M, F	Pyrrolidone carboxylic acid salt	4	5
365	0/17	3	Tablets	52 (33-66)	M, F	Asp.HCL	4	6
365	0/39	3	Granules	40 (20-59)	M, F	Asp.HCL	8	7
365	1/278	3	Tablets	28 (20-38)	F	Asp.HCL	26	8
365	4/17	3	Tablets	71 (56-88)	nd	Lactate Citrate	6	9
365	4/22	nd	Tablets	62	M, F	Hydroxide	12	10
384	1/25	6	Ent.coated	21	F	Chloride	4	11
384	2/21	Divided	Ent.coated	63 (42-73)	M, F	Chloride	6	12
400	2/20	nd	Ent.coated	46 (26-65)	M, F	Chloride Oxide	8	13
476	18/50	2	Capsules	30 (21-50)	M, F	Oxide	8.5	14
480	2/12	nd	nd	16 (11-21)	M, F	Asp.HCL	12	15
480	2/37	2	Granules	28.5 ± 4.5	F	Asp.HCL	4	16
500	2/20	3	Capsules	57	M, F	Oxide	12	17
576	0/5	3	Tablets	54 (38-75)	M, F	Oxide	6	18
970	Adaptation to doses	1-3	Tablets	50	M, F	Hydroxide	3x3	19
1095	8/8	nd	Tablets Granules Capsules	nd	M, F	Asp.HCL	1	20

* Referred to elemental Mg; nd = no data; M = males; F = females; # Asp. = aspartate.

1 Classen *et al* (1986) 2 Schimatschek *et al* (1997) 3 Schimatschek *et al* (2001) 4 Stendig-Lindberg *et al* (1993)
5 Fehlinger *et al* (1988) 6 Cappuccio *et al* (1984) 7 Plum-Wirell *et al* (1994) 8 Spätling and Spätling (1988)
9 Gullestad *et al* (1991) 10 Rasmussen *et al* (1989) 11 Ricci *et al* (1991) 12 Bashir *et al* (1993)
13 Nadler *et al* (1992) 14 Marken *et al* (1989) 15 Rueddel *et al* (1990) 16 Spätling *et al* (1998)
17 Daly *et al* (1990) 18 Spencer *et al* (1994) 19 Widman *et al* (1993) 20 Muehlbauer *et al* (1991)

7. CHARACTERISATION OF RISK

Diarrhoea induced by easily dissociable Mg-salts or compounds like Mg-oxide is completely reversible within 1 to 2 days and does not represent a significant health risk in subjects with intact renal function. Poorly dissociable Mg salts (e.g. phytates) have a lower, if any, potential to induce diarrhoea.

While the UL is expressed as a daily intake it should be noted that most of the studies used in its derivation involved daily intake obtained from two or more doses. Therefore the UL should apply to daily intake of Mg consumed on two or more occasions. This is of greater importance for the sulphate salt than for other readily dissociable salts of Mg, given the additional osmotic effect of sulphate ion.

No UL could be established for 1-3 year old children. Although the incidence of diarrhoea is generally higher and its effects potentially more significant in this age group than in older children or adults, there is otherwise no basis for considering that they are more susceptible to the laxation effects of Mg.

Toxic hypermagnesaemia, presenting e.g. with hypotension or muscular weakness, is only seen at oral Mg doses greater than 2,500 mg, i.e. doses exceeding the UL by a factor of more than 10.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF PANTOTHENIC ACID

(EXPRESSED ON 17 APRIL 2002)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Pantothenic acid, sometimes designated as vitamin B_5 (in some text books also as B_3), is N-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-β-alanine. D-Pantothenic acid (MW 219.23) is the only occurring natural form. Free pantothenic acid and its sodium salt are chemically unstable, and therefore the usual pharmacological preparation is the calcium salt (calcium pantothenate). The alcohol, panthenol, is a synthetic form which can be oxidised *in vivo* to pantothenic acid. It is included in the list of substances that may be used in the manufacture of foods for particular nutritional uses and in food supplements (the legal measure on food supplements is expected to be adopted in the immediate future). Panthenol is widely used in cosmetic products.

Calcium pantothenate, commercially available as the D-isomer or D,L-racemate, is stable in neutral solution, and destroyed by heat, and at alkaline or acid pH. In most dietary sources, and in biological tissues, pantothenic acid is present as coenzyme A and 4'-phosphopantetheine.

2. NUTRITIONAL BACKGROUND AND METABOLISM

Pantothenic acid was first shown to be an essential factor in 1933 for the growth of yeast and in curing of (deficiency-induced) dermatitis in chickens. Pantothenic acid plays a central role in intermediary metabolism as part of the coenzyme A (CoA) molecule and as part of the pantotheine functional group in the acyl-carrier protein (Acyl-CP). This vitamin serves therefore as a cofactor in acyl-group activation and transfer in fatty acid and carbohydrate metabolism, as well as in a wide range of (other) acylation reactions (see Fox, 1984 and Plesofsky-Vig, 1996 for reviews). The synthesis of CoA is regulated by pantothenate kinase, which is under control of the end products (CoA and acyl-CP).

Pantothenic acid is widely distributed among foods, especially high concentrations are found in yeast and organ meat (liver, kidney), but eggs, milk, whole grain cereals and vegetables (e.g. broccoli) are good sources. In most foods it is present in bound form (as CoA), requiring enzymatic treatment for analysis of total contents. Ingested pantothenic acid is first hydrolysed to pantotheine and subsequently to free pantothenic acid by pantotheinase in the intestinal lumen. Although in earlier studies simple diffusion was reported to be the main transport system, there is now ample evidence that transport is effected in mammals through a saturable, sodium dependent transport system in the jejunum (Fenstermacher and Rose, 1986; Stein and Diamonds, 1989). The intestinal flora can produce pantothenic acid and animals practising coprophagy such as, rats and mice, can use faeces as a "dietary" source. It is not yet clear whether there is also direct uptake in the colon.

Data on bioavailability of pantothenic acid from foods in humans is limited. In one study (Tarr *et al*, 1981) availability of pantothenate from an average American diet was assessed by comparing urinary excretion levels after controlled feeding (during 5 weeks) of an average American diet, containing 11.5 mg pantothenate, and a formula diet supplemented with 6.0 mg pantothenate (total intake 8.2 mg), respectively. An average "relative" bioavailability of 50% (range: 40-61%; n=6) was calculated, assuming 100% availability from the synthetic form. About 60% of an oral dose of 10 and 100 mg/day, respectively, was excreted as intact pantothenic acid (Fry *et al*, 1976). Urinary excretion (normal range between 2-7 mg [9-32 μmol]/day) reflects dietary intake, although a wide range of individual variation has been noted.

In blood, pantothenic acid occurs both in plasma and in red blood cells. Whole blood concentrations are reported to be ca. 2 μmol/L, and is considered to reflect status, although there are only few data available to substantiate this conclusion.

Deficiency in humans is rare because of the widespread availability of pantothenic acid in the usual diet. In underfed World War II prisoners of war in the Philippines painful burning sensations in their feet ("burning feet syndrome") and numb toes have been ascribed to a pantothenic acid deficiency. Symptoms reported from an experimentally induced deficiency in humans using the antagonist ω-methylpantothenate in combination with a pantothenic acid deficient diet, include non specific symptoms such as headache, fatigue, insomnia and paresthesia of hands and feet. Increased insulin sensitivity and a decreased eosinopenic response to ACTH, and loss of antibody production, have also been reported (see Plesofsky-Vig, 1996). In animals diet-induced deficiency symptoms are hypertrophy of the adrenal cortex and increased resistance to viral infections in rats; hypoglycaemia, gastrointestinal symptoms and convulsions in dogs, and dermatitis and poor feathering in chickens.

There are only limited data on the pantothenate requirements and the SCF could not establish a recommended intake. Average intakes in adults are about 4-7 mg/day, with a range of 3-12 mg/day. Such intakes were considered adequate to prevent deficiency, including during pregnancy and lactation (SCF, 1993). Mean (97.5 percentile) intake from food in Great Britain (UK 1986/87 survey; Gregory *et al*, 1990) were 6.3 (10.5) and 4.5 (7.7) mg/day for men and women, respectively; and 6.6 (11.2) and 5.1 (9.1) mg/day, respectively, from all sources (food and supplements). For Ireland mean intakes from all sources (food and supplements) were reported as 6.5 (12.5) mg in men and 5.3 (14.4) mg in women, and from food sources only as 6.1 (10.4) and 4.3 (7.2) mg/day, respectively (IUNA, 2001).

Recently the Institute of Medicine Committee (IOM, 1998) estimated the level of adequate intake (AI) for pantothenic acid at 5 mg/day for adults, based upon replacement of the amount lost by urinary excretion.

3. HAZARD IDENTIFICATION

In the studies on pantothenic acid which were reviewed it was not specified whether D or D,L forms were used and therefore the conclusions drawn relate to both isomers.

3.1. Data from studies in animals

For mice and rats, a subcutaneous LD_{50} has been reported for pantothenic acid of 2.7 g/kg bw and 3.4 g/kg bw, respectively; the oral LD_{50} for mice was 10 g/kg bw, death due to respiratory failure (Unna and Greslin, 1941). Following repeated oral dosing in rats (50-200 mg/day for 190 days), in dogs (50 mg/kg bw/day for 6 months), and in monkeys (1 g/day; 250-400 mg/kg bw/day for 6 months) no toxic effects were reported (Unna and Greslin, 1941).

According to the "Cosmetic ingredient review" (1987) on panthenol and pantothenic acid no teratogenic or foetotoxic effects are known for rats fed calcium pantothenate before mating and throughout gestation. No abnormal chemical, histochemical and histological abnormalities were observed in the liver, adrenal, duodenum and tibia of the young rats at birth, born from females receiving 100 μg or 1 mg calcium pantothenate daily during pregnancy (Everson *et al*, 1954; Chung *et al*, 1954). In the offspring of the group of rats treated with 50 mg/day, which were fed with the same daily dose as soon as they were weaned, a normal development was observed and weight gain comparable to the control group (Unna and Greslin, 1941).

No toxicological effects have been reported for D- and D,L-panthenol in subchronic oral toxicity studies in rats with dosages between 20-200 mg/day for 90 days; and with 2 mg/day for 6 months (studies cited in the "Cosmetic ingredient review", 1987)

3.2. Data from studies in humans

No data have been reported on pantothenic acid or panthenol toxicity in humans. A Medline and Toxline search from 1966 on did not reveal any report on adverse effects after oral intake of pantothenic acid or panthenol.

High dosages were used in a placebo-controlled, double-blind trial on the potential beneficial effect of pantothenic acid in treatment of patients with arthritic symptoms (General Practitioner Research Group, 1980). In this study 94 patients were treated for 8 weeks with dosages of calcium pantothenate, starting with 500 mg/day in the first two days, then 1 g/day for the next three days, 1.5 g/day the following four days, and 2 g/day from day 10 until the end of the trial (47 patients treated; 46 on placebo). In this study

no side effects of treatment were noted, while some evidence was obtained for a beneficial effect on pain and disability in the subgroup of rheumatoid arthritis patients. Other therapeutic trials, such as in wound healing, using dosages between 0.2-0.9 g/day also reported no adverse (nor beneficial) effects (Vaxman, 1996).

In one study in children with attention deficit disorders (n=41) treated with 1.2 g pantothenic acid per day, in combination with 3 g nicotinamide, 3 g ascorbic acid and 0.6 g pyridoxine, increased serum transaminase levels were reported for 17 children, treated for 12 weeks (Haslam *et al*, 1984). Whether this hepatotoxic effect was related to the high dose of pantothenic acid, or to the combination with the high doses of nicotinamide, vitamin C and pyridoxine, cannot be concluded from this study, and therefore, this study cannot be used in risk assessment of pantothenic acid. Occasional diarrhoea and water retention might occur at daily dose levels of 10-20 g/day, as found in studies on stress protection and prevention of greying of hair (studies mentioned in a review by Fox, 1984, and in a Cosmetic Ingredient review on the safety of panthenol and pantothenic acid, 1987).

4. DOSE RESPONSE AND DERIVATION OF TOLERABLE UPPER INTAKE LEVEL (UL)

Owing to the lack of systematic oral dose response intake studies and the very low toxicity of pantothenic acid (calcium pantothenate or panthenol) no LOAEL and NOAEL can be established and no numerical UL can be derived.

5. CHARACTERIZATION OF RISK

Pantothenic acid apparently has a very low toxicity and minor adverse gastrointestinal effects such as occasional diarrhoea and water retention occurred only at very high intake levels (10-20 g/day). Average intakes in adults range between 3-12 mg/day, and this intake level is considered as adequate. Few data on distribution of intakes from dietary and supplement sources are available. In Ireland the 97.5 percentile of intakes from all sources (food and supplements) was reported 12.5 mg in men and 14.4 mg in women, and from food sources only as 10.4 mg and 7.2 mg per day, respectively (IUNA, 2001). For the UK the 97.5 percentile of intakes reported for all sources (food and supplements) was 11.2 mg in men and 9.1 mg in women, and from food sources only as 10.5 mg and 7.7 mg per day, respectively (Gregory *et al*, 1990).

Although it is not possible to derive a numerical UL for pantothenic acid evidence available from clinical studies using high doses of pantothenic acid indicates that intakes considerably in excess of current levels of intake from all sources do not represent a health risk for the general population.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF NICOTINIC ACID AND NICOTINAMIDE (NIACIN)

(EXPRESSED ON 17 APRIL 2002)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Niacin is the term used to describe two related compounds, nicotinic acid and nicotinamide, both of which have biological activity. Niacin is not strictly speaking a vitamin because it is formed from the metabolism of tryptophan, and is not *per se* essential to the body, providing that there is an adequate supply of the essential amino acid tryptophan (Horwitt *et al*, 1981). Niacin is the precursor for two cofactors, NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate), which are essential for the functioning of a wide range of enzymes involved in redox reactions.

The condition that is characteristic of a deficiency of both tryptophan and preformed niacin is pellagra, which was originally recognised in 1735 and is characterised by spinal pains, “magenta tongue”, digestive disturbances and subsequently erythema with drying and expurgation of the skin. Various nervous manifestations, such as spasms, ataxic paraplegia and mental disturbances occur in severe cases. The deficiency disease occurred in Italy, Southern France, Spain and in the southern states of the United States of America where over 170,000 cases were reported annually between 1910 and 1935. In the 1930s the recognition of the essential role of nicotinic acid in relation to a related condition, canine black-tongue, and the essential role of nicotinamide in the cofactors NAD and NADPH led to the recognition of the nature of the deficiency, and the establishment of niacin as a vitamin (Smith *et al*, 1937). Short-term (3 weeks) niacin deficiency in the elderly may lead to an increase in serum triglycerides in some subjects (Ribaya-Mercado *et al*, 1997).

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

The co-enzymes NAD and NADPH are involved in a large number of redox reactions essential for the normal functioning of mammalian cells. In addition, NAD is the source for ADP-ribose, which is used in repairing DNA breakage caused by mutagens and other toxins.

Niacin is present in food largely as bound forms that require hydrolysis to release the free nicotinamide or nicotinic acid prior to absorption. In animal tissues niacin is present mainly as the coenzymes NAD and NADP (Henderson, 1983; Turner and Hughes, 1962). There are negligible concentrations of free nicotinic acid in crops such as cereals. Boiling releases most of the total niacin present in sweet corn as nicotinamide (up to 55 mg/kg) but very little as nicotinic acid (<5 mg/kg) (Kodicek *et al*, 1974; Mason *et al*, 1973). The niacin in cereals such as wheat, barley and oats does not give free nicotinic acid or nicotinamide on cooking (Mason *et al*, 1973). Roasted coffee contains higher concentrations of free nicotinic acid (160-400 mg/kg) (Smith, 1963).

Nicotinamide may be obtained from the diet where it is present primarily as NAD and NADP, which are hydrolysed in the intestine and the resulting nicotinamide absorbed either as such, or following its hydrolysis to nicotinic acid. In addition, the niacin in cereals is present as a glycoside of nicotinic acid, which undergoes limited hydrolysis *in vivo* and is essentially not absorbed from the gastrointestinal tract and is not bioavailable (Yu and Kies, 1993). Nicotinic acid itself is rapidly absorbed from both the stomach and the upper small intestine (Bechgaard and Jespersen, 1977). The conversion of nicotinic acid to nicotinamide occurs subsequent to its formation as a pyridine nucleotide; nicotinic acid reacts with 5-phosphoribosyl-1-pyrophosphate to form the nicotinic acid mononucleotide, which then condenses with ATP to form the nicotinic acid analogue of NAD, which is subsequently converted to NAD by a reaction with glutamine and ATP. In contrast, nicotinamide is converted to the pyridine nucleotide simply by reaction with phosphoribosyl-1-pyrophosphate. The

cofactor NAD is converted to NADP by reaction with ATP. Nicotinamide can be formed from NAD via enzymatic cleavage to nicotinamide and adenosine diphosphate ribose.

The major pathway of metabolism of nicotinamide is by methylation in the liver to form N^1-methyl nicotinamide via reaction with methionine (as a methyl donor) and ATP. N-Methyl nicotinamide does not have biological activity and is a polar, water-soluble excretory product. It may be further oxidised in the 6 position of the pyridine ring to give N^1-methyl-6-pyridone-3-carboxamide. High doses of nicotinic acid are excreted in the urine, as nicotinic acid and its glycine conjugate (nicotinuric acid) (Figge *et al*, 1988; Stern *et al*, 1992).

Because of the metabolic formation of niacin from tryptophan, the dietary requirements for niacin are complex and related to the dietary content of both tryptophan and niacin (neglecting niacin in cereals, which is largely not bioavailable). By convention the total niacin equivalents in the diet is taken as the sum of preformed niacin plus 1/60 of the tryptophan content (Horwitt *et al*, 1981; SCF, 1993). There is no absolute requirement for preformed niacin in the diet, and the 1993 SCF evaluation recommended intakes of niacin equivalents between 9 and 18 mg/day. However, the SCF report stated "it is likely there is no requirement for any preformed niacin in the diet under normal conditions and that endogenous synthesis from tryptophan will meet requirements".

Intake data are available for a number of European countries (Table 1), which indicate that average intakes are about twice those recommended in the 1993 report of the SCF. The data also show that food supplements (that contain nicotinamide) represent a minor source of intake, even at the 97.5th percentile of intake.

Table 1. The daily intakes of niacin equivalents in EU countries (mg/day)

	Population	n	Method	Supplements*	Mean or Median	97.5%
Austria[a]	individual	2488	24h recall	Not defined	30.6	66.3
Germany[b]	men	856			15.0	
	women	1138			11.6	
Germany[c]	men	1268		-	39.2	28.8
	women	1540		-	27.8	20.5
	men	240		+	39.5	
	women	347		+	27.8	
Italy[d]	Household	2734	7-day record	+	19	31
Netherlands[e]	Household	5958	2-day record	-	16.7	36.7
UK[f]	men	1087	7-day record	-	39.9	62.2
	women	1110		-	28.5	46.4
	men	1087		+	40.9	67.4
	women	1110		+	30.3	51.2
Ireland[g]	men		7-day record	-	27.1	46.7
	women			-	18.6	32.3
	men			+	28.2	60.0
	women			+	20.7	44.0

Results are for intake as preformed niacin and from metabolism of dietary tryptophan.
* + data included supplements; - data excluded supplements.
[a] Elmadfa *et al* (1998)
[b] Heseker *et al* (1992)
[c] Mensink and Ströbel (1999)
[d] Turrini (INRAN)
[e] Hulshof (1999) (preformed only)
[f] Gregory J *et al* (1990)
[g] IUNA (2001) (preformed only)

3. HAZARD IDENTIFICATION

All of the available data on hazard identification and characterisation relate to studies following the administration of either nicotinic acid or nicotinamide.

3.1. Data from studies in animals

There is a very limited animal toxicity database on either nicotinic acid or nicotinamide, with the majority of the available studies published before 1960. Weight loss, convulsions and death were reported in dogs given nicotinic acid at 2 g per day for 20 days, but not in dogs treated with up to 1 g per day for 8 weeks (Chen *et al*, 1938), or in dogs given sodium nicotinate at a dose of 1 g per kg body weight for 63 days (Unna, 1939). Toxicity was not found in rats given 1 g per day of sodium nicotinate in the diet for 40 days (Unna, 1939), whereas nicotinamide inhibited the growth of rats when given at 1% in the diet for 28 days (Handler and Dann, 1942). Suppression of growth in rats was reported when nicotinamide was incorporated into the drinking water to give an intake of 0.62 g/day (Petley and Wilkin, 1992). A study has reported that nicotinamide was not carcinogenic when mice were given a 1% nicotinamide solution as drinking water for their life-span (Toth, 1983). Data on genotoxicity have not been identified.

Niacin deficiency results in birth defects (Chamberlain and Nelson, 1963) and impaired viability; nicotinamide is transferred actively across the placenta (Hill and Longo, 1980; Kaminetzky *et al*, 1974) and into breast milk (Deodhar *et al*, 1964). However there are only limited data on the effects of excessive niacin either *in utero* or during neonatal development. Abnormal neural tube closure defects and other abnormalities were reported when chick egg white was replaced with a solution containing 20 mg of nicotinic acid (Hansborough, 1974) but such experiments are not of value for hazard identification. The limited data available from animal studies did not indicate that either nicotinamide or nicotinic acid was teratogenic, but these were observations from old studies (cited in Schardein, 2000) that would not be considered adequate for risk assessment purposes.

3.2. Data from studies in humans

The principal identification of hazards associated with excessive intakes of niacin have arisen from studies in which high doses of nicotinic acid have been used for its therapeutic effects in lowering blood cholesterol and blood hyperlipidaemias. The most comprehensive study was that conducted by the Coronary Drug Project Research Group (1975). A number of hazards have been reported to be associated with high doses of nicotinic acid. These have been summarised by the US National Academy of Sciences Institute of Medicine in their evaluation of dietary reference intakes.

In addition, nicotinamide has been investigated as a method for reducing the risk of the development of diabetes (Knip *et al*, 2000). Studies have shown that nicotinamide can afford protection in an animal model of immune mediated insulin-dependent diabetes (Reddy *et al*, 1990), and it has been investigated in a number of clinical trials, some of which are still ongoing.

3.2.1. Vasodilatory effects (flushing)

Vasodilation is commonly seen in patients given high doses of nicotinic acid for the treatment of hyperlipidaemias. Very large single doses cause hypotension, although tolerance develops to this effect after several days of continued high dose intake. In general, flushing is a mild and transient effect although in many clinical trials it has resulted in patients withdrawing from treatment. The flushing activity appears to be related to the presence of a carboxyl group on the pyridine nucleus since compounds lacking this function, including nicotinamide, are not associated with facial flushing (Bean and Spies, 1940). Flushing is associated with periods of rapid rises in blood concentrations, and sustained-release formulations were developed for the use of nicotinic acid in the treatment of hypercholesterolaemia, in order to minimise this side-effect. Flushing is produced via prostaglandin D_2 release (Morrow *et al*, 1989 and 1992) and a “niacin flush-test” has been used as a method of investigating essential fatty acid metabolism (Glen *et al*, 1996). Tolerance develops due to decreased formation of prostaglandin D_2 on repeated dosage (Stern *et al*, 1991). Although flushing is not a clearly adverse effect and single oral doses of 100 mg do not alter heart rate or blood pressure, some patients in the study of Spies *et al* (1938) reported dizziness after oral nicotinic acid (doses not defined). Theoretically if flushing occurred in the elderly, it could exacerbate mild postural hypotension, and could increase the risk of falls, which are a common cause of morbidity in the elderly. This risk relates to taking supplements containing nicotinic acid (not nicotinamide), especially if taken on an empty stomach.

3.2.2. Gastrointestinal effects

Gastrointestinal effects such as dyspepsia, diarrhoea and constipation are common in patients with hypercholesterolaemia given high doses of nicotinic acid (3 g/day - especially as the sustained-release formulation; Knopp *et al*, 1985).

3.2.3. Hepatotoxicity

Severe and potentially life-threatening hepatotoxicity has been associated with treatment of patients with 3-9 g nicotinic acid per day for periods of months or years for the treatment of hypercholesterolaemia. Severe cases show liver dysfunction and fulminant hepatitis and may even proceed to encephalopathy requiring liver transplantation. Many of the patients showing hepatotoxicity were taking the slow release formulation of the compound, so that in contrast to the flushing discussed above, the development of hepatic toxicity is a function of long-term chronic exposure to relatively constant levels rather than the fluctuating levels and rapid rises which produce flushing.

3.2.4. Glucose intolerance

Nicotinic acid (3 g/day) has been reported to impair glucose tolerance in otherwise healthy individuals treated for hypercholesterolaemia.

3.2.5. Other effects

There have been rare reported cases of a range of effects including blurred vision, macular oedema and increased plasma homocysteine concentrations in patients given high doses of nicotinic acid. These effects were reported at doses similar to those producing hepatic dysfunction, and were reversible upon cessation of high dose treatment. (See below).

There has been a single report of a possible association with congenital malformation in women taking nicotinamide during early pregnancy (Nelson and Forfar, 1971). On the basis of their retrospective survey of drug and vitamin prescriptions during pregnancy in 1369 mothers, the authors reported that a significantly ($P<0.05$) higher proportion of women with infants showing abnormalities took nicotinamide in the first 56 days (5/458), compared with mothers of normal babies (1/911). In contrast no such relationship was found during later phases of pregnancy or over the whole of the pregnancy. The paper did not report the doses of nicotinamide taken. This finding is in contrast to the results of the large multicentre study on vitamins and the prevention of neural tube defect (MRC Vitamin Study Research Group, 1991). In that study 1817 women with high risk for producing a baby with neural tube defect were randomised into 4 groups; one group received folic acid, one group a multivitamin preparation (that did not include folate but contained 15 mg/day of nicotinamide), one group was given both preparations and one group received neither preparation. Although the study focussed on neural tube defects, any foetal malformation was recorded together with other pregnancy outcomes, and there was no difference in incidence between the multivitamin preparation and placebo.

4. DOSE-RESPONSE ASSESSMENT

4.1. Nicotinic acid

4.1.1. Vasodilatory effects (flushing)

Low doses of nicotinic acid may produce mild but noticeable flushing when taken on an empty stomach (Hathcock, 1997) and this represents the adverse effect detected at the lowest doses. An early study (Smith *et al*, 1937) reported that a single oral dose of 60 mg nicotinic acid produced marked flushing, which was not associated with changes in heart rate or blood pressure. Spies *et al* (1938) reported flushing in 5% and about 50% of subjects given single oral doses of 50 mg and 100 mg nicotinic acid, respectively. The dose-response for flushing was examined further by Sebrell and Butler (1938) who gave 3 groups of 6 subjects daily dose of 10, 30 or 50 mg nicotinic acid for 92 days as single oral doses given in solution added to tomato juice and consumed with the mid-day meal; flushing was reported intermittently by 4, 2 and 0 of the subjects given 50, 30 and 10 mg, respectively. The response is possibly related to periods of rapid increase in plasma concentrations of nicotinic acid, because the response is greater after intravenous dosage and is blunted if taken orally with food (Bean and Spies, 1940). Rash, pruritus and a sensation of warmth was reported following the consumption of pumpernickel bagels, accidentally made to contain 190 mg nicotinic acid per bagel (MMWR, 1983) and following the consumption of cooked meat containing 225 mg/100 g (Press and Yeager, 1962). This hazard does not seem to be related to nicotinamide. The facial flushing associated with low doses of nicotinic acid can be prevented by co-administration of an inhibitor of prostaglandin synthesis such as aspirin (although this is not always recommended - Schuna, 1997).

4.1.2. Gastrointestinal effects

Gastrointestinal effects such as dyspepsia, diarrhoea and constipation are common in patients given high doses of nicotinic acid for hypercholesterolaemia. Ruffin (cited in Sebrell and Butler, 1938) reported

nausea and vomiting in 2 out of 10 subjects given 1 g of nicotinic acid. Spies *et al* (1938) reported nausea and vomiting in subjects given oral doses of 300-1500 mg of nicotinic acid. Nausea is a common adverse effect in the studies of patients given 3 g of nicotinic acid daily for hypercholesterolaemia.

4.1.3. Hepatotoxicity

The first report of hepatotoxicity associated with the administration of nicotinic acid was in a study in dogs (Chen *et al,* 1938), which compared the toxicity of nicotine with nicotinic acid. In that study 2 dogs were given either 145 or 133 mg/kg bw/day nicotinic acid orally and both developed convulsions and excreted blood in their faeces about 2-3 weeks after treatment started. *Post mortem* observations included gastrointestinal adhesions, "fatty metamorphosis" of the liver and neuronal damage.

The first case-report of hepatotoxicity of nicotinic acid in humans was in a 23 year old man who developed jaundice after taking 3 g per day for 72 weeks (Rivin, 1959). Subsequent case-reports included a man who had taken 3 g per day for 6 months (Pardue, 1961), and a woman who developed pruritus and jaundice after taking 3 g nicotinic acid (together with 3 g vitamin C and 100 mg pyridoxine for a psychological disturbance) per day for 2.5 years (Einstein *et al*, 1975). A survey of 66 patients treated with nicotinic acid, of whom 51 had taken 3 g/day for 12 months or more, found a high incidence of abnormal liver function tests (23 patients) while on treatment, with 2 patients developing jaundice (Berge *et al*, 1961).

Approximately one-third of the 1119 patients in the study of the Coronary Drug Project Research Group (1975), who received 3 g/day nicotinic acid for up to 5 years, were reported to have elevated serum glutamate-oxaloacetate transaminase (SGOT) and alkaline phosphatase levels.There have been a number of reports of individual cases of patients with severe hepatotoxicity resulting from the use of nicotinic acid for hypercholesterolaemia or hypertriglyceridaemia. Four cases of liver disease were associated with doses of 2.5 g of sustained-release nicotinic acid daily for 5 months, 1.5 g of sustained-release nicotinic acid per day for 3 months, 2.25 g of sustained-release nicotinic acid per day for an unrecorded period, and 2 g of sustained-release nicotinic acid daily for an unrecorded period (Coppola *et al*, 1994). In all cases, cessation of nicotinic acid administration resulted in resolution of the liver symptomatology. A single case report gave some insight into the dose-response relationship for sustained-release nicotinic acid since symptoms of anorexia, fatigue and persistent nausea arose approximately one month at the end of a sequence of dose escalation from 1 g/day through 3 g/day for one month and finally 4 g/day for one month (Lawrence, 1993). A rapid reversal of the symptoms was found at 3 weeks after discontinuation of the nicotinic acid therapy.

Rader *et al* (1992) reviewed the available cases of hepatotoxicity and side-effects from conventional and sustained-release nicotinic acid and concluded that adverse effects were frequently seen shortly after an abrupt change from unmodified to sustained-release preparations. Their paper summarised both the dose and the duration of therapy in the different cases of hepatic toxicity and showed that in general toxicity was associated with doses of 3 g/day or more, although there were 2 cases who took less than 1 g/day for short periods (0.75 g conventional nicotinic acid per day for less than 3 months; 0.5 g sustained-release nicotinic acid for 2 months).

A comparison of an immediate release formulation and a sustained-release formulation of nicotinic acid in two groups of 23 patients with low density lipoproteinaemia studied the sequential effect of 0.5, 1, 1.5, 2 and 3 g per day for period of 6 weeks each. The therapeutic efficacy was similar for the two formulations but there were interesting differences in the side-effect profiles. About 39% of subjects on the immediate release formulation withdrew before completing the 3 g/day dose due to vasodilatory symptoms and fatigue, whereas 78% of subjects in the sustained-release group withdrew before completion of the study, primarily due to gastrointestinal tract symptoms, fatigue and changes in serum aminotransferases, indicative of hepatic dysfunction. Interestingly, the lowest dose of 0.5 g/day appeared to be better tolerated with the sustained-release preparation than the immediate release primarily because of vasodilatory symptoms (McKenney *et al*, 1994).

The study of McKenney *et al* has been criticised because the dosage regimen of twice daily administration was considered to minimise the tolerability of the protocol and give the greatest potential for side-effects. The authors of the critique (Kennan *et al,* 1994) report that there was only a 5% drop-out rate as a result of intolerance and toxicity after one year in a study of 1119 subjects receiving 3 g (1 g three times a day) of immediate release nicotinic acid. The study of McKenney *et al* was also criticised because of the high top dose administered since drop-out rates of only 3-4% had been reported in studies where the maximum dose was 2 g/day.

The results of a multicentre study on the long-term safety and efficacy of a sustained-release preparation of nicotinic acid were reported by Guyton *et al* (1998). Nicotinic acid doses, ranging from 0.5-3 g were given once a day at bedtime to a total of 269 patients for a period up to 96 weeks. The average dose given at the end of the study was 2 g/day with a range from 1-3 g, which indicates the poor tolerability of doses greater than 2 g/day. The principal adverse effect was flushing which resulted in 4.8% of the participants discontinuing the study (although they were advised that they could take aspirin to reduce this symptom). Those individuals who showed flushing had an average of one episode of 1.2 hours duration every 4-5 weeks. A total of 9 patients showed elevated transaminase levels of at least 2 times the upper limit of normal. However 5 of these patients were on a combination therapy including nicotinic acid plus nystatin or a bile acid sequestrant. In 5 of the cases the transaminase elevation resolved while treatment with nicotinic acid continued and without a reduction in dose. Therefore this study demonstrates only mild hepatotoxicity in a group of subjects given controlled doses of sustained-release nicotinic acid.

Dalton and Berry (1992) describe a single case of a woman who presented with hepatotoxicity after taking crystalline nicotinic acid for a period of 2 years and sustained-release formulation for a period of only 2 days prior to admission. Her symptoms on admission to hospital included hypothermia, hypotension and metabolic acidosis, and the authors suggested that this may have been a result of the change from conventional to sustained-release nicotinic acid associated with prolonged flushing and possibly significant transcutaneous heat loss. This observation is ironic, since the sustained-release formulation was primarily developed to minimise the skin flushing reaction associated with conventional nicotinic acid (Rader *et al*, 1992). Some studies have suggested that sustained-release formulations of nicotinic acid produce a greater incidence of hepatotoxicity (Christensen *et al*, 1961; Knopp, 1989; Mullin *et al*, 1989; Henkin *et al*, 1990), although this is not a consistent observation in all studies (Gibbons *et al*, 1995).

Gray *et al* (1994) reported that the daily intakes of nicotinic acid in 42 elderly diabetic patients who developed hepatic dysfunction (2.33 ± 0.15 g/day) were significantly higher than the doses for the remaining 854 subjects (1.64 ± 0.03 g/day) who did not develop hepatic dysfunction.

Effects on prothrombin time have been reported in patients taking sufficient nicotinic acid to cause hepatic toxicity. Elevated prothrombin times have been reported in a small number of cases, which were associated with only mild elevation of transaminase levels so that blood-clotting disorders may become the limiting sign of hepatotoxicity in some cases. Three cases reported by Dearing *et al* (1992) were receiving 2.0, 2.0 and 3.0 g of nicotinic acid daily.

In contrast to the studies that have reported abnormal liver function in patients treated with nicotinic acid, a small study in the group of 30 patients with hyperlipidaemia who were given slow release nicotinic acid at 1 g/day for 2 months and then 2-3 g/day for 10 months reported a low incidence of symptoms other than skin flushing (which had an incidence of 26.7% - mostly at the start of the treatment period). There was no evidence of hepatic abnormalities as indicated by changes in serum aminotransferases, alkaline phosphatase or antipyrine test results (Chojnowska-Jezierska and Adamska-Dyniewska, 1998).

A large number of studies have defined the efficacy and tolerability of both conventional and sustained- or controlled-release nicotinic acid in the treatment of hypercholesterolaemia and hyperlipidaemias. The data from these studies provide adequate evidence of the hazard identification and some evidence of dose-response characterisation. A major problem with the use of such data for establishing an upper level is that the doses investigated were restricted to those that showed clinical efficacy in the conditions being treated (mostly 3 g/day), and there are few data available at lower levels (Rader *et al*, 1992). Hodis (1990) reported a case of acute hepatic failure, which was ascribed to treatment with 500 mg per day nicotinic acid, however there was no repeat challenge or other data to support causation (other than an absence of other recognised reasons).

4.1.4. Glucose intolerance

Although hyperglycaemia is a relatively rare side-effect associated with high doses of nicotinic acid, it can be of clinical significance. Administration of 3 g of nicotinic acid per day for 10-14 days to volunteers resulted in an increase in fasting blood glucose and immuno-reactive insulin in serum (Miettinen *et al*, 1969). An increase in blood glucose concentrations, glycosuria, elevated serum ketone bodies, and an increase requirement for hypoglycaemic medication were reported in 6 patients with diabetes mellitus, who were receiving between 1 g and 3 g of nicotinic acid daily for a period of 2 weeks or more (Molnar

et al, 1964). Gray *et al* (1994) reported a high incidence of hyperglycaemia in elderly hyperlipidaemic patients who had been treated with high doses of nicotinic acid (average dose 1.7 g/day). Schwartz (1993) described a patient who was hospitalised with severe hyperglycaemia following treatment with 3 g of nicotinic acid per day for 4 months; administration of insulin and oral hypoglycaemics reversed and stabilised the blood glucose levels.

4.1.5. Other effects and overall dose-response relationships

Thrombocytopaenia, which resolved on cessation of nicotinic acid treatment, was reported in a single patient who developed hepatitis 10 years after the initiation of nicotinic acid treatment (Reimund and Ramos, 1994).

The plasma concentrations of homocysteine were increased by 55% in patients with peripheral arterial disease who were treated with nicotinic acid (Garg *et al*, 1999). The 52 patients were a subgroup from a multicentre study in which patients were given increasing doses of 100, 500 and 1000 mg per day over periods of 3-4 weeks (in order to identify patients who tolerated nicotinic acid), following which the subjects were randomised to receive either placebo or nicotinic acid (up to 3 g per day). The plasma concentrations of homocysteine were measured at baseline, at randomisation and at 18 and 48 weeks after randomisation. Plasma homocysteine increased from 13.1 ± 0.5 μM at baseline to 15.3 ± 0.8 μM at randomisation. After randomisation the levels increased further in those receiving nicotinic acid (to about 20 μM at 18 and 48 weeks; n=25 and 24, respectively), but decreased in those on placebo (to about 12 μM at 18 and 48 weeks; n=21 and 22, respectively). The clinical significance of this is unclear, but elevated plasma homocysteine is a recognised risk factor for coronary artery disease.

Severe reversible cystoid macular oedema was reported in 3 patients receiving high-doses of nicotinic acid (Gass, 1973). A survey of 116 patients who had received nicotinic acid (3 g or more per day) for treatment of hyperlipidaemia and a similar number of patients who were not treated with nicotinic acid revealed an increased incidence of decreased vision associated with sicca syndromes, eyelid oedema or macular oedema (Fraunfelder *et al*, 1995).

Because the majority of the data arise from studies designed to investigate the hypolipidaemic action of nicotinic acid, most of the data relate to doses of 1 g/day or more. In consequence, there are few data available on the tolerability and toxicity of doses less than 500 mg/day. In general the main adverse effect reported at intakes below the 500 mg/day has been flushing which is generally self-limiting in relation to continuation of treatment or intake of nicotinic acid.

High dose nicotinic acid (0.5-2.25 g daily) has been used for the treatment of severe hypercholesterolaemia in children. A retrospective review of 21 such cases reported similar adverse effects to those found in adults, with 6 children showing reversible dose-related elevations in serum transaminases, and 8 children who discontinued treatment because of flushing, abdominal pains and/or elevated serum transaminase levels. Hepatitis was reported in subjects with very high doses on a mg/kg bw/day basis (50, 67, 41, 34, 48 and 39 mg/kg bw/day) (Colletti *et al*, 1993).

In a study in the USA on elderly male veterans (age 62 ± 9 years) the doses administered averaged 1.7 g/day with a mean duration of intake of 13 ± 10 months (Gray *et al,* 1994). Almost one-half of the subjects discontinued treatment because of adverse effects with poor glycaemic control occurring in 41% of patients with diabetes mellitus. Probable and possible nicotinic acid-induced hepatotoxicity occurred in 2.2 and 4.7% of the patient group. These data indicate that the spectrum of toxicity is similar in elderly and young adults.

The side-effect profile of wax matrix sustained-release nicotinic acid was studied in groups of younger (<50 years) and older (50-70 year old) hypercholesterolaemic subjects. The study was a randomised double-blind placebo controlled design of 8 weeks duration with doses of 1.0-2.0 g/day. Clinically significant side-effect included flushing, itching, tingling, upper gastrointestinal symptoms, constipation, diarrhoea, dizziness, palpitations and blurred vision; the overall incidence of adverse effects was similar in the two difference age groups (Keenan *et al,* 1992).

4.2. Nicotinamide

4.2.1. Vasodilatory effects (flushing)

The flushing reported with nicotinic acid does not occur following nicotinamide, either given as an intravenous injection (Bean and Spies, 1940) or when it is given orally at high-doses to patients with diabetes (Knip *et al*, 2000).

4.2.2. Gastrointestinal effects

Gastrointestinal effects are rare following high-dose treatment with nicotinamide (Knip *et al*, 2000). Nausea was reported in a single subject who had taken nicotinamide 3 g daily followed by 9 g per day for several days (Winter and Boyer, 1973).

4.2.3. Hepatotoxicity

Only one patient has been reported to have developed hepatitis after nicotinamide alone, and this subject had been given 3 g daily followed by 9 g per day for several days (Winter and Boyer, 1973); other subjects who developed liver disease after nicotinamide had also received prolonged treatment with nicotinic acid (see Rader *et al*, 1992).

Increased serum transaminase levels were reported for 17 out of 41 children with attention deficit disorders treated for 12 weeks with daily doses of 3 g nicotinamide, in combination with 1.2 g pantothenic acid, 3 g ascorbic acid and 0.6 g pyridoxine (Haslam *et al*, 1984). Whether this hepatotoxic effect was related to the high dose of nicotinamide, or to the combination with the high doses of pantothenic acid, vitamin C and pyridoxine, cannot be concluded from this study, and therefore, this study cannot be used in risk assessment of nicotinamide.

The supplementation trials on the use of nicotinamide to prevent or delay the development of diabetes mellitus have not reported hepatitis as an adverse effect (Knip *et al*, 2000); however these have involved smaller number of subjects, have been of shorter duration and at lower doses than the trials on the use of nicotinic acid for the treatment of hypercholesterolaemia. Ten newly diagnosed Type 1 diabetic patients were given 1 g/day for 45 days (Mendola *et al*, 1989), and compared over the following year with a group who were treated with placebo; the authors reported that no adverse effects were observed when physical, biochemical and haematological parameters were considered (no details of the tests were given and the main aim of the paper was to study efficacy). A group of 35 patients, aged 6 to 18 years, were given either placebo (n=17), or up to 1.5 g/day of slow-release nicotinamide (n=18) for 12 months (Chase *et al*, 1990); various tests, including measurement of serum transaminases, alkaline phosphatase and bilirubin, were performed after 4 and 12 months, and remained normal in all subjects. No adverse effects were reported in a group of nine Type 1 diabetic patients with ketosis given 3 g of nicotinamide per day, three of whom were treated for up to 12 months, compared to 7 similar patients given placebo (Vague *et al*, 1987).

Major long-term studies in patients with Type 1 diabetes mellitus, at dosages of 2-3 g of nicotinamide per day, have been undertaken recently (ENDIT - see Pociot *et al*, 1993; IMDIAB III - see Pozzilli *et al*, 1995; DENIS - see Lampeter *et al*, 1998). The ENDIT (European Nicotinamide Diabetes Intervention Trial) has reviewed the safety data on nicotinamide before starting the clinical phase, but no results of the trial have yet been published (Pociot *et al*, 1993; Knip *et al*, 2000). The IMDIAB III study involved a double-blind trial in which 28 newly diagnosed patients with Type 1 diabetes mellitus were given 25 mg/kg bw of nicotinamide daily for 12 months, and a similar number treated with placebo; no adverse effects were reported and biochemical parameters including liver and kidney function were normal during follow-up (the publication describes the measurement of bilirubin). The DENIS trial (Deutsche Nicotinamide Intervention Study) was a study in young children (average age 3 years) at high risk of developing Type 1 diabetes mellitus in which 25 subjects were randomised to receive nicotinamide (1.2 g per m^2 per day), and 30 to receive placebo; the trial continued for 3 years and during this period all biochemical markers (including alanine aminotransferase, aspartate aminotransferase and bilirubin) were in the normal range.

4.2.4. Glucose intolerance

Nicotinamide has been studied in relation to reducing the risk of the development of diabetes mellitus; none of the studies (see above) has reported a worsening of symptoms in the treated groups.

4.2.5. Other effects and overall dose-response relationships

There have been no other adverse effects reported following the administration of nicotinamide in trials in patients with diabetes. Determination of the NOAEL from the intervention trials is difficult, because of the different dosage regimens employed. Studies have used fixed doses of 1 g/day (Mendola *et al*, 1989), 1.5 g/day (Chase *et al*, 1990), 3 g/day (Vague *et al*, 1987), 25 mg/kg bw/day (IMDIAB III trial) and 1.2 g/m^2/day (DENIS trial). These different doses can be calculated on a body weight basis using the data on body weights or ages in the different publications; the doses approximate to 17 mg/kg bw/day (Mendola *et al*, 1989; average age 18.3 years), 37 mg/kg bw/day (Chase *et al*, 1990; average age 12.5 years), 43 mg/kg bw/day (Vague *et al*, 1987; adults), 25 mg/kg bw/day (IMDIAB III trial; ages

in the range 5-35 years) and 50-40 mg/kg bw/day (DENIS trial; ages 3-12 years). The lowest of these values (25 mg/kg bw/day) was from one of the largest published studies, and this has been used as the NOAEL for nicotinamide.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL

Because of the difference in adverse effect profiles, different upper levels should be developed for nicotinic acid and nicotinamide.

5.1. Nicotinic acid

The more severe forms of toxicity of nicotinic acid, as described above, occur principally at doses of greater than 500 mg/day. The limiting adverse effect at lower doses is flushing, and this has been reported at much lower intakes than the other adverse effects. The most severe and potentially life-threatening adverse effects, such as hepatotoxicity, occur at doses one order of magnitude higher than have been reported for flushing. The dose of free nicotinic acid reported to produce flushing consistently in clinical studies is 50 mg/day (Sebrell and Butler, 1938; Spies *et al*, 1938). Spies *et al* (1938) reported a 5% incidence of flushing after a single oral dose of 50 mg nicotinic acid and a 50% incidence at 100 mg. The available data indicate that flushing would be unlikely to occur repeatedly in subjects given less than 50 mg/day, but occasional flushing was reported by Sebrell and Butler (1938) at a dose of 30 mg of nicotinic acid daily. A tolerable upper intake level for nicotinic acid of 10 mg/day is based on the available data indicating occasional flushing at 30 mg per day, using an uncertainty factor of 3 to allow for the fact that a slight effect was reported, and that the study was performed in a small number of subjects, but taking into account the steep dose-response relationship. This upper level is 300-fold below the dose frequently used clinically for the treatment of hypercholesterolaemia (3 g/day) and which is associated with a high incidence of serious adverse reactions. The only reports of flushing associated with the ingestion of nicotinic acid with food have occurred following the addition of free nicotinic acid to food prior to consumption. Although flushing might be considered a minor health effect, it has been used as the basis for setting the upper level for nicotinic acid, because of concerns about the possibility of a transient hypotensive episode, especially in the elderly.

The upper level of 10 mg/day for free nicotinic acid is not applicable during pregnancy or lactation because of inadequate data relating to this critical life stage. The upper levels for intake by children and adolescents have been derived on the basis of their body weights:

Age (years)	Tolerable Upper Intake Level (UL) for nicotinic acid (mg per day)
1-3	2
4-6	3
7-10	4
11-14	6
15-17	8

5.2. Nicotinamide

Nicotinamide does not produce the flushing response that has been used as the basis for the upper level for nicotinic acid. There has been only one reported case of hepatotoxicity in a patient receiving high-dose nicotinamide (however, nicotinamide has not been subject to extensive clinical trials [at 3 g per day or more] for use as a hypolipidaemic agent).

No significant adverse effects have been reported in trials on the possible benefits of nicotinamide in patients with or at risk of diabetes, which have used doses up to the equivalent of 3 g per day, for periods up to 3 years. The NOAEL from these studies is approximately 25 mg/kg bw/day. This value represents the lowest reported dose in a number of recent trials of high quality, many of which used sensitive markers of hepatic function and glucose homeostasis, and included a range of age groups, with some subjects treated with up to 50 mg/kg bw/day. An uncertainty factor of 2 has been used to allow for the fact that adults may eliminate nicotinamide more slowly than the study groups, many of which were children, and that data for children would not reflect the full extent of intersubject variability that could occur in an older population. The upper level for nicotinamide is established at 12.5 mg/kg bw/day or approximately 900 mg/day for adults.

The upper level of 900 mg/day for nicotinamide is not applicable during pregnancy or lactation because of inadequate data relating to this critical life stage. The upper levels for intake by children and adolescents have been derived on the basis of their body weights:

Age (years)	Tolerable Upper Intake Level (UL) for nicotinamide (mg per day)
1-3	150
4-6	220
7-10	350
11-14	500
15-17	700

6. RISK CHARACTERISATION

The form of niacin generally used in vitamin supplements and for addition to foods is nicotinamide. This form does not produce flushing and seems to be of low toxicity compared with nicotinic acid.

The upper level for free nicotinic acid has been derived from data on flushing following administration of a single oral dose given in solution added to tomato juice and consumed with a meal. Flushing has not been reported for the bound forms of nicotinic acid that are present in foods.

The upper levels do not apply to the use of nicotinic acid or nicotinamide under clinical supervision for the treatment of hypercholesterolaemia and hyperlipidaemias or reducing the risk of the development of diabetes.

There are inadequate data on the safety of nicotinic acid or nicotinamide during pregnancy or lactation, and therefore the upper level for adults does not apply to these life stages. However, it is noted that the adverse effect produced by low doses of free nicotinic acid is of a mild and transient nature and there are no reports of increased susceptibility to this effect during pregnancy or lactation. With regard to nicotinamide, there is no indication that intakes within the range currently consumed in foods, including fortified foods, in European countries are associated with any risk during pregnancy or lactation and there is evidence, at least from one study, that an additional 15 mg is without adverse effect on pregnancy outcome.

7. RECOMMENDATIONS

There is a need for reproductive toxicity studies on both nicotinic acid and nicotinamide.

The upper level for nicotinic acid has been based on the possibility that the flushing detected at higher doses in young subjects could result in transient hypotensive episodes in the elderly. This possibility should be investigated.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF IODINE

(EXPRESSED ON 26 SEPTEMBER 2002)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address : http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Iodine is a reactive Periodic Group VII element (halogen) existing in the valency states -1 to +7 but not occurring free in nature. Iodides and iodates, its mineral forms, occur ubiquitously in igneous rocks and soils, most commonly as impurities in saltpetre and natural brines. It is liberated by weathering and erosion, particularly following glacial erosion during the ice ages, and, being water-soluble, it leaches by rainwater into surface water, the sea and oceans, leaving behind mountainous regions with soils low in iodide. Liberated elemental iodine evaporates into the atmosphere because of its volatility and is precipitated by rainfall onto the land surface. The iodides in the sea accumulate in sea weeds, sea fish and shellfish. On land small amounts of iodide are taken up by plants, which have no essential nutritional requirement for this element, the plants being subsequently ingested by herbivores. In many areas of the world the surface soil becomes progressively poorer in iodide through these leaching processes (Whitehead, 1984).

Iodine is an essential dietary element for mammals being required for the synthesis of the thyroid hormones thyroxine (T4, 3,5,3',5'-tetraiodothyronine), containing 65% by weight of iodine, and its active form T3 (3,5,3'-triiodothyronine), containing 59% by weight of iodine, as well as the precursor iodotyrosines. The only natural sources for humans and animals are the iodides in food and water. Examples of anthropogenic sources are medicinal products, sanitising solutions and iodophores.

2. NUTRITIONAL BACKGROUND

2.1. Levels in the environment and food

Atmospheric iodine derives from vaporisation from seawater and is present at levels of 3-50 ng/m^3, the average global value being 10-20 ng/m^3 (WHO, 1988; NNT, 2002). Unpolluted surface water contains <3 µg iodide/L, drinking water <15 µg/L but in the US the average drinking water contains 4 µg iodide/L (WHO, 1988). Sea water levels amount to 50 µg iodide/L.

The iodide content of foods and total diets differs depending on geochemical, soil, and cultural conditions. The major natural food sources are marine fish (mean 1220 µg/kg, up to 2.5 mg I/kg), shellfish (mean 798 µg/kg, up to 1.6 mg I/kg), marine algae, seaweed (1000-2000 µg/kg) and sea salt (up to 1.4 mg I/kg). In industrialised countries the most important sources of iodides are dairy products, e.g. whole cow's milk (mean 27-47 µg/kg), UK winter milk (mean 210 µg/kg), UK summer milk (90 µg/kg), eggs (mean 93 µg/kg), and grain and cereal products (mean 47 µg/kg depending on the soil). Other food sources are freshwater fish (mean 30 µg/kg), poultry and meat (mean 50 µg/kg), fruits (mean 18 µg/kg), legumes (mean 30 µg/kg) and vegetables (mean 29 µg/kg) (WHO, 1996; Souci *et al*, 2000; MAFF, 2000; EGVM, 2002).

Milk and dairy products contain relatively high amounts derived from iodinated cattle feed supplements, from iodophor medication, iodine-containing sterilizers of milking equipment, teat dips and udder washes. Some of the iodide in cereal products derives from iodate-containing dough conditioners. Other sources of iodide in food are iodised salt (Germany: 15-25 mg I/kg as KIO_3; Austria 20 mg I/kg as KI; Switzerland 25 mg I/kg as KI), bread and sugar supplemented with iodide in some countries, and iodine-containing herbicides/fungicides. Cooking reduces the iodine content of food, frying by 20%, grilling by 23% and boiling by 58% (WHO, 1996).

Non-food sources are iodine-containing medication, topical medicines, antiseptics (povidone-iodine), X-ray contrast media (~5000 mg/dose yielding 1-4 g in cholecystography, >10 g in urography), iodised oil for oral

or i.m. use, mineral dietary supplements (up to 190 mg iodide/dose), tablets or capsules of seaweed-based dietary supplements (0.045-5 mg iodide/dose) and kelp tablets as dietary supplement (up to 57 mg iodide/dose) (Pennington, 1990). Marine macroalgae produced in China, Japan, the Philippines, North and South Korea are products grown in aquaculture from brown, red and green algae and can have an extremely high iodine content, particularly in marketed products derived from dried material (up to 6500 mg iodine/kg dry product). A product known as Kombu-powder contains about 0.5% iodine (BGVV, 2001).

2.2. Intake estimates

The intake of iodine generally corresponds to the amount entering the local food chain from the geochemical environment, and is normally low from food. If seaweed is consumed or if iodine-containing mineral supplements or medicinal products are ingested, it can rise to several mg/day (WHO, 1988; US Food and Nutrition Board, 2001). Individual intake from air may average 0.5 µg/person/day (NNT, 2002), that from water (assuming a consumption of 1.5-2 L/day) about 8-30 µg/person/day (WHO, 1988), that from food about 3-75 µg/serving (US Food and Nutrition Board, 2001). Ingestion of marine food or processed food containing iodised salt, calcium iodate, potassium iodate or cuprous iodide also increase the iodine intake.

Breast milk was reported in the 1980s to contain 12 µg/L in Eastern Germany, 27 µg/L in Italy, 95 µg/L in France, the median US value being 178 µg/L (Gushurst *et al*, 1984). More recent reports quote for Germany 36 µg/L in 1992, 86 µg/L in 1994 and 95 µg/L in 1995-6 (Meng and Schindler, 1997). In general breastfeeding women produce daily 500-800 mL (average 780 mL) breast milk (SCF, 1993).

According to NHANES III the US median intake of iodine from dietary supplements was 140 µg/male or female adult. In 1986 some 12-15% of the US population were taking dietary iodine supplements (US Food and Nutrition Board, 2001). According to FDA surveys the daily US median adult iodine intake was 240-300 µg for males and 190-210 µg for females, the highest intake of any life stage and gender for the 95th percentile excluding supplements being 1 mg/day and including supplements 1.15 mg/day (US Food and Nutrition Board, 2001).

In Germany the median daily iodine intake varied from about 64-118 (mean 45.3) µg I/day for males aged 4-75 years and from 59-114 (mean 44.2) µg I/day for females aged 4-75 years (Schneider *et al*, 1995). For infants aged 6 months, children and young adults up to the age 18 years the mean iodine intakes varied from 31-64 µg/day for males and from 28-56 µg/day for females (Alexy and Kersting, 1999). In those taking iodine supplements once/week the corresponding mean levels were 124 µg I/day for males and 109 µg I/day for females compared to 107 µg I/day for males and 102 µg I/day for females not taking any supplements (Mensink and Ströbel, 1999). In Denmark the median intake was about 119 µg I/day for males and 92 µg I/day for females. In The Netherlands the median intake was about 145 µg/day for males and 133 µg/day for females (TNO, 1992). In Great Britain the median dietary intake from all sources was 226 µg/day for males and 163 µg/day for females, the 97.5th percentile reaching 434 µg/day in males and 359 µg/day in females. Survey data in young children aged 1½-4½ years show for high milk consumers in winter 247 µg/day to 309 µg/day, suggesting that some pre-school children are likely to have intakes exceeding the JECFA PMTDI (EGVM, 2002).

Dietary iodine absorption and incorporation is reduced by smoking, thiocyanates, isothiocyanates, nitrates, fluorides, Ca, Mg and Fe in food and water (Ubom, 1991). Glucosinolates (goitrogenic thioetherglycosides yielding on hydrolysis a thioglucose and the aglycones isothiocyanate, nitrils or thiocyanate) and goitrins block the incorporation of iodine into the tyrosine precursors of thyroid hormones and suppress thyroxine secretion (Cornell University, 2001). Degradation of cyanoglycosides (liberating cyanide on enzymatic hydrolysis in the gut which is subsequently metabolised to thiocyanate), glucosinolates and goitrins present in vegetables like brassica, crucifera, rape, cabbage, cauliflower, broccoli, Brussels sprouts, kale, kohlrabi, turnips, maize, lima beans, bamboo shoots, peanuts, walnuts, sweet potatoes, millets and cassava (linamarin is the cyanogenic glycoside in cassava) block the thyroidal uptake of iodine after ingestion thereby decreasing the production of thyroid hormones. Soya flour also inhibits the absorption of iodide by interference with the enterohepatic circulation of thyroxine. Hence goitre and hypothyroidism have appeared in infants fed entirely on soya-based infant formula. The latter is now enriched in the EU to a minimum content of 5 µg iodine/100 kcal intake. Water from polluted wells contains humic substances which also block the iodination process. Vitamin A-, Se-, Zn-, Cu-, Fe- and vanadium-deficiency can result in hypothyroidism and may exacerbate the effects of preexisting iodine deficiency. Large amounts of absorbed iodine, e.g. from radiological contrast media, from iodide liberated from erythrosine, from water purification tablets, from amiodarone (an antiarrhythmic drug), from skin disinfectants and dental disinfectants, also reduce iodine uptake causing the production of iodine deficiency symptoms.

2.2.1. Iodine excess

Excessive intake of iodine can occur as a result of ingestion of large amounts of seaweed, kelp, marine fish, ground beef containing thyroid tissue, iodised water, bread or salt and iodide-containing dietary supplements. The ingestion of iodine-rich algal products made from marine macroalgae grown in aquaculture in the Far East, particularly dried products, can lead to dangerously excessive iodine intakes, if such products contain more than 20 mg I/kg dry matter and the exposed population lives in an area of endemic iodine deficiency. This would not apply to countries with traditional adequate dietary iodine intake (BGVV, 2001).

Excessive intakes can also follow the ingestion of iodide-containing pharmaceuticals for the treatment of asthma, bronchitis, cystic fibrosis, chronic obstructive pulmonary disease, and of goitre, after the use of amiodarone for the treatment of arrhythmias, of iodine-containing topical antiseptics, mouthwashes and vaginal solutions, and the treatment of burns and wounds with povidone-iodine. However the degree of absorption and incorporation of iodine from these sources is not known. Much circumstantial evidence links excessive iodine intake with the risk of increased incidence of autoimmune thyroiditis but environmental contaminants may also play a part (NNT, 2002).

2.3. Nutritional status

The nutritional status, and consequently the iodine requirements, of a population group can be assessed in various age groups by analysing the following indicators:

1. the fraction of an oral dose of ^{131}I concentrated in the thyroid gland;

2. the average daily iodine turnover (uptake and release) calculated following i.v. administration of ^{131}I;

3. the urinary iodine excretion as determined in 24-hour samples, measured in µg/L or µg/g creatinine, representing more than 90% of the dietary intake. The minimum European urinary excretion should amount to 100 µg/day (DGE, 2002), deficiency being indicated by iodine levels of <50 µg/L (50 µg/g creatinine). US data show 138-155 µg/L for adult males and 110-129 µg/L for adult females. Using a median 24-hour urine volume of 0.9 mL/hr/kg bw for children aged 7-15 years and a median 24-hour urine volume of 1.5 mL/hr/kg bw for adults, and assuming 92% bioavailability, allows calculation of the iodine intake from such urinary measurements (US Food and Nutrition Board, 2001);

4. the goitre incidence rate and size, the latter determined from ultrasound measurements rather than palpation. In mild Iodine Deficiency Disease (IDD) goitre prevalence in school children is 5-20% with a mean urinary iodine excretion of >50 µg/g creatinine. In moderate IDD it is up to 30% with a mean urinary iodine excretion of 25-50 µg/g creatinine. In severe IDD goitre prevalence is >30% with a mean urinary iodine excretion of <25 µg/g creatinine;

5. the iodine balance estimates are only of limited use. The techniques are crude and the control of intake assessment is limited because some iodine sources always remain unknown (US Food and Nutrition Board, 2001);

6. the serum levels of TSH (thyroid stimulating hormone), Tg (thyroglobulin), T4 and T3 are the soundest parameters providing an indirect measure of iodine nutritional status. The best parameter is the TSH serum level especially if hypothyroidism is to be detected in pregnant women and neonates. Estimates can be performed on blood spots. The normal serum TSH range is 0.1-5 mU/L. The sensitivity can be increased by previous stimulation with TRH. An exaggerated response to TRH suggests an inadequate hormone availability, hypothyroidism and iodine deficiency. Elevated serum Tg levels are useful for detecting metastases of differentiated thyroid cancer, hyperplasia of the thyroid and IDD. The serum level of Tg on adequate iodine intake is 10 ng/mL. Serum levels of T4 and T3 are less sensitive and unreliable for estimating iodine nutritional status. The normal T4 serum level is 100 nmol (80 µg)/L but is lower in IDD. The T3 serum level is normally 1.8 nmol (1.2 µg)/L but is lower in fasting individuals or those suffering from malnutrition (US Food and Nutrition Board, 2001).

2.4. Nutritional requirements

The recommended mean population intake for iodine is 100-150 µg/day (WHO, 1996). This is adequate to maintain normal thyroid function, growth and development. In the presence of goitrogens in the diet iodine intake should be raised to 200-300 µg/day. Vegetarians, sufferers of milk allergy, lactose intolerance, fish allergy or persons on low salt diet are liable to develop dietary iodine deficiency and therefore need iodine supplements. Pregnant and breastfeeding women need a higher iodine intake

because the higher renal blood flow increases the urinary loss of iodine. Various international and national authoritative bodies have established requirements for iodine intake (SCF, 1993; US Food and Nutrition Board, 2001; DGE, 2002): In Germany and Austria the recommended adult daily intake is 200 µg and during pregnancy 230 µg, in Switzerland it is 150 µg and in pregnancy 200 µg. The SCF recommends as average requirement for adults 100 µg/day, as Population Reference Intake for adults and in pregnancy 130 µg/day, and in lactation 160 µg/day (SCF, 1993).

2.5. Metabolic fate of iodine and interrelationship with thyroid hormones

The biological function of the thyroid hormones T4, T3 and of iodotyrosines encompasses the regulation of energy metabolism and endocrine function by cellular oxidation, calorigenesis, thermoregulation, intermediate metabolism, protein and enzyme synthesis, nitrogen retention, gluconeogenesis and pituitary gonadotropins. Thyroid hormones also play a role in the intestinal absorption of glucose and galactose, in lipolysis and in the uptake of glucose by adipocytes, in the integrity of the connective tissue, and are necessary for optimum cellular metabolism particularly during early growth, development and maturation of most organs especially the brain. The target organs are the developing brain, muscle, heart, pituitary and the kidney. Additional functions of the thyroid hormones include a beneficial effect on mammary dysplasia and fibrocystic breast disease, support of the myeloperoxidase of leucocytes in the inactivation of bacteria and support of the immune response, while iodine lack may be associated with an increased incidence of gastric cancer (US Food and Nutrition Board, 2001).

The biological action of T3 and T4 is mediated through T3 receptors which bind free T3. These receptors are situated on inner mitochondrial membranes and in the nucleus. The nuclear receptors increase transcription for membrane protein and enzyme synthesis. These receptors belong to a superfamily together with steroid hormones, vitamin D and retinoid receptors, and are especially found in the pituitary, the liver and neonatal testis but are rare in the spleen. T3 and T4 may interact at receptor and gene expression level with sex hormones. They may upregulate hepatic oestrogen receptor levels in the rat. They also regulate the same subset of genes involved in lipid homoeostasis (NNT, 2002).

Ingested inorganic iodine and iodate are reduced to iodide in the gut and almost completely absorbed by the small intestine. T3, T4 and the drug amiodarone are absorbed intact, while the metabolism of iodinated X-ray contrast media, e.g. lipiodol, is not entirely clear (US Food and Nutrition Board, 2001). The bioavailability of oral inorganic iodide is >90%, that of oral T4 about 75%, some 15% of ingested iodide is taken up by the thyroid within 24 hours. Iodine can be absorbed dermally from topically applied material, the absorbed iodide is distributed in the extracellular fluid.

Some 30% of absorbed iodide is concentrated in the thyroid, the excess being excreted by the kidneys in the urine. About 80% of iodine stored in the thyroid is in the form of iodinated tyrosine, some 20% as thyronines and 1% as iodide (NNT, 2002). Minor tissue sites for iodide uptake from blood plasma are the salivary glands, the choroid plexus, the mammary gland, the kidneys, and the gastric mucosa. Iodide is able to cross the placenta. The biosynthesis of the biologically active thyroid hormones T3 (triiodothyronine), the most active, T4 (thyroxine), and the hormonally inactive T1 (monoiodo-3)- and T2 (diiodo-3,5)- derivatives of the precursor amino acid tyrosine utilises the circulating plasma iodide. T3 is produced by the deiodination of T4 in the liver and kidney of man and probably also in the thyroid of the rat. Plasma iodide is actively taken up by the basal membrane of the thyroidal follicular cells using a sodium-dependent, carrier-mediated pathway and concentrated 20-50 times in these cells. These follicular cells synthesise intracellularly the thyroglobulin (Tg), a glycoprotein of molecular weight 660,000. This Tg meets the iodide at the apical surface where the intracellular iodide is oxidised by thyroperoxidase (TPO), a Se-containing enzyme, in the presence of H_2O_2 to an iodonium ion which simultaneously attaches to the tyrosyl functional groups of Tg. Further action of TPO leads to the formation of T1, T2, and the coupling of 2 T2 to give T4 or of T1+T2 to give T3, all these thyronines remaining attached to the Tg. The iodinated Tg is stored extracellularly in the colloid of the thyroid follicles, about 1/3 of the iodine being present in T3 and T4, the rest in T1 and T2. When needed, T3 and T4 are released into the circulation from Tg by endosomal and lysosomal cellular proteases.

Thyroid function is regulated by a feedback process in which thyrotropin-releasing hormone (TRH) of the hypothalamus and thyroid-stimulating hormone (TSH) of the anterior pituitary are released in response to a decrease in circulating T3 and T4 levels. TSH stimulates within minutes the secretion of thyroid hormones, causes an increased iodide uptake and an increased Tg breakdown. Iodothyronine secretion, including T3 and T4, is also controlled by interaction between growth factors, their receptors and signal transition pathways. Epidermal and insulin-like growth factor also stimulate follicular cells (NNT, 2002). Iodine interacts with selenium and possibly with vanadium (EGVM, 2002).

Persistent action of TSH causes hypertrophy and hyperplasia of the thyroid gland, reduces the colloid and the stored iodine. TSH secretion of the anterior pituitary is stimulated also by TRH, a protein of molecular weight 28,000. TRH secretion is stimulated by α-noradrenergic impulses and inhibited by dopaminergic impulses but is also responsive to circulating levels of T3 and T4. The autonomous regulation of thyroidal iodine metabolism occurs independent of TSH (Forth *et al*, 1987).

T1, T2, T3 and T4 are metabolised by specific deiodinases, a family of selenoproteins, the freed iodide entering either the plasma pool or being reutilised by the follicular cells. T4 is produced only by the thyroid gland, T3 is primarily produced (80% in man) by extrathyroidal deiodination in liver and kidney, brain, pituitary and brown fat and some (20% in man) by deiodination in the thyroid. In rats the deiodination of T4 takes place mainly in the thyroid (NNT, 2002).

TPO is inhibited by thioamides, the deiodinases forming the active hormone T3 are inhibited by thiouracil, propylthiouracil, propranolol, and glucocorticoids or may be genetically deficient. Monkey TPO is less sensitive to inhibition than rat TPO. Deiodinases show reduced activity in Se-deficiency with consequent impaired hormonal activity (Forth *et al*, 1987). Deiodinase activity is lower in human liver than in the rat.

Circulating T3 and T4 are in a reversible equilibrium attached to binding proteins synthesised by the liver, e .g. thyroxine binding globulin (Tg), transthyretin (prealbumin) (TTR), albumin and lipoproteins. In humans T4 is mainly bound to Tg, in rodents to TTR (NNT, 2002). Glucuronidation of T3 and T4 is less important in man. Less than 1% of T3 and T4 is free in plasma (NNT, 2002). Most biological action in the target tissues is probably mediated by T3 receptors. The bound T4 is enzymatically deiodinated to its active form T3, the liberated iodine entering the serum pool as iodide and being either reused by the thyroid or being excreted in the urine. T3 being less tightly bound enters cells more easily. In neonatal animals, in protein starvation, liver and kidney disease, and pyrexia T4 is mostly converted to T3.

The synthesis of normal quantities of thyroid hormones requires an adequate dietary intake of iodide but excess intakes may inhibit thyroid function by either inhibition of iodide organification (Wolff-Charkoff effect) or by inhibition of thyroglobulin proteolysis with reduction in hormone secretion. Plasma concentrations above 20-30 µg I/100 mL inhibit organic iodine uptake of the thyroid and intrathyroidal iodine is transformed from its inorganic form into organic iodine derivatives. Thiocyanate and perchlorate reduce thyroidal iodine transport and inhibit the conversion of the inorganic form of intrathyroidal iodide into its organic form, causing its discharge into the extracellular fluid (Forth *et al*, 1987).

The average adult thyroid contains about 8-15 mg iodine, the total body iodine amounts to about 10-20 mg of which 70-80% is found in the thyroid, some also appearing in muscle and the eye. The thyroid store at birth is 0.1 mg. Some 70% (100-150 µg) of ingested iodide are excreted daily by adults on adequate iodine intake in the urine with partial reabsorption occurring in the renal tubules, about 20% (15-20 µg) are excreted in the faeces, about 5-10 µg appear in the sweat, saliva and the bile (US Food and Nutrition Board, 2001). Lactating women excrete 10-15% of the daily iodine intake into breast milk (Saller, 1998). In a 5-day old infant urinary excretion varies from 2.8-11 µg/100 mL. In Germany adult urinary iodine excretion varies from 20-65 µg/day, in Denmark males excrete 64 µg/day and females 73-100 µg/day (Vitti *et al*, 1999). The renal iodide clearance is 34 mL/minute.

The placenta traps maternal excess serum iodide and transfers maternal T4 to the foetus until the foetus produces its own T4. Therefore iodine supply to the mother must be adequate to prevent foetal goitre formation (Glinoer *et al*, 1995).

Administration of iodide to the rat also causes transient inhibition of intrathyroid organification of iodine and reduces hormone synthesis. Escape from this effect occurs through reduction in iodide transport mechanism until intrathyroid concentration of iodide is below the level necessary to maintain biosynthesis inhibition.

Many environmental agents interfere with thyroid function, the most prominent effect being the development of goitre but decreases in T3 and T4 may also alter brain maturation and testis development. Direct toxic effects include 1) inhibition of iodide transport into and uptake by the thyroid; 2) inflammation and degeneration of follicular cells; 3) damage to DNA of follicular cells; 4) accumulation of iodotyrosines in the gland. Indirect toxic effects manifest themselves by 1) changes in plasma transport of hormones e.g. by displacement from TTR; 2) increased iodotyrosine excretion by increased activity of hepatic microsomal enzymes; 3) inhibition of iodotyrosine deiodinases; 4) interference with the intestinal absorption of T3 and T4 with faecal loss; 5) interference at the level of TSH or TTR (NNT, 2002).

2.6. Iodine deficiency

Several mechanisms compensate for low dietary iodine intake. If these mechanisms are insufficient clinical symptoms of iodine deficiency appear. These clinical effects are seen at all stages of development and are particularly noticeable in the foetus, the neonate and the infant as goitre, this being the commonest cause of human thyroid disease. Inadequate iodine intake and the resulting IDDs are widespread in Europe and the developing countries of Asia, Africa and South America. They arise from a depletion of the thyroid iodine stores of the body with consequent fall in daily T4 and T3 production and their plasma levels, which trigger an increased secretion of TSH and hyperactivity of the thyroid coupled with thyroid epithelial cell hyperplasia, goitre formation, and faster iodine turnover. Simultaneously, tests show an increased uptake of ^{131}I (WHO, 1996). About 1600 million people are at risk of iodine deficiency disorders worldwide because they inhabit iodine-deficient environments. IDD is a public health problem in 118 countries. In Europe about 140 million are at risk. Worldwide some 700 million have goitre. Thyroid hypofunction can also be induced by thyroiditis and exposure to antithyroid compounds.

2.6.1. Iodine deficiency disorders (IDD) in adults

This is associated with goitre, low serum T4 and suboptimal brain function. In some areas apathy and low capacity for initiative and decision making is also seen. Goitrous enlargement of the thyroid gland occurs at intake levels of <50 µg I/day. It is the common feature of iodine deficiency (WHO, 1996). Goitre is initially diffuse but later becomes nodular with appearance of autonomous nodules, which may cause hyperthyroidism by production of T4 uncontrollable by TSH. The appearance of large goitres may cause obstruction of the trachea and the oesophagus and increases the risk of thyroid disease and thyroid cancer.

Hypothyroidism (myxoedema), another form of IDD, also results from hormone deficiency and is associated with reduced metabolic rate, cold intolerance, weight gain, puffy face, oedema, hoarse voice and mental sluggishness.

2.6.2. Foetal iodine deficiency

This results from maternal iodine deficiency. It is accompanied by higher rates of stillbirths, abortions and congenital abnormalities. Low maternal T4 levels (<25 µg/mL) are correlated with adverse pregnancy outcome, perinatal mortality and cretinism. The major hazard is endemic cretinism associated with iodine intakes of <25 µg/day. It is characterised by very low serum T4, T3, and a very high serum TSH (40-50 mU/L). The more common neurological type is characterised by mental deficiency, deaf mutism, spastic diplegia, the less common myxoedematous type by apathy, hypothyroidism, puffy features, growth retardation, delayed bone maturation, retarded sexual maturation and dwarfism. Endemic cretinism can disappear spontaneously without supplementary iodization measures but usually needs preventive treatment by iodised oil injection before pregnancy. Congenital hypothyroidism can occur despite adequate dietary intakes of iodine. Its incidence is 1/3000 to 1/4000 and is due to congenital maldevelopment or aplasia of the thyroid. In the US and in many European countries all neonates are screened by blood spot tests for TSH or T4 levels in order to detect any cases of congenital hypothyroidism due to thyroid aplasia (US Food and Nutrition Board, 2001).

2.6.3. Neonatal iodine deficiency

This is associated with increased perinatal and neonatal mortality and more frequent congenital abnormalities. It constitutes a threat to early brain development with consequent physical and mental retardation and possible later depressed cognitive and motor performance. This is a more serious socio-economic risk for children than the incidence of cretinism.

2.6.4. Iodine deficiency in children

Mild deficiency is associated with goitre in 5-20% of school children, appearing more frequently in girls, and is accompanied by a median urinary iodine concentration of 43.5 nmol I/nmol creatinine. School performance and IQs are impaired even if allowance is made for confounding factors. Growth is reduced and psychomotor development lags behind normal children noticeable already from age 2.5 years onward. Moderate iodine deficiency is associated with a median urinary iodine level of 21.5-43.5 nmol I/nmol creatinine, and a goitre frequency of 30%. Severe iodine deficiency is associated with a median urinary iodine level of <21.5 nmol I/nmol creatinine, with >30% goitre frequency and 1-10% incidence of endemic cretinism (WHO, 1996). A metaanalysis of 18 studies has shown that iodine deficiency alone may reduce the mean IQ scores by 13.5 points (US Food and Nutrition Board, 2001).

2.6.5. Iodine deficiency in animals

In animals reproductive, neurological and other defects are the important consequences of iodine deficiency. Natural iodine deficiency in farm animals, e.g. cattle and sheep, causes failure in reproduction, retarded or arrested foetal development with consequent foetal resorption, early foetal deaths, spontaneous abortions, stillbirths, as well as prolonged gestation and parturition, placental retention and low hormone levels. Maternal hypothyroidism before the onset of foetal thyroid secretion together with subsequent foetal hypothyroidism leads to reduced neuroblast multiplication. Maternal hypothyroidism in early pregnancy in the rat causes reduced pup weight and number of embryos, reduced brain weight and reduced transfer of maternal T4.

Sheep on experimental iodine deficient feed of 5-8 µg I/day/40 kg bw showed more abortions, stillbirths and lower foetal weight, reduced or even complete absence of wool growth, retarded bone development, skull deformities, reduced brain weight, reduced brain cell numbers and brain DNA content. The same deficiency effects were seen in marmosets on 0.3 µg I/day/340 g bw (WHO, 1996).

2.6.6. Control of IDD

In Europe about 100 million and worldwide about 700 million individuals are affected by goitre. Of these some 1 million in Europe are also affected by impaired mental development compared to >11 million cases of cretinism worldwide (Vitti *et al*, 1999). This constitutes a major public health problem. Combative measures are the introduction of iodised salt, iodised bread or iodised oil, the use of iodine supplements in the feed of cattle and pigs to raise the iodine level of milk and meat, and the preventive use of iodised oil by injection (1 mL contains 480 mg I) to all females in the human population in areas of severe IDD up to age 40 years and all males in the human population in areas of severe IDD up to age 20 years in areas with poor control over iodine intakes of the population. If needed, injections should be repeated in 3-5 years. More recently iodised walnut or soya bean oil have been introduced as alternative oral treatment to supplementation of generally available dietary items with iodine.

2.6.7. Reported beneficial effects

Iodine supplements have been claimed to assist in the treatment of weight loss, rheumatism, ulcers, hair loss, maintenance of healthy arteries, nervous tissue and nails (EGVM, 2000). Iodine caseinate has been proposed as treatment for fibrocystic breast disease at doses of 70-90 µg I/kg bw (Murray and Pizzorno, 1998).

3. HAZARD IDENTIFICATION

3.1. Toxic effects in animals

Excess iodine intake in animals leads to acute inhibition of iodine uptake. Laboratory animals, poultry, pigs and cattle have a high tolerance to large iodine intakes. Animal data are of limited value because of species differences in basal metabolic rate and in iodine metabolism (US Food and Nutrition Board, 2001). The non-obese diabetic mouse (NOD)-42^{h4} develops spontaneously more frequent and severe autoimmune thyroiditis if iodine is added to the drinking water probably as a response to an increase in iodinated Tg (NNT, 2002).

3.1.1. Acute and subacute studies

The acute oral LD_{50} in rats for NaI is 4340 mg/kg bw (3320 I^-), the oral LD_{100} for KI in the mouse is 1862 mg/kg bw (1425 mg I^-) (Clayton and Clayton, 1981). Amounts of 200-500 mg/kg bw can cause death in experimental animals (SCOGS, 1975).

Two strains of chickens (CS and OS), genetically susceptible to autoimmune thyroiditis, were given either 20 or 200 mg KI/L in their drinking water for the first 10 weeks of their lives. At both levels the incidence of the disease was increased as shown histopathologically and also by measurements of T3, T4 and thyroglobulin antibody titres (Bagchi *et al*, 1985).

Groups, each of 20 rats, were fed diets containing 0 or 1000 mg KI/kg diet (39 mg I^-/kg bw) for 19 weeks. No tumours of the thyroid were found either in controls or in treated animals. The exposure period in this inadequate study was too short for any carcinogenic effect to be detected (Hiasa *et al*, 1987).

3.1.2. Reproduction and teratogenicity studies

Groups of female rats were given in their diet 0, 500, 1000, 1500 and 2000 mg KI/kg diet throughout gestation, lactation and weaning. Pup survival was reduced from 93% in controls to 16% in rats treated

at the top dose and milk secretion was diminished. There were no adverse effects on ovulation rate, implantation rate and foetal development (Ammermann *et al*, 1964).

Pregnant rats were given 11 mg KI/day in their drinking water (37 mg/kg bw/day) and the brain enzymes of pups investigated. Glutamate dehydrogenase was increased transiently, succinate dehydrogenase decreased transiently. Phosphofructokinase and malate enzymes increased but hexokinases were unaffected. Serum T4 levels were unchanged compared to controls (Morales de Villalobos *et al*, 1986).

Mares given 48-432 mg I/day during pregnancy and lactation produced foals with disturbed metabolism. The long bones of the legs of the foals showed osteopetrosis. Serum phosphate and alkaline phosphatase levels were increased (Silva *et al*, 1987).

3.1.3. Chronic studies

Metaplasia of the thyroid was reported in rats given potassium iodide in their drinking water for two years. This was thought to occur through a non-genotoxic proliferation dependent mechanism (EGVM, 2002).

3.1.4. Genotoxicity studies

The mutagenicity data for iodide are generally negative (EGVM, 2002)

3.2. Effects in humans

3.2.1. General observations on response to excess iodine

Disturbed thyroid gland activity as a result of excessive iodine intake may manifest itself either as a goitre, as hypothyroidism with/without goitre, or as hyperthyroidism (0.01-0.6% in populations on iodine prophylaxis, 0.25% in West Germany [JECFA, 1989]), the outcome depending on the initial and current iodine status and current thyroid dysfunction. Other effects may be sensitivity reactions (0.4-5%) (JECFA, 1989) and poisoning through ingestion of large quantities of iodine. Modest excessive iodine intake causes a temporary increase in iodide uptake by the thyroid with formation of more organic iodine and large hormone stores. Somewhat larger excessive intake inhibits the iodide release from thyrotoxic thyroids or from TSH stimulated glands and in 0.01-0.06% of exposed people leads to hypothyroidism. Greater excessive intake inhibits the formation of iodinated tyrosine, lowers the T4 and T3 plasma levels and raises the plasma TSH (Wolff-Charkoff iodide effect). These effects may be transient and in many individuals the thyroid can escape this Wolff-Charkoff effect. Individuals not escaping the Wolff-Charkoff effect develop goitre and become hypothyroid. The inhibiting effects of excess iodide occurs via unknown organic compounds, probably iodolipids (Cavalieri, 1997). TSH effects are blunted while the Wolff-Charkoff effect occurs. Other effects include the down-regulation of iodide transport, a raised ratio of iodotyrosines to iodothyronines in Tg, inhibition of pinocytosis and proteolyis with reduced hormone secretion (EGVM, 2000). The Wolff-Charkoff effect is the basis for the treatment of thyrotoxicosis with iodide. Very high intakes of iodide saturate the active transport system thereby preventing the uptake of radioactive iodine isotopes.

If excess intake occurs during pregnancy, the foetal thyroid is unable to escape the Wolff-Charkoff effect. The newborn therefore develops a goitre, is hypothyroid and may suffer possible tracheal compression. Alternatively, the condition may regress spontaneously postnatally after several months.

Some subpopulations such as those suffering from autoimmune thyroid disease, from IDD or nodular goitre with autonomous functioning nodules are sensitive to external iodine supply. They tend to respond adversely to levels of iodide which are without adverse effects in the general population. These persons may develop thyroiditis, goitre, hypothyroidism, hyperthyroidism, sensitivity reactions, papillary thyroid cancer and acute effects following exposure to iodide. Iodine-induced hypothyroidism occurs particularly in underlying thyroid disease especially in women (Braverman, 1990).

There is much circumstantial evidence linking excess iodine intake with an increased risk of autoimmune thyroiditis. It is more prevalent in the US than in Europe because the US population has a higher iodine intake (250-500 µg I/day). It is also more prevalent in areas with adequate iodine intake. The existing homoeostatic mechanisms control the tolerance to the widely changing dietary iodine levels except in a subsection of the population which develop thyroid dysfunction and autoimmunity on increased iodine intake. This occurs in IDD areas on introduction of iodine prophylaxis. T-cells from individuals with chronic Hashimoto's disease proliferate in the presence of iodinated Tg (NNT, 2002). Hashimoto's thyroiditis is associated with defective intrathyroidal organic binding of iodide leading to hypothyroidism at pharmacological iodide doses.

Hyperthyroidism can occur after an increase in iodine intake (iodine-induced thyrotoxicosis) usually in association with IDD but can also occur with non-toxic goitre. It is generally associated with nodular goitre and thyroid autonomy especially in elderly persons with IDD. Autonomy arises from mutation with activation of the TSH receptor or Gsα protein. The condition lasts about 1-6 months. It occurs also in euthyroid individuals from the use of iodinated compounds rather than from iodide, with 50% developing goitre but no exophthalmos. The mechanism is unclear (EGVM, 2000).

3.2.2. Excessive intake from food

In the normal human thyroid there is no real evidence that moderate acute excess iodine intake decreases thyroidal uptake of iodine largely because the variable dietary intakes do not appear to affect the serum levels of the thyroid hormones, the TSH level or the size of the thyroid gland. The normal amounts of iodine occurring in food do not cause goitre, thyrotoxicosis or iodine acne, only if intakes rise beyond the 10-fold normal value. Acute iodine excess increases thyroid hormone synthesis 10-20 fold while chronic iodine excess increases synthesis only 2-4 fold. Chronic intake of moderate or large doses of iodine decreases the serum level of thyroid hormones, increases TSH serum levels, increases the TSH response to TRH, and increases the size of the thyroid gland. In a random trial in Wales some participants received 500 µg iodide in addition to their normal daily intake of 250 µg. Some of those receiving the additional iodine showed significantly elevated TSH levels compared to the placebo controls (Chow *et al*, 1991).

Excessive intakes can cause an increase in thyrotoxicosis and Hashimoto's disease (with autoantibodies against thyroid proteins), but can also reduce the incidence of toxic nodular goitre and diffuse non-toxic goitre. It can also induce hypothyroidism in autoimmune glands. These changes are not seen in Japanese people despite an average intake of 50-80 mg I/day. In these circumstances urinary iodide excretion would increase to 20 mg/day or more.

3.2.3. Excessive intake from anthropogenic sources

In iodine-induced hyperthyroidism excess hormone is produced. This occurs especially in areas with endemic goitre or IDD, when iodine supplementation of the diet is introduced. In normal circumstances or at intakes <5 mg/day by populations which had no previous experience of iodine deficiency the incidence of hyperthyroidism or toxic nodular goitre is rare (Nagataki, 1987). In populations with preexisting IDD 5-8% may get transient hyperthyroidism and thyrotoxic goitre even at normal levels of iodine intake (150-200 µg/day) but always in response to dietary iodine supplementation. These effects occur usually in individuals aged 40-50 with nodular goitre or autonomous thyroid tissue (hormone production not controlled by TSH) or in individuals under 40 with undiagnosed Graves' disease. If the introduction of iodised salt to combat IDD is accompanied by poor monitoring of the quality of the iodised salt and of the iodine intake of the affected population cases of iodine-induced hyperthyroidism will occur. This was noted in Zimbabwe and the Democratic Republic of Congo (Delange *et al*, 1999). Intake of water containing 1 mg I^-/L caused impaired iodotyrosine formation in 13% of individuals. In asthmatics and bronchitics treated with KI some 0.5% developed myxoedema and 0.2% showed slight thyroid enlargement. In cystic fibrosis patients treated with saturated KI solution some 15% developed goitre, 5% hypothyroidism and 5% goitre plus hypothyroidism.

3.2.4. The effect of pregnancy

Pregnancy is goitrogenic therefore intakes of >100-200 µg I/day are required to prevent the development of goitre and to keep the serum levels of free T4 and T3 stable. Intakes of <50 µg I/day during pregnancy lead to hypothyroidism and the development of goitre in the mother and the newborn (Glinoer *et al*, 1995). Pregnant and breastfeeding women need a higher iodine intake because of increased urinary loss of iodine.

Excessive prenatal maternal iodine exposure of the order of 12-1650 mg iodide/day (0.2-27 mg/kg bw/day) from expectorant mixtures consumed during pregnancy was associated with 8 congenital goitres and hypothyroidism in infants but a clear causal relationship could not be demonstrated (Carswell *et al*, 1970). Maternal multiple topical applications of povidone-iodine (1% free I) have produced hypothyroid infants (Danziger *et al*, 1987). Similarly, maternal rectal irrigation with povidone-containing solutions have produced hypothyroid infants (US Food and Nutrition Board, 2001).

Pregnant women with concomitant excessive thyroid stimulation due to iodine deficiency, diagnosed by reduced free serum T4, reduced urinary I excretion, increased serum Tg, T3/T4, TSH and increased thyroid volume, if untreated, developed goitre in 18% of cases and the neonates had larger thyroids. When treated with either 100 µg KI/day or 100 µg KI + 100 µg L-T4/day TSH levels returned to normal, Tg decreased, and no goitre developed, while in the newborn Tg was also lowered and thyroid volume remained normal (Glinoer *et al*, 1995).

3.2.5. Goitre and thyroid cancer

Some 70% of the epithelial tumours of the thyroid are papillary carcinomas, 15% are follicular carcinomas, >5% are anaplastic carcinomas, while some 5-10% arise from medullary calcitonin-producing C-cells. The papillary carcinomas are less aggressive while the follicular carcinomas have a worse prognosis. Carcinomas are more frequent in females than males, occur especially in the aged and the mortality ranges from 0.2-0.7/100,000 females. Thyroid cancer incidence is increasing in many countries, particularly Norway and Denmark, but mortality rates are decreasing (NNT, 2002). The incidence shows great geographical variation between and within countries indicating an influence of exogenous factors. In man the only well established cause of thyroid cancer is external radiation to the thyroid (NNT, 2002). Goitre predisposes to thyroid papillary cancer as diffuse hyperplasia may be followed by nodular hyperplasia, benign tumour formation and eventual follicular papillary cancer, the risk being related to the presence of goitre and not the functional state of the thyroid. There is no animal evidence for this cancerogenic effect of goitre. The effect of iodine prophylaxis on the incidence of thyroid cancer in an IDD area of Argentine was examined by comparing the incidence in the 15 years before introduction of iodised salt with the incidence in the next 16 years. The incidence of papillary carcinoma increased but there was no effect on the incidence of follicular or medullary cancer. The papillary carcinomas were associated with a higher occurrence of lymphocytic thyroiditis (Harach and Williams, 1995).

Low dietary iodine intake may produce an increased gonadotrophic stimulation possibly leading to a hyperoestrogenic state with greater production of oestrogens and oestradiol. This may increase the risk of breast, endometrial and ovarian cancer (Stadel, 1976).

3.2.6. Acute exposure

Suicides have occurred with Lugol's solution, causing burning of mouth, gastrointestinal irritation, abdominal pain, ulceration, hyperthyroidism, haemolytic anaemia, acute renal failure with tubular necrosis, delirium, stupor and collapse. Tincture of iodine ingestion can cause vomiting, abdominal cramps, diarrhoea, anuria, fever, weak pulse, cardiac irritability, cyanosis, coma and death. Ingestion of 1184-9472 mg I causes death within 48 hours.

Doses of 2000-3000 mg iodine (30-40 mg I/kg bw) are probably lethal to humans but survival has been reported after ingestion of 10-15 g. Exposure to iodine vapour causes lung, eyes and skin irritation. Iodide in expectorant mixtures has been used at doses of 3.3 mg/kg bw mostly without adverse reactions. Iodine intakes >10 mg/day from drugs or accidental poisoning is toxic for some individuals (WHO, 1988).

The intake of foods or seasonings made from algae or seaweed containing more than 20 mg iodine/kg dry mass could damage health (BGVV, 2001)

Thirty two individuals, of which 22 had Hashimoto's thyroiditis and 10 normal controls were given a single dose of 2.0 mg iodide and the effect on the uptake of ^{131}I was measured and compared with the uptake before treatment. Patients with thyroiditis had their I uptake reduced by 54%-99%, normal persons had a reduction of 8-54%. Thus iodide aggravated some thyroid disease (Paris *et al*, 1961).

3.2.6.1. Sensitivity reactions to iodine

Iodide can also give rise to sensitivity reactions such as urticaria, angiooedema, polymyalgia, conjunctivitis, coryza, iodide fever, headache, salivary gland enlargement, cerebral symptoms and hypotension. Iododerma, eosinophilia, pruritic rashes, vesicular eruptions and fungoid eruptions may also occur (WHO, 1988). Some 3.2% of individuals treated with ^{131}I-labelled protein developed sensitivity reactions. Following amiodarone treatment about 0.4% developed erythema nodosum. In individuals with hyperthyroidism treated with iodide some 1.75% developed fever. In asthmatics/bronchitics treated with KI about 5% showed swollen salivary glands, 3% had runny noses, 2% headaches and 15% gastrointestinal complaints. In individuals treated with contrast media for urography (I content 4935-5150 mg/dose) some 1.7% experienced acute allergic reactions and 1.5% suffered from hives, sneezing, nasal congestion, pruritus and facial oedema, diffuse rash, hypotension, collapse, asthma, laryngeal oedema, grand mal seizures and parotid swelling.

3.2.7. Subchronic exposure

For persons with autonomous thyroid tissue intakes of 100 µg/day posed no risk (Joseph *et al*, 1980) but 200 µg/day caused thyrotoxicosis in some people resident in an IDD region (Stewart, 1975). Iodide supplementation of 1500 µg/day had a significant inhibitory effect on thyroid function in normal men (Meyers *et al*, 1985). An evaluation of the oral doses at which adverse effects were reported showed

21 cases out of 1256 (1.7%) reported in the literature at doses <1.0 mg/day, while 49 cases out of 1256 (3.9%) occurred at doses of ≤10 mg/day. Of these some had underlying thyroid disease which may have affected their response to the extra iodine supplied (Pennington, 1990).

Normal subjects receiving 50-250 mg iodide/day for 10-14 days were reported to show subtle changes in thyroid function. These consisted of small but significant decreases in serum levels of T4, T3 and concurrent small compensatory increases in basal serum TSH concentrations and exaggerated serum TSH responses to i.v. TRH (Vagenakis *et al*, 1973; Saberi and Utiger, 1975). The dietary intakes of iodine were not recorded in these studies.

Men who drank iodised water providing iodine doses of 0.17-0.27 mg/kg bw/day for 26 weeks reported no adverse effects (Morgan and Karpen, 1953).

Pharmacological doses of iodide of 1000 mg/person/day administered to 4 normal euthyroid volunteers for 11 weeks caused small but significant decreases in serum levels of T4 and T3 and compensatory increases in basal serum TSH levels and the responses elicited by TRH (Jubiz *et al*, 1977).

The ingestion of about 3 mg iodine/day for 6 months during daily mouth-rinsing with an iodine-containing mouthwash had no effect on thyroid function (Ader *et al*, 1988).

The ingestion of 200 mg/day of erythrosine (I-rich food colour) for 2 weeks caused a small increase in basal and TRH-stimulated TSH secretion. The urinary iodine excretion was about 1200 µg/day (Gardner *et al*, 1987).

A study was designed to determine the effectiveness of oral doses of iodide (199, 300, 600, 1000 µg/day) in suppressing ^{131}I uptake in groups of 4-10 children of different ages (1-3, 4-6 and 9-11 years) with clinically normal thyroid function as protection in the event of radio-iodine fall-out following a nuclear incident. In the group 1-3 years old, a decrease in uptake occurred with 300 µg/day within 2 weeks, and a further fall occurred when the intake was subsequently increased to 600 µg/day. Suppression of uptake was also analysed in relation to the iodide dose expressed as µg/m^2 body surface area/day. The maximum suppression of uptake occurred within 2 weeks with 1500-2000 µg/m^2. Doses of 100 µg/m^2 slightly increased uptake. The NOEL for any effect on the uptake of ^{131}I by children aged 1-11 years was 100 µg/m^2/day. No toxic effects were noted at any of the doses used, however the study used only small groups, exposure was short and the groups may not have included susceptible individuals. No indication of the iodine intake from the children's daily diet was given, and therefore it is not possible to calculate the total intake. A dose of 100 µg/m^2 is equivalent to about 170 µg/day (in addition to intake from food) for an adult (assuming 1.7 m^2 body surface area) (Saxena *et al*, 1962).

The studies of Paul *et al* (1988), Gardner *et al* (1988), and Chow *et al* (1991) are also subchronic studies and these are described in detail in Section 4 (Dose-response assessment).

3.2.8. Chronic exposure

Chronic exposure to iodine causes iodism. The symptoms resemble coryza as well as salivary gland swelling, gastrointestinal irritation, acneform dermatitis, metallic taste, gingivitis, increased salivation, conjunctivitis and oedema of eye lids (Goodman and Gilman, 1970). Some consider 2 mg iodide/day (0.03 mg/kg bw) excessive but the Japanese appear to consume 50-80 mg/day (0.8-1.3 mg/kg bw) without adverse effects (Mertz, 1986).

In a study on 37 patients with chronic lung disease, treated with 1000-2000 mg iodine/day for a mean 2.2 years, some 13 became clinically hypothyroid but in 7 of these patients normal thyroid function returned on withdrawal of iodine medication (Jubiz *et al*, 1977).

The introduction of iodised bread in The Netherlands raised the daily intake by 120-160 µg iodine resulting in an increase in hyperthyroidism (Van Leeuwen, 1954) The use of winter milk in the UK raised the iodine intake of women to 236 µg/day and of men to 306 µg/day and was associated with a peak incidence of hyperthyroidism (Nelson and Phillips, 1985). In 32 young adult Swiss with simple goitre (and urinary I excretion of 32 µg/day) given 200 µg I/day only one case of transient hyperthyroidism appeared which showed a serum T4 of 14 µg/100 mL, a serum T3 of 293 ng/100 mL, suppressed TSH, tachycardia and weight loss (Baltisberger *et al*, 1995).

When iodine intake was increased by iodide tablets, iodised bread and iodophors in Tasmania to 200 µg/day the incidence of hyperthyroidism rose from 24 to 125/100,000 in subjects >40 years suffering

from multinodular goitre and preexisting heart disease over a period of 10-12 years (Connolly *et al*, 1970). In Tasmania the incidence of nodular goitre and toxic nodular goitre was eliminated in persons with normal thyroids. Those which developed hyperthyroidism also had autoimmune antibodies (Adams *et al*, 1974). A clinical survey of 30 hyperthyroid patients observed in Tasmania after the introduction of bread fortified with iodate detected 8 patients with autonomous thyroid nodules but no thyroid stimulating antibodies, 16 patients without localised autonomy but with antibodies and 6 patients without either localised autonomy or antibodies. Serum TSH was 0.15 mU/mL or less in all cases. Hence this crop of hyperthyroidism cases was due to the latent hyperthyroidism associated with the presence of toxic nodules or thyroid stimulating antibodies (Adams *et al*, 1975).

The introduction of iodised salt (30-90 mg I/kg) in Zimbabwe, an area of moderate to severe IDD, led to an increase in cases of hyperthyroidism, normally rare among African populations, as shown by a review of local hospital records of relevant laboratory tests for free T3, T4 and serum TSH levels. The annual incidence of hyperthyroidism rose from about 90 to about 163 during two years after the introduction of iodised salt (30-90 mg/kg). A review of some 235 patients diagnosed as thyrotoxicosis showed an incidence of Graves' disease of 27% and of toxic nodular goitre of 58%. Patients were mostly females with a mean age of 50 years. Some 14 deaths occurred from heart failure with atrial fibrillation and some embolic episodes. Urinary iodine levels had risen from a median 20 µg/L to 238 µg/L. The problem of iodine-induced hyperthyroidism appeared to have lasted for about 2 years (Todd *et al*, 1995).

Similar reports of iodine-induced biochemical and overt clinical hyperthroidism in IDD areas have come from the Democratic Republic of Congo and 7 other African countries after the introduction of iodised salt. These measures reduced considerably the goitre prevalence in school children and urinary iodine levels indicated the elimination of IDD (Delange *et al*, 1999).

In a 5-year study using iodinated drinking water (1 mg/L) supplied to 750 male and female prison inmates no hyper- or hypothyroidism, no sensitisation reactions and no iodism were noted. The average dose was 30 µg/kg bw. There was a statistically significant decrease in ^{131}I uptake and an increase in protein-bound iodine (PBI) of the thyroid. One-hundred and seventy seven women inmates delivered 181 infants showing no thyroid-related adverse effects. Four hyperthyroid women became more hyperthyroid. The difficulties with this study were the imprecise estimates of intakes from the diet and fluid consumption of the participating individuals as well as the variable exposure time but the group size and duration of exposure were adequate (Stockton and Thomas, 1978).

4. DOSE/RESPONSE ASSESSMENT

A study on the effects of doses of 250, 500 or 1500 µg iodide/day for 14 days on thyroid function was carried out in 9 euthyroid men (mean age 34 years) and 23 euthyroid women (mean age 32 years) with 5 age-matched controls. The parameters examined were PBI, total serum iodine, T4, T3, TSH, integrated 1-hour serum TSH response to an intravenous dose of 500 µg TRH, and 24-hour urinary iodine excretion. The dietary intake of iodine was estimated from the urinary iodine excretion to be approximately 200 µg/person/day making the total ingested doses approximately 450, 700 or 1700 µg iodide/day. The estimated dose of 1700 µg/day increased the total serum iodine without affecting the PBI, significantly decreased serum T4 and T3 and increased TSH levels, whilst 700 and 450 µg/day did not affect significantly these values. Only 1700 µg/day increased the TSH response to TRH (in women more than in men). The TSH response to TRH was also increased, though not significantly, in the individuals receiving 700 µg iodide/day. No biochemical effects were detected with 450 µg of iodide/day; however this study used only small groups, extended over only 2 weeks and the dietary iodine intake was not determined analytically but was estimated (Paul *et al*, 1988).

In another study groups of 10 males (mean age 27 years) were treated for 2 weeks with either 500, 1500 or 4500 µg iodide/day. The dietary intake was estimated from urine iodine excretion to have been approximately 300 µg/person/day making the total ingested doses approximately 800, 1800 or 4800 µg iodide/day. Serum levels of T3, T4, TSH, PBI, and total iodide, the TSH response to intravenous TRH and 24-hour urinary excretion of iodide were measured before treatment and again on day 15. Serum T4 levels decreased significantly after ingestion of 1800 µg and 4800 µg/day but did not change after 800 µg/day. Serum T3 levels did not change at any dose. Serum TSH levels remained unchanged in those receiving 800 µg/day but increased in those receiving 1800 µg and 4800 µg/day. The TSH response to TRH was significantly enhanced with all iodide doses administered. No adverse effects were reported and no significant symptoms of thyroid dysfunction were noted. Again only small groups of subjects were studied, only males were examined, exposure was rather short and the actual dietary intake of iodine was not determined analytically but estimated (Gardner *et al*, 1988).

A study on the effect of supplementation of normal dietary intakes (about 250 µg I/day) with 500 µg/day iodide, giving a total iodide intake of approximately 750 µg iodide/day, or a placebo for a period of 28 days, on the serum levels of free T4 and TSH was carried out in women selected from a general practice in Cardiff. The groups studied were aged 25-54 years and thyroid antibody positive (subclinical Hashimoto's thyroiditis) (n=20) or antibody negative (n=30), or aged 60-75 years and from an area with adequate dietary iodine supply (n=29) or from an area that was previously iodine deficient (n=35). The study was described as a randomised placebo-controlled trial, but it is not clear whether the study was of crossover or parallel group design. Small decreases in T4 levels and small increases in TSH levels, indicating mild biochemical hypothyroidism, occurred in all iodide-supplemented subjects of all groups. None of the groups on supplemental iodide showed any incidence of hyperthyroidism. Following iodide supplementation TSH levels increased above the normal level of 5 mU/L in 3 of the 60-75 year old subjects, while the raised TSH levels increased even further in 2 antibody-positive subjects (Chow *et al*, 1991).

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The parameters altered in these dose-response studies included an elevation of serum TSH levels in response to iodine intake and the enhanced response in TSH levels to TRH stimulation. They were all of a biochemical nature and not associated with any clinical adverse effects. However, elevated serum levels of TSH are not necessarily clinically adverse, but could be regarded as indicators of an existing risk of induced hypothyroidism. There is uncertainty whether the subtle changes observed, such as an enhanced response to TRH, would have significant adverse biological consequences even if sustained over longer periods, because all observed values remained within the normal ranges for the parameters determined. It remains uncertain whether chronic exposure to these small doses would have any relevant clinical consequences in normal euthyroid individuals.

An UL can be established on the basis that the noted biochemical changes in TSH levels and the TSH response to TRH administration were marginal and unassociated with any clinical adverse effects at estimated intakes of 1700 and 1800 µg/day.

Although the studies on which these UL estimates are based were all only of short duration, involved only a small number of individuals, and lacked precision of the actual total dietary intakes, their results were supported by the study covering a 5-year exposure at approximately similar iodide intake levels of 30 µg/kg bw/day (equivalent to approximately 1800 µg iodide/day) in which no clinical thyroid pathology occurred. An UF of 3 is thus considered adequate and provides an UL for adults of 600 µg/day.

The UL of 600 µg is also considered to be acceptable for pregnant and lactating women based on evidence of lack of adverse effects at exposures significantly in excess of this level.

Since there is no evidence of increased susceptibility in children, the ULs for children were derived by adjustment of the adult UL on the basis of body surface area (body weight $^{0.75}$).

Age (years)	Tolerable Upper Intake Level (UL) for Iodine (µg per day)
1-3	200
4-6	250
7-10	300
11-14	450
15-17	500

In the US the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes of the Food and Nutrition Board together with Health Canada are pursuing a joint project which proposes a tolerable upper level of intake for iodine for adults of 1100 µg/day (US Food and Nutrition Board, 2001). WHO has suggested a provisional maximal tolerable daily intake of 1 mg/day from all sources, equivalent to 17 µg/kg bw (WHO, 1988). In countries with long-standing IDD the intake should not exceed 500 µg/day to avoid the occurrence of hyperthyroidism. In France the Expert Committee on Human Nutrition has suggested an UL of 500 µg I/day in countries with long-standing IDD to avoid the occurrence of hyperthyroidism (AFSSA, 2001).

6. CHARACTERISATION OF RISK

Data from European populations indicate that the intakes of iodine from all sources in adults are unlikely to exceed the UL. For example, in the UK where iodine intake is considered to be high relative to other European countries, the 97.5 percentile intake in men is 434 µg/day.

In the UK survey data in young children aged 1½-4½ years have shown that iodine intakes may vary from 87-309 µg/day, with almost all iodine deriving from the consumption of milk. High winter milk consumers may ingest up to 247-309 µg/day. The UK COT considered that the intake of iodine at the concentrations that have been found in cow's milk is unlikely to pose a risk to health even in those children who are high level consumers (COT, 2000). The SCF agrees with this and notes that an UL is not a threshold of toxicity but may be exceeded for short periods without an appreciable risk to the health of the individuals concerned.

Ingestion of iodine-rich algal products, particularly dried products, can result in dangerously excessive iodine intakes.

These ULs do not apply to IDD populations, as these are more sensitive to iodine exposure.

The UL is not meant to apply to individuals who are being treated with iodine under medical supervision.

7. RECOMMENDATIONS FOR FUTURE WORK

1. More precise definition of the safety levels for iodine-sensitive individuals relative to those with normal thyroid function.

2. Better data on the iodine intake and thyroid status of children aged 1-3 years.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF PREFORMED VITAMIN A (RETINOL AND RETINYL ESTERS)

(EXPRESSED ON 26 SEPTEMBER 2002)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Vitamin A is a micronutrient essential to most mammalian species. The term vitamin A describes a group of lipid soluble compounds related metabolically to all-*trans*-retinol. In the diet, vitamin A is found in products of animal origin, as retinyl esters, mainly retinyl palmitate. Other esters (oleate, stearate, myristate), and retinol contribute to the dietary vitamin A intake. The forms most commonly found in vitamin supplements or enriched food, are retinyl acetate, retinyl palmitate and retinol. These vitamin A compounds, together with their metabolites, and synthetic derivatives that exhibit the same properties, are called retinoids.

Some carotenoids (α- and β-carotenes, β-cryptoxanthine) can be cleaved into retinol, via an enzymatic process, which occurs mainly in the small intestine, and is readily saturated. The toxicity of carotenoids differs from that of retinoids, and the risks of high intakes of carotenoids are not linked to the adverse effects of retinoids. Consequently, this report will deal only with the effects of retinoids, on the assumption that the pro-vitamin A properties arising from dietary intakes of carotenoids will not contribute significantly to the toxicity of high intakes of vitamin A.

Vitamin A can be expressed on a weight basis as Retinol Equivalents (1 RE = 1 μg retinol) or in International Unit (IU). Both units take into account the vitamin A potency of various esters, according to the conversion factors indicated in the following table:

Molecule	Vitamin A activity in International Units (I.U.)	Vitamin A activity in Retinol Equivalent (R.E.)
Retinol (1 mg)	3330	1000
Retinyl acetate (1 mg)	2900	870
Retinyl palmitate (1 mg)	1830	550

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

Dietary vitamin A is absorbed in the upper part of the small intestine, by mechanisms similar to those of lipid absorption. Retinyl esters undergo hydrolysis by pancreatic lipase (short chain esters) and an enzyme in the intestinal brush border (long chain esters). The released retinol is incorporated into mixed micelles and absorbed into enterocytes where it is bound to an intra-cellular protein called CRBPII (cellular retinol binding protein II). The intracellular retinol is re-esterified (largely with palmitic acid), packaged into chylomicrons and released into the general circulation via the lymphatic system. The chylomicrons in the general circulation are hydrolysed by plasma lipoprotein lipase and chylomicron remnants that are rich in retinyl esters are taken up by tissues, which possess specific receptors, mainly the liver. Remnants are degraded within the hepatocytes, and the released retinol is transferred to stellate cells for storage after re-esterification.

The liver is the major storage site for vitamin A, which is mainly localised in lipid droplets of hepatic stellate cells (also known as Ito cells or lipocytes). These droplets, which never fuse into a large vacuole, may almost fill the cell, which thus has a very high, but not unlimited, storage capacity.

In normal conditions, vitamin A is mobilised from the liver stores as retinol, bound to a specific carrier protein, the Retinol-Binding Protein (RBP), and released into the plasma. This mobilisation is highly regulated and ensures a homeostatic control of the plasma retinol concentration, which is maintained at a concentration of about 2 µmol/L, except during extreme hypo- or hyper-vitaminosis A. The usual plasma concentrations of retinoic acids, the active metabolites of retinol, are much lower (about 10 nmol/L), and their regulatory control is not well understood. The retinol-RBP complex is stabilised by forming a complex with transthyretin (pre-albumin).

Retinol bound to RBP enters target tissues, by a mechanism that may involve a specific membrane receptor, although such a receptor has been described only in the pigmented epithelium of the retina. Another hypothesis to explain the specific and regulated delivery of vitamin A to tissues proposes a particular lipid composition of some membrane areas. After internalisation, retinol is usually bound to the intra-cellular binding protein CRBP.

Intracellular retinol can undergo a number of different pathways of metabolism. It can be esterified within the tissue, probably to generate a limited local *in situ* store. Retinol can undergo metabolic activation by oxidation of the side chain into retinal and retinoic acids, which can in turn be further oxidised, probably by cytochrome P450, into 4-hydroxy metabolites (Collins and Mao, 1999). Another metabolic pathway is conjugation with glucuronic acid, which leads to retinoyl- and retinyl glucuronides, the enhanced polarity of which results in their elimination in faeces and urine (reviewed in Olson, 1999). Nearly all of the metabolically inter-related retinoid compounds may exist in an all-*trans* form or as *cis*- or di-*cis*- isomers (mainly at the 9, 11 and 13 positions). Numerous enzymes are involved in these various metabolic pathways, and the relative involvement of each of them is not yet completely understood. Of particular importance are the enzymes involved in the conversion of retinol to retinal and then to retinoic acid, because these enzymes may act as regulators of tissue retinoic acid levels. The reversible interconversion of retinol into retinal can be catalyzed both by the group of short-chain dehydrogenase/reductase and by alcohol dehydrogenases. Conversely, the irreversible oxidation of retinal into retinoic acid can be due to aldehyde dehydrogenases or members of the cytochrome P450 family (Duester, 1996).

Retinal, the initial oxidised metabolite of retinol, is the chromophore of rhodopsin, a visual pigment of the cone cells of the pigmented epithelium of the retina. The photo-induced isomerisation of 11-*cis*-retinal into all-*trans*-retinal is the initial event of the photo-transduction cascade, which ends by the production of a signal to the ocular nerves.

Retinoic acids, both all-*trans*-retinoic acid (TRA) and its 9-*cis* isomer (9CRA) act as regulators of genomic expression; the 13-*cis* isomer (13CRA), which is present in plasma at similar concentrations to TRA, is probably not involved in the actions of vitamin A. Retinoic acids are able to bind to specific nuclear receptors known as RAR and RXR receptors: RARs can bind either TRA or 9CRA, while RXRs bind only 9CRA. Upon ligand binding these nuclear receptors bind to specific response elements on DNA, and thus regulate gene expression. The system is complex due to the existence of several isoforms for RAR and RXR, and because the activated receptors dimerise with themselves, with each other, or with other members of the same superfamily of receptors (namely vitamin D receptor, thyroid hormone receptor, PPAR) before they bind to the DNA response elements. The number of genes known to be regulated by retinoic acids is continually increasing. Retinoic acids are considered as the molecular species responsible for all the functions attributed to vitamin A, with the exception of vision.

Of particular importance in the setting of an upper level is the role of retinoic acids during morphogenesis and embryonic development. It has long been recognised that abnormal fetal development is associated with either insufficient or excessive intakes or vitamin A and related compounds. The role of retinoic acid and its receptors in ontogenesis was confirmed by the finding that RAR-null mutant mice (Lohnes *et al*, 1994) died *in utero* or shortly after birth, and exhibited congenital abnormalities. Moreover, RARs and RXRs show specific spatio-temporal patterns of expression in all developing systems during embryonic development, which suggests that retinoic acid signalling is involved in most, if not all, morphogenetic and patterning processes (Morriss-Kay and Sokolova, 1996).

Vitamin A deficiency is rare in the Western world, but is a major problem in developing countries. Specific symptoms associated with deficiency include visual problems such as night blindness and xerophthalmia that may end in irreversible blindness. Other reported effects include growth retardation in children, skin disorders, impaired immune function and congenital malformations of the eyes, lung, cardiovascular and urinary systems if deficiency occurs during pregnancy. However, in humans, these latter symptoms are often associated to a multi-nutrient deficiency and the exact role of vitamin A remains to be ascertained.

In 1992, the Committee determined a population reference intake (700 μg/day for men and 600 μg/day for women) (SCF, 1993). This recommendation is met by the intakes of the general population in developed countries. Intake data for various European countries (Table 1) indicate that the mean intakes are well above the population reference intakes, whereas the median intakes are at or slightly below the population reference intakes. The difference between the mean and median values indicates a skewed distribution of intakes, which arises from the non-uniform distribution of preformed retinol in the food supply, and very high intakes by consumers of foods such as liver.

Table 1. The daily intakes of preformed retinol (retinol and retinyl esters) in EU countries (μg/day)

	Population	n	Method	Supplements	Mean	97.5%
Austria[a]	men + women	2488	24 h recall	Not defined	1120	4230
Germany[b]	men	854			660	
	women	1134			530	
Germany[c]	men	1268		-	2010	4100
	women	1540		-	1710	3440
	men	240		+	2020	
	women	347		+	1790	
Italy[d]	household	2734	7-day record	+	759	4377
Netherlands[e]	household	5958	2-day record	-	891	3230
UK[f]	Men	1087	7-day record	-	1226 (602)	6564
	women	1110		-	1058 (463)	5698
	men	1087		+	1277 (618)	6671
	women	1110		+	1133 (491)	5779

Results are for intake as preformed retinol
[a] Elmadfa *et al* (1998)
[b] Heseker *et al* (1992) - median not mean value; data reported as preformed retinol
[c] Mensink and Ströbel (1999) - these values include retinol equivalents from carotenoids
[d] Turrini (INRAN) - as retinol
[e] Hülshof and Kruizinga (1999)
[f] Gregory *et al* (1990) - values are the mean with the median in parentheses

3. HAZARD IDENTIFICATION

Many cases of acute hypervitaminosis A has been reported in the past 60 years. They have been extensively reviewed by Bauernfeind (1980) who quoted an exhaustive compilation of 385 individual cases up to 1975 (Köerner and Völlm, 1975). These cases mainly concern anecdotal ingestion by adults of large amounts of shark or polar bear liver, suspected to provide more than 2 mg/g of retinol equivalents. More than 100 cases of iatrogenic hypervitaminosis A were reported in children in France and Spain in the early 60s, probably potentiated by high doses of vitamin D. Excessive dosages of vitamin A may result in a number of adverse effects, including skin disorders, nausea, vomiting, bone pain, plus teratogenicity due to retinol and its metabolite TRA (which has been the focus of most previous risk assessments).

The following adverse effects are reviewed separately:

1. Bulging fontanelle in infants/intracranial pressure
2. Hepatotoxicity
3. Effects on bone metabolism
4. Effects on lipid metabolism
5. Teratogenicity.

3.1. Bulging fontanelle in infants/Intracranial hypertension

The prevention of vitamin A deficiency in developing countries has involved the administration to infants and children of a single large dose of vitamin A, or a series of large doses with an interval of 1 month or more between consecutive doses. The development of reversible bulging fontanelle (BF) has been reported in a number of these studies. BF is a clinical symptom, which can be observed in infants at examination. Usually, it is not accompanied by an elevation of intracranial pressure (Agoestina *et al*, 1994), probably because the increased volume of the cerebro-spinal fluid can expand, using

the fontanelles and the un-fused cranial sutures (Humphrey *et al*, 1998; WHO, 1998). The effect is age-dependent with higher doses being without effect in 6 or 9 month old infants (Stabell *et al*, 1995) compared with 6-17 week old infants (De Francisco *et al*, 1993; Baqui *et al*, 1995). Vitamin A induced BF is always rapidly reversible (usually in less than 2 days). BF exclusively concerns a distinct and sensitive sub-population that is neonates and infants of both genders, from birth to 6 to 8 months of age. A recent study by Humphrey *et al* (1998) clearly shows that vitamin A-induced BF is not associated with adverse growth or developmental sequelae.

BF may represent the infant form of the headaches that are frequently reported during hypervitaminosis A in adults, and which may possibly arise from increased intracranial pressure (although it is actually not measured). In older children or in adults, the increased volume of the cerebro-spinal fluid can be linked to an increased intra-cranial pressure (Babikian *et al*, 1994). Excessive vitamin A intakes have been described as one among many possible causal factors of symptomatic intracranial hypertension (Pasquariello *et al*, 1977; Tibbles *et al*, 1972; Gangemi *et al*, 1985).

3.2. Hepatotoxicity

Relatively few chronic toxicity studies on animals have been reported. Leo *et al* (1982) showed that rats receiving 120 μg RE of vitamin A daily for 8 weeks resulted in proliferation of the endoplasmic reticulum and mitochondria enlargement, symptoms which were enhanced when the animals received ethanol simultaneously. An earlier study (Randall, 1951, cited by Santos, 1987) in which rats were fed 5 days a week during 10 months with 3 to 15,000 μg RE of vitamin A/kg of diet did not show any change in body growth and haematological parameters, but liver examination was not performed. Subsequent animal studies have confirmed the effects of vitamin A on the liver and the nature and extent of hepatic damage (Shintaku *et al*, 1998). Lettinga *et al* (1996) have shown that feeding rats during one week with 75,000 μg RE of vitamin A/kg diet (approximately 1500 μg RE per animal, daily) led to activation of Kupffer cells and proliferation of hepatic stellate cells. These events are known to be initial steps of the development of an experimental fibrosis in rats.

The available data on humans are exclusively case reports, either describing a single case, or gathering information obtained in a given hospital. For obvious ethical reason, no experiments have been carried out on humans. In most of the reported cases, the toxicity has been linked to the intake of high doses of vitamin A, over long time periods. Evidence of the causal link between the vitamin and the hepatotoxicity was the improvement of symptoms after withdrawal of vitamin A, and the fact that other possible etiologic causes had been ruled out by experienced clinicians and adequate assays. Furthermore, hepatotoxicity was very frequently associated with elevated retinol and retinyl esters in serum, and histology revealed hepatic stellate cell hyperplasia.

Hepatotoxicity is one of the most severe outcomes of chronic intake of high dosages of vitamin A (Bauernfeind, 1980; Geubel *et al*, 1991; Kowalski *et al*, 1994). The first symptoms of hypervitaminosis A are not hepatic; they vary greatly, according to the severity of the disease, and often include headache, bone and joint pain, nausea and dry skin. Vitamin A induced hepatotoxicity can be diagnosed clinically using signs of hepatomegaly, chronic hepatic disease, ascites, icterus, oedema, oesophageal varices or dermatological lesions. Serum transaminase levels are usually moderately enhanced, and there is often a slight anicteric cholestasis. Histological features of vitamin A-induced hepatotoxicity include hepatic stellate cell hyperplasia and hyperproliferation, as well as collagen diffusion within the space of Disse, which can evolve in a portal hypertension (Guarascio *et al*, 1983; Geubel *et al*, 1991; Jacques *et al*, 1979).

In many cases, the hepatotoxicity is reversible after the withdrawal of vitamin A, which results in slow (up to several years) normalisation of the biochemical indexes. However, in some patients the liver disease progresses after vitamin A withdrawal from steatosis or fibrosis into micronodular cirrhosis, development of which can be fatal. The hepatotoxicity can be potentiated by various pathological conditions, including hypertriglyceridemia (Ellis *et al*, 1986), chronic alcohol intake (Leo and Lieber, 1999), and pre-existing liver disease (Russell *et al*, 1974). It is difficult to estimate quantitatively the proportion of vitamin A-induced hepatotoxicity which is reversible upon withdrawal. In the most comprehensive available set of data, 41 patients were diagnosed with a vitamin A-induced hepatic pathology, at various levels of severity; nine (22%) died in less than 2 years following diagnosis and progression of the disease was demonstrated in 3 others.

Mechanisms of hepatic effects are linked to overload of the storage capacity of the liver for vitamin A. The liver then becomes unable to take up newly-absorbed retinyl esters, and possibly releases retinol unbound to RBP. Non-specific delivery of retinol, which has surface-active properties, may produce membrane damage and lysosomal rupture (Ellis *et al*, 1986).

In addition, the vitamin A-loaded hepatic stellate cells may fill the sinusoidal space and thus obstruct the blood flow and create portal hypertension (Russell *et al*, 1974; Hruban *et al*, 1974). Hepatic stellate cells are also involved in the synthesis of extracellular matrix proteins, and upon activation, they exhibit a "myofibroblast-like" phenotype (Davis *et al*, 1987; Svegliati-Baroni *et al*, 2001), producing collagen type III, and promoting fibrosis and potential cirrhosis.

3.3. Bone metabolism

Histopathological changes in animal bone following very high vitamin A doses (up to 13,500 μg RE per animal) have been reviewed in Hathcock *et al* (1990) and have been shown to lead to bone fragility and spontaneous fractures (Nieman and Obbink, 1954). Similar bone lesions have been described in rats following retinoic acid administration (Dhem and Goret-Nicaise, 1984), and in the rabbit following intra-articular injection of 30,000 μg RE of retinyl palmitate (Lapadula *et al*, 1995).

Several isolated cases of skeletal problems in children with severe hypervitaminosis A have been reported (reviewed in Biesalski, 1989). Bone symptoms involve a decrease in density, osteoporotic changes and cortical thickening of the long tubular bones, leading to retarded growth. Freudenheim *et al* (1986) measured bone mineral content in a 4 year clinical trial in women receiving or not receiving calcium supplementation. Dietary intake was determined by a single 24-hour record and used to determine any dietary factors affecting the results. A highly significant effect of vitamin A was reported at only one site, the ulna, and only in women taking calcium supplements; this finding is difficult to interpret as it seems to have arisen largely due to a single individual with a very high vitamin A intake (4300 μg RE) and who showed very rapid bone loss. Sowers and Wallace (1990) reported no relationship between vitamin A intake or serum retinol concentration and radial bone mass or fracture history in a group of 246 postmenopausal women. A brief report by Theiler *et al* (1995) suggested that chronic vitamin A intoxication in adults might be related to osteoarthritis. Houtkooper *et al* (1995) analysed the influence of various factors, including nutrient intake, on annual rates of change in bone mineral density in a group of 66 pre-menopausal women who were taking calcium supplements. There was a slight loss of bone, measured at a number of sites, during the 18 months of the study, which was within the measurement errors of the techniques available. At one of the measured sites there was an indication that high intakes of vitamin A were associated with less loss of bone. No association was found between serum retinyl esters and reduced bone density in the 1988-1994 United Kingdom National Health and Nutrition Survey (Ballew *et al*, 2001), although serum retinyl esters reflect recent intake and are not a good indicator of vitamin A status.

There were no changes in serum markers of skeletal turnover (bone-specific alkaline phosphatase, N-telopeptide of type 1 collagen and osteoclastin) in a group of 40 male volunteers given 7.6 mg vitamin A as retinyl palmitate per day for 6 weeks (Kawahara *et al*, 2002). Such serum measurements were considered by the authors to be sensitive markers of bone turnover as they show larger and more rapid changes to therapeutic treatments than would be found with measurements of bone mineral density. However the authors concluded that whether long-term vitamin A supplementation might have adverse skeletal effect remains to be determined.

A nested case-control study (Melhus *et al*, 1998) has investigated the vitamin A intake (which was divided into 4 bands of <0.5, 0.5-1.0, 1.0-1.5 and >1.5 mg/day) by 247 women with a hip fracture and 873 controls from a group of 66,651 Swedish women in a mammography study cohort. The dietary intake of pre-formed retinol, was associated, in a dose-dependent manner, with a higher risk of hip fracture; both univariate and multivariate analysis showed a significant ($P<0.01$) 1.5- to 1.6-fold increase in risk per mg retinol consumed daily. An associated cohort study indicated that similar intakes of retinol reduced bone density (Melhus *et al*, 1998). Analysis of data from the Nurses' Health study in the US (Feskanich *et al*, 2002) reported 603 hip fractures a total of 72,337 women who had been studied for up to 18 years, with an increased risk attributable to total retinol (vitamin A) intake and retinol intake but not beta-carotene intake. The total vitamin A and retinol intakes were divided into quintiles and there was a significantly elevated relative risk of 1.48 and 1.89 respectively in the highest quintiles of intake (>3000 and >2000 μg RE per day respectively) compared with the lowest quintiles. Multivariate analysis revealed highly significant trends of increased risk for both total vitamin A ($P=0.003$) and retinol ($P\leq0.001$) for total intake (food plus supplements) but not for food only ($P=0.24$ and 0.05 respectively). The lower statistical power for the food only data may have been related to differences in the precision of the intake estimates, which were based on a semi-quantitative food frequency questionnaire, and brand-specific information on vitamin preparations. Hormone replacement therapy appeared to reduce the relative risk in post-menopausal women.

These findings may have a mechanistic explanation, related to a possible effect of retinoic acid in regulating the expression of genes, since both osteoblasts and osteoclasts express RARs and RXRs (Saneshige *et al*, 1995). Retinoic acid inhibits osteoblast differentiation (Cohen-Tanugi and Forest, 1998) and stimulates osteoclast formation and bone resorption (Scheven and Hamilton, 1990; Kindmark *et al*, 1995). A molecular interaction of vitamin A and vitamin D could also be responsible for the antagonism of vitamin A towards the action of vitamin D reported in rats (Rohde *et al*, 1999). A recent trial on 9 human healthy volunteers receiving either 15 mg of retinyl palmitate (8250 μg RE), or 2 μg of 1,25$(OH)_2D_3$ vitamin D, or a mixture of both, indicated that retinyl palmitate antagonizes the rapid calcium response to physiological levels of vitamin D (Johansson and Melhus, 2001). These data suggest that excessive vitamin A may increase bone resorption and decrease bone formation (Binkley and Krueger, 2000).

3.4. Lipid metabolism

Several reports suggest that retinoic acids increase plasma triacylglycerol concentrations in humans. Long-term intakes of moderate doses of retinol have been shown to increase circulating concentrations of both triacylglycerols and cholesterol (Cartmel *et al*, 1999). A population of 2297 subjects, with a moderate risk of skin cancer (actinic keratoses), received 7500 μg RE/day of retinol for approximately 4 years in a placebo-controlled trial. The treated group exhibited a small (2-3%) increase in cholesterol concentration. Serum cholesterol is a known risk factor for cardiovascular diseases, and even a small increase in concentration would represent an increase in risk.

3.5. Teratogenesis

The teratogenic effects of retinoic acids, the active oxidized metabolites of vitamin A, have been known for a long time and documented both in animals and in humans. Children exposed *in utero* to isotretinoin (13CRA) exhibit a pattern of congenital malformations, known as “the retinoic acid syndrome”, which includes defects of the craniofacies (small or absent external ears and auditory canals, cleft palate, micrognathia, low set ears), of the central nervous system (micro- or anopthalmia, cerebellar or cortical defects, microcephaly), of the thymus and of the cardiovascular system (transposition of the heart vessels, aortic arch hypoplasia, ventricular septal defects) (Lammer *et al*, 1985; Chan *et al*, 1996; Sinning, 1998). The risk of these defects was 25 times higher in the exposed children, and even greater when neuropsychological dysfunctions were assessed (Adams and Lammer, 1991). This last outcome could be related to an abnormal development of specific brain structures, which has been documented in rodents (Holson *et al*, 1997a, b and c). Most of these anatomical defects appear to be associated with alterations in the migration of cells from the neural crest (Morriss-Kay *et al*, 1993). The gestational period at which exposure occurred is of critical importance in the generation of these effects. In animals, the extent and nature of the defects resulting from the same dose of the same retinoid varies according to the gestational day of exposure (Holson *et al*, 1997a, b and c; Shenefelt, 1972). In humans, the critical period seems to be between the second and the fifth week of pregnancy, although it is generally stated that caution should be taken from the very beginning and up to the 60th day of pregnancy.

Birth defects similar to those observed following therapeutic use of isotretinoin or other retinoids have been described in approximately 20 women who had ingested vitamin A during the early weeks of their pregnancies (reviewed in Biesalski, 1989). These were separate case reports, in which the exposure to vitamin A occurred via supplements. Although the data represent a series of anecdotal cases, they confirm the link between excessive vitamin A intake and teratogenesis, which has been clearly documented in various animal species, including mice, rabbits, rats (Piersma *et al*, 1996) and non human primates. Large species differences in susceptibility exist, for example, the teratogenicity of 13CRA in mice is 20-fold less than in the Cynomolgus monkey, and 100-fold less than in humans (Hummler *et al*, 1990; Public Affairs Committee of the Teratology Society, 1990). The Cynomolgus monkey is the most sensitive animal species studied to date.

An important question is whether the pre-existing body stores affect the intake-response relation for teratogenicity. It is possible that the risk of the malformations could be higher in individuals with high body loads, but data from experiments on rats do not support this hypothesis. Biesalski *et al* (1996) did not find teratogenicity in rats fed a very high vitamin A diet before mating, and Piersma *et al* (1996) reported that the background level of circulating retinol or liver vitamin A did not affect the teratogenic potential of a single dose of retinyl palmitate. This indicates that a high vitamin A dose would have a similar teratogenic potential in a woman with good liver stores and in a vitamin A-deficient mother.

Several epidemiological studies have been designed to investigate the relationship between vitamin A intake and teratogenesis in humans. Five case-control studies have been published since 1990, in which the intake of vitamin A has been estimated retrospectively, both in controls and in mothers of malformed babies (Martines-Frias and Salvador, 1990; Werler *et al*, 1990; Botto *et al*, 1996; Mills *et al*, 1996; Shaw *et al*, 1997). The design of these studies varies, especially as regards the classification of the observed malformations, the numbers of women studied and the statistical power, and the collection of data on vitamin A consumption. The studies and data are summarised in Table 2.

Table 2. Epidemiological case-control studies that investigated the association between vitamin A intake and foetal malformations

Population		Results		Comments	Ref.
Cases (n)	**Controls (n)**	**Exposure to vitamin A**	**Odds ratio (95% confidence interval)**		
11,193	11,293	>3,000 µg RE/day >12,000 µg RE/day	1.1 (0.5 - 2.5) 2.7 (0.8 - 11.7)	only 11 cases and 4 controls at high exposure level	Martines-Frias and Salvador, 1990
2,658	2,609	during the 1st month	2.5 (1.0 - 6.2)	no information on vitamin A doses well-characterized neural crest-malformations	Werler *et al*, 1990
		2nd month	2.3 (0.9 - 5.8)		
		3rd month	1.6 (0.6 - 4.5)		
158	3026	Use of multivitamin Supplement	0.57 (0.33 - 1.00)	focus on conotruncal defects only	Botto *et al*, 1996
548 (NTD)	573	>2,400 µg RE/day from supplements	NTD: 0.91 (0.31 - 3.68) others: 1.05 (0.51 - 2.18)	Consumption of liver does not increase the risk	Mills *et al*, 1996
387 (others)		>3,000 µg RE/day from food and supplements	NTD: 0.73 (0.40 - 1.53) others: 0.92 (0.40 - 2.11)		
426	432	0-2999 µg RE/day	1.0 (reference)	NTD (neural tube defects) only; vitamin A from food and supplements	Shaw *et al*, 1997
16	12	3000-4499 µg RE/day	1.4 (0.6 - 2.8)		
6	7	>4500 µg RE/day	0.9 (0.3 - 2.5)		

NTD: neural tube defect.

Two prospective studies have been performed. Rothman *et al* (1995) recruited more than 22,748 pregnant women into a prospective study in which their intake of vitamin A, through both diet and supplements, was assessed by questionnaire for each of the 12 weeks since their last menstrual period. Information on pregnancy outcomes was obtained through obstetricians or mothers, without direct examination of the children. The birth defects that were reported in 339 children were then classified, and 121 malformations appeared to be of cranial-neural-crest origin. Rothman and colleagues reported that a daily intake exceeding 3000 µg RE of supplemental vitamin A significantly increased the risk of malformations. The percentages of babies with cranial-neural-crest defects were 0.52 and 1.06 in women with intakes from food of 0-1500 µg RE/day and more than 3000 µg RE/day respectively. A greater difference was found when the comparison was based on reported intakes from supplements with values of 0.46 and 2.21 percent in women with intakes of 0-1500 µg RE/day and more than 3000 µg RE/day respectively (giving a prevalence ratio of 4.8 with 95 percent confidence intervals of 2.2 to 10.5). These differences were based on small number of babies in each of the high intake sub-groups, i.e. 2 babies after >3000 µg RE/day from food and 7 babies after >3000 µg RE/day from supplements. Analysis of the potential vulnerable period in babies whose mothers had an intake >3000 µg RE/day showed that the prevalence of cranial-neural-crest defects was 4.8%, 3.8% and 0% when the high intake was only during 2 weeks before conception (n=2/42), only before week 7 of pregnancy (n=3/80) and only after week 6 (n=0/70) respectively. The overall conclusion of the study (see quantitative considerations below) was different from those of the retrospective studies. This paper has been criticised, particularly in relation to possible

misclassification of the malformations. Only 76.5% of the pregnancy outcomes were assessed by physicians, and the remainder were based on information provided by the mother. The birth defects were classified independently by two researchers, who were blind to the intake estimates, using a classification scheme that focussed on cranial-neural-crest and other likely vitamin A related defects.

The second prospective study (Mastroiacovo *et al*, 1999) collected data on 423 babies exposed for at last one week during the first 9 weeks of pregnancy to high doses of vitamin A (3000 μg RE or more) and evaluated the outcome data. The mothers were recruited following referral to 13 European Teratology Information Services for advice about the possible risk associated with their high intakes of vitamin A. In this study, only a low incidence of major malformations was reported (3 out of 311 exposed pregnancies). Based on the prevalence rate in the study of Rothman *et al* (1995) a total of 7 babies would have been predicted to have suffered malformations. Although the confidence intervals for this result overlapped with the confidence intervals for the data from the study of Rothman *et al* (1995), the authors pointed out that the cases were at intakes of 7500, 9000 and 15,000 μg RE per day, and that no abnormalities were reported in 120 women with intakes reported to exceed 15,000 μg RE per day. No evidence was found of an increased risk of major malformation in babies exposed to vitamin A in early pregnancy when compared to those exposed later (i.e. after the 9th week of pregnancy) (rate ratio: 0.28 [95% confidence intervals 0.06-1.23]). There was no evidence of increased risk when the data for the group exposed to vitamin A were compared to a group referred for advice because of exposure to non-teratogenic compounds (rate ratio: 0.50 [CI 0.14-1.76]). The possibility of misclassification of the malformations was not directly considered, but all cases of congenital anomaly, neonatal problems or prolonged stay in hospital were followed up with the attending paediatrician.

A clinical trial has been carried out in Hungary (Dudas and Czeisel, 1992) in which a supplement of 1800 μg RE vitamin A did not increase the incidence of foetal malformations. However this study does not negate the findings of Rothman *et al* (1995) because of the low dose used; also no conclusions can be drawn with respect to the incidence of neural tube defects, because folic acid was administered simultaneously with vitamin A.

4. DOSE-RESPONSE ASSESSMENT

4.1. Bulging fontanelle in infants/Intracranial hypertension

The available data on bulging fontanelle (BF) come from intervention studies on large human populations of healthy infants (more than 100 subjects), with a placebo group, in which all the events of BF have been recorded. The route of exposure (*per os*) and the chemical form and intake conditions (and therefore the bioavailability) were similar in all studies. The data only concern the effects of acute or sub-acute dosages.

Vitamin A-induced BF is reported regularly, and usually occurs in a small proportion of treated infants less than 6 months of age. The proportion affected increases when the same infants receive further doses. The administered dose-effect relationship is clearer when the effect of cumulative dose is considered. BF has been reported in young infants given doses of 15,000 μg RE at 6, 10 and 14 weeks of age (De Francisco *et al*, 1993), or 7500 μg RE at 6, 12 and 17 weeks of age (Baqui *et al*, 1995). Conversely, BF was not reported when 30,000 μg RE was given at both 6 and 9 months.

4.2. Hepatotoxicity

In humans, the available data clearly suggest that the occurrence of toxic symptoms depends both on the vitamin A dose taken on a regular basis, and on the duration of this intake. The most extensive report (Geubel *et al*, 1991), included 41 cases, but reliable intake information was available on only 29 patients who had a mean daily intake of 28,770 μg RE (range, 6,000-120,000 μg RE). The duration of high intake averaged 7.17 ± 1.21 years (range 0.2-15 years). Interestingly, these authors reported that the most severely affected subjects, i.e. those with cirrhosis (n=13), had consumed significantly more vitamin A, both daily and in total, than the patients without cirrhosis. The lowest continuous daily consumption in patients with cirrhosis was 7500 μg RE/day taken over 6 years. A similar case (7500 μg RE/day for 6 years) has been reported more recently (Kowalski *et al*, 1994), in which progressive liver failure led to death of the patient. Cases of hepatotoxicity have not been reported below 7500 μg RE/day, and it can be hypothesized that this value might be the upper threshold of the storage capabilities of the liver. It is not known if a dose lower than 7500 μg RE/day could induce hepatotoxicity if taken for more than 6 years, but such low intakes may not been considered by physicians when they attempted to identify the cause of their patient's liver disease.

Differential sensitivity to vitamin A-induced hepatotoxicity has been considered by several authors. On a weight basis, it does not seem that children (more than one year old) are more sensitive than adults (reviewed in Hathcock *et al*, 1990). In elderly people (64-88 years old) plasma retinyl esters and retinol values were correlated to their supplemental vitamin A intakes (up to 14,100 μg RE/day for 5 years), but not to liver function tests (Stauber *et al*, 1991).

4.3. Bone metabolism

The risk for hip fracture in Swedish women (Melhus *et al*, 1998) is doubled for retinol intake greater than 1500 μg RE/day as compared to intakes less than 480 μg RE/day. Based on univariate analysis, the relative risks at intakes of 500-1000 μg/day, 1000-1500 μg/day and >1500 μg/day, compared with individuals with intakes <500 μg/day, were 0.93 (0.61-1.41), 1.27 (0.80-2.02) and 1.95 (1.15-2.11) respectively. The intake was from dietary sources and therefore it is possible that the effects detected may have arisen from unrecognised confounding; however the mechanistic data on the actions of retinoic acid on bone metabolism are consistent with the reported relationship. An intake of 1500 μg RE/day is close to the PRI (600 μg RE/day for women) and lower than the actual intakes for a substantial proportion of the population (see Table 1). A similar dose response relationship was reported by Feskanich *et al* (2002) in data from a large cohort of women in the US, studied over a period of 18 years. The cohort was divided into quintiles for total vitamin A intake (<1250, 1250-1699, 1700-2249, 2250-2999, >3000 μg RE daily) and also for retinol intake (<500, 500-849, 850-1299, 1300-1999, >2000). Significant trends were apparent between relative risk and the intakes from food and supplements of total vitamin A and also of retinol. A significant increase in relative risk was reported using a multivariate analysis for the two highest quintiles of retinol intakes (1300-1999 and >2000 μg RE/day) compared with the lowest quintile (<500 μg RE/day). The trend analyses for retinol from food and supplements (P≤0.001) compared with food only (P=0.05) indicates an important contribution from supplements and this would be less likely to be affected by dietary confounding than the data from the study of Melhus *et al* (1998). Therefore, both of these major epidemiology studies indicate an increased risk of bone fracture over an intake range similar to that normally consumed from food and supplements (Table 1).

4.4. Lipid metabolism

Patients given 7500 μg RE/day for approximately 4 years exhibited a small (2-3%) increased in cholesterol concentration (Cartmel *et al*, 1999). A similar study, conducted on 146 patients with retinitis pigmentosa during 12 years failed to show any adverse effect of 4500 μg RE/day (Sibulesky *et al*, 1999).

4.5. Teratogenesis

Several methods have been used to assess the dose-response relationship for the risk of high intakes of vitamin A during pregnancy.

4.5.1. Analysis of the relationship between vitamin A intake and the occurrence of birth defects

Dose-response relationships could be examined on the basis of the published case reports, but the available information is usually limited, and relates to supplemental intake only. The 18 cases reviewed in Biesalski (1989) had ingested daily between 5400 and 45,000 μg RE, over periods of several weeks or months, usually starting before pregnancy. Based on the data from studies in pregnant animals, teratogenesis could result from either a single dose or a limited number of doses, and there is a report of congenital malformation after ingestion of a single dose of approximately 300,000 μg RE (Mounoud *et al*, 1975). The absence of anecdotal case reports at lower intakes of vitamin A cannot be taken as evidence of an absence of risk: babies with cranial-neural-crest defects are born to women with normal intakes of vitamin A, and clinicians would not suspect a cause-effect relationship at doses close to normal intakes. Establishment of the dose-response relationship requires the analysis of data from epidemiology studies.

4.5.2. Analysis of the results of epidemiological studies

No association has been found in the majority of case-control studies between daily doses of vitamin A of 3000 μg RE or less and foetal malformation. However, in each of these studies, the number of women consuming high amounts of vitamin A was too limited to give a reliable estimate of a safe intake value. It is possible that a meta-analysis could be of value, but to combine the data for high exposure individuals would probably require access to the original databases.

The prospective study of Rothman *et al* (1995) was large enough to stratify the population according to the vitamin A intake. Moreover, the origin of the vitamin A intake (supplement or food) was available for all subjects. The authors found that the women taking daily more than 4500 μg RE of total vitamin A (from food and supplement) had a 3.5 times higher risk of giving birth to a child with cranial-neural-crest defects, than mothers ingesting less than 1500 μg RE/day. When the analysis was restricted to the supplemental intake of vitamin A only, the relative risk for mothers ingesting more than 3000 μg RE/day was 4.8 higher than those ingesting 1500 μg RE/day. The authors fitted a regression curve to their data, which indicated a rise in the ratio of prevalence of birth defects associated to the cranial-neural crest at doses greater than 3000 μg RE/day of vitamin A (food and supplement). The conclusions of the study remained the same when several potential confounding factors were considered.

The quantitative conclusion from the Rothman's study was that 3000 μg RE/day of supplemental vitamin A can be considered as a threshold for teratogenicity, and this has been discussed extensively. Khoury *et al* (1996), for example reported that 2400 μg RE/day of supplemental vitamin A did not increase the risk of birth defects, but this report, which lacks details does not contradict the conclusion from the study of Rothman *et al* (1995). Similarly, in the study of Duda and Czeisel (1992) a dose of 1800 μg RE/day was given to pregnant women without observing any increase in birth defects, but there are methodological uncertainties in the report, and the data are consistent with the conclusion of Rothman *et al* (1995). The study that contradicts the dose-response model reported in the paper of Rothman *et al* (1995) is that of Mastroiacovo *et al* (1999). These authors reported only three cases of malformations out of 423 pregnancies (311 births) exposed to vitamin A, at levels above 3000 μg RE/day. Although the number of women recruited was considerably smaller than in the Rothman *et al* (1995) study, they all had high intakes because they had been referred for advice about the possible risk associated with their high intakes. The number of women exposed to more than 6000 μg RE/day was twice as high as in the Rothman's study. Moreover no malformations were observed in the babies from 120 women who were exposed to more than 15,000 μg RE/day. The main weakness of this study is its statistical power: the sample size had only 80% power to detect an increased risk higher than 2.76. However, it seems reasonable to conclude from the data of Mastroiacovo *et al* (1999) that a daily intake of 3000 μg RE/day would be associated with a low or negligible risk of teratogenicity.

4.5.3. Analysis based on circulating concentrations of active metabolites

A "metabolic" approach has been proposed based on the hypothesis that the plasma concentrations of retinol and its metabolites following different doses of vitamin A are predictive of the teratogenic risk.

This approach was developed using data from animal studies. Ritchie *et al* (1998) quantified the teratogenic potencies of retinoids on cultured rat embryos, and compared them with circulating concentrations of the same metabolites *in vivo* after administration of a teratogenic dose of vitamin A. The hypothesis was that malformations would only be induced if the threshold concentrations were exceeded. Their conclusion was that plasma retinol was the best predictor of teratogenicity, and that an intake of 7500 μg RE/day of vitamin A would be unlikely to generate teratogenic plasma concentrations of retinoids. However, they pointed out several pitfalls in this analysis. Species differences, protein binding and transfer to the embryo were not taken into account and this prevented recommendation of this method to predict the teratogenicity of vitamin A in humans.

Data on Cynomolgus monkeys (Wiegand *et al*, 1998, and unpublished results) indicate that a dose of 2250 μg RE/kg body weight daily as retinyl palmitate from the 16th to the 27th day of gestation did not produce any malformations of the pups, as compared with controls fed a diet providing 300 μg RE/kg body weight. The authors extrapolated these data to humans on the basis that the dose-responses for the teratogenicity of isotretinoin (CRA) appeared similar in Cynomolgus monkeys and humans, and that there is similar conversion of CRA to TRA in both species. They conclude that a daily intake of 9000 μg RE should be considered non-teratogenic in humans.

A similar method has been used in humans by Miller *et al* (1998), who determined the baseline circulating concentrations of retinoic acids, and then compared them to the increases observed following intakes of known doses of vitamin A. Plasma concentrations in pregnant women with normal pregnancy outcomes, were 0.81 to 2.40 ng/mL for TRA, 0.81 to 4.90 ng/mL for 13CRA and 0.97 to 7.86 ng/mL for 4-oxo-13CRA (Miller *et al*, 1998). These authors concluded that that a meal containing 3000 or 9000 μg RE of vitamin A would only slightly increase the peak plasma concentrations of TRA and of 13CRA; the peak values would still be within the range of the reference values. Other authors have shown that 15,000 μg RE/day for a period of 20 days increased the level of retinoic acids or metabolites by a factor 2 to 7 (Eckhoff and Nau, 1990). Buss *et al* (1994) reported higher plasma concentrations;

after a single dose of 45,000 μg RE, the maximum plasma concentration of TRA increased 35 times if a water miscible supplement was given but only 1.6 times after the same dose in cooked liver. Increases in the peak concentrations of 13CRA and 4-oxo-13CRA acid were 26 and 10 times, after supplement intake, and 10 and 5 times after liver intake, respectively. This study showed that the matrix in which the vitamin is present can influence the plasma concentration profiles of retinol and its active metabolites. However, a recent study (van Vliet *et al*, 2001) showed higher serum levels of TRA when 15,000 μg RE of vitamin A had been taken in liver paste than in an oil-based supplement. These two studies indicate that the rate of absorption may influence the plasma concentration-time curves of the active metabolites.

This method has a number of problems that prevent its use for deriving safe levels of vitamin A intake. Important issues requiring resolution include:

i. The nature of the ultimate teratogenic metabolite(s) of vitamin A, and the importance of circulating TRA 13CRA and 9CRA. Numerous derivatives of retinoic acid can be found in plasma: TRA can be isomerized *in vivo* to 9CRA and 13CRA, oxidized to 4-oxo-TRA and then converted to oxo-13CRA, conjugated with glucuronic acid and metabolised by other minor pathways. Moreover, it is likely that retinoic acid metabolites can be generated locally within tissues, so that circulating concentrations may not reflect tissue levels.

ii. The metabolic inter-conversions of the different bioactive metabolites of retinol. The teratogenic potency of different retinoic acid derivatives is difficult to assess unequivocally, because they are interconnected by metabolic pathways, for which there are large interspecies differences.

iii. The link between circulating concentrations and foetal exposure including the unique conformation of the human placenta. Embryotoxic doses of vitamin A in rabbits are associated with low plasma but high embryonic concentrations of TRA (Tzimas *et al*, 1996). The limited teratogenicity of 13CRA in mice may be due to the very low placental transfer of this derivative in this animal species. Conversely, 13CRA is a potent teratogen in humans, probably due to its metabolism to TRA, either before or after placental transfer (Creech-Kraft *et al*, 1989). The placental transfer differs between the chemical structures of closely related retinoic acid derivatives, as shown by various embryo/maternal plasma ratio, reported by Nau (1995). Thus, the differing placental structure between animal species, including human, is likely to be a critical parameter in the teratogenicity of vitamin A, although this has been poorly addressed until now.

The correlation between the occurrence of a given retinoic acid metabolite and the teratogenic potency has not been established clearly, even in animal studies. Studies on rats or mice had suggested that there was a correlation between the AUC (area under the concentration-time curve) for these metabolites in plasma and the teratogenicity of retinoids (Nau, 1990). However, it is likely that the duration of the exposure at potentially teratogenic concentrations is also of crucial importance, because the embryo should be exposed during a time long enough to generate malformations (possibly 12 to 24 hours in the human species; Ritchie *et al*, 1998). Tembe *et al* (1996) reported different plasma concentration-response relationships in studies on the plasma kinetics and teratogenicity of TRA in rats given a single dose or the same total amount in divided doses over a period of a few hours. This study raises further doubts about risk assessments based on circulating plasma concentrations of TRA and its metabolites.

Because of these difficulties, the concentrations and/or the kinetics of the circulating retinoids following the intake of a given vitamin A dose cannot be used at the present time as the basis for deriving a tolerable upper intake level.

4.6. Selection of effect(s) on which to base the upper level

A number of adverse effects have been reported at intakes of preformed vitamin A above the population reference intake. The lowest doses reported to produce the different effects are:

Bulging fontanelle	7500 μg RE (as a single dose in infants)
Hepatotoxicity	7500 μg RE/day for 6 years
Bone density/fracture	1500 μg RE/day (trend analyses do not show a threshold)
Lipid metabolism	7500 μg RE/day for 4 years (but a minor change only)
Teratogenicity	>3000 μg RE/day (based on Rothman *et al*, 1995)

It is clear from this that the hazards and their associated doses are different for different groups of the population. In addition the severity of the adverse effect varies from minor to irreversible.

The associations between vitamin A and bone mineral density and the risk of bone fracture (Melhus *et al*, 1998; Feskanich *et al*, 2002) were reported at lower intakes than the other adverse effects listed above. The associations were based on the analysis of data for women in Sweden aged 40-76 years, and for women in the US aged 34-77 years. Middle aged and elderly women probably represent the most sensitive group for such effects. However, the dose-response arose from normal dietary intakes and non-prescription use of supplements, so that it is not possible to establish a clear tolerable upper intake level. Both studies indicate that intakes as low as 1500 μg RE/day might pose a risk. Whether the same dose-response would apply to men is not known. The statistical analyses showed a relationship after multivariate analysis and the possible impact of correction for confounding on the data is of concern, especially at such low relative risks.

Previous evaluations of the risk of high intakes of vitamin A have concentrated on teratogenicity, because this is an irreversible form of toxicity that occurs at low intakes. As indicated above, a tolerable upper intake level based on this effect would also allow for other adverse effects, with the possible exception of changes in bone mineral density and the risk of bone fracture.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL

Determining an upper level for preformed vitamin A is difficult, because any proposal has to take into account the narrow margin between the population reference intake and the intakes associated with adverse effects.

The findings on bone density and the risk of fracture were reported at lower daily intakes than other adverse effects. However, it was considered that the currently available data did not provide sufficient evidence of causality, and were not appropriate for establishing a tolerable upper level.

The teratogenic potential of vitamin A has received the most attention in previous evaluations, probably because of the severe and irreversible nature of this form of toxicity. A clear dose-response has been provided in the paper of Rothman *et al* (1995), but this was derived by fitting a curve to the available data that included a very large number of subjects from the at risk group of the population, but only a few cases at intakes above the suggested threshold of 3000 μg RE/day. The study of Mastroiacovo *et al* (1999), indicated that the threshold could be at higher intakes. In consequence the establishment of a clear threshold for teratogenicity is difficult, but a cautious approach would be to use the low value from the study of Rothman *et al* (1995). An uncertainty factor is not considered necessary, because the data from other studies indicated that the true threshold for an effect could be higher than this value. Based on these studies a tolerable upper level of 3000 μg RE/day is suggested for all women of child-bearing age (because the risk occurs very early in pregnancy). This value is 2.5-fold lower than the daily intake that might cause hepatotoxicity in women during chronic intake. The study of Rothman *et al* (1995) estimated the intakes of preformed vitamin A from all sources, and therefore the tolerable upper level applies to intakes from both foods and supplements.

Although teratogenicity is only relevant to women of child-bearing age, the upper level of 3000 μg RE/day is appropriate for men, and for infants and children after correction for differences in metabolic rate, because it is 2.5-fold lower than the lowest daily intake that has been associated with hepatotoxicity during chronic intake. This upper level does not apply to postmenopausal women, who represent the group at greatest risk of bone fracture, because it may not provide an adequate margin of safety in relation to the possible decrease in bone density and the risk of bone fracture. Further data to clarify the possible contributions of confounding to the reported increase in risk of bone fracture would provide greater confidence in a true cause-effect relationship at such low levels of intake.

Because the tolerable upper intake level relates to the risk of hepatotoxicity as well as effects produced during reproduction, it applies to intakes during pregnancy and lactation.

The tolerable upper level for children is based on the value of 3000 μg RE/day for adults, with correction for differences in basal metabolic rate compared to adults using scaling according to body surface area (body weight$^{0.75}$).

Age (years)	Tolerable Upper Intake Level (UL) for preformed vitamin A (retinol and retinyl esters) (µg RE/day)
1- 3	800
4 - 6	1100
7- 10	1500
11-14	2000
15- 17	2600
Adults+	3000

+ Women of child-bearing age and men (see text for advice concerning postmenopausal women).

6. RISK CHARACTERISATION

1. The tolerable upper level applies to both dietary and supplemental intakes of vitamin A.

2. The 97.5 percentile intake for adults in most of Europe is greater than 3000 µg RE/day.

3. Because alterations of embryogenesis may occur following a single or a small number of doses of vitamin A, for women of child bearing age the upper level should be compared with intake estimates that reflect short-term, rather than long term exposure.

4. The current recommendations that women who are planning to become pregnant or who are pregnant should not consume cooked animal livers (SCF, 1992) should be maintained.

5. Because the tolerable upper level may not adequately address the possible risk of bone fracture in particularly vulnerable groups, it would be advisable for postmenopausal women, who are at greater risk of osteoporosis and fracture, to restrict their intake to 1500 µg RE/day.

6. Because the current intakes may exceed the tolerable upper level, careful consideration should be given to the appropriateness of the enrichment of human foods with vitamin A, and to the potential effects on human exposure of the addition of vitamin A to animal feed.

7. RECOMMENDATIONS

1. The possible link between bone density, the risk of fracture and vitamin A intake should be reviewed when further data become available.
2. Ideally, resolution of the issue of bone mineral density and the risk of fracture should be studied by a prospective study, in which the effects of age on the risk, and also of confounding variables are taken into account in the study design. It is recognised that such a study would require a very large population and prolonged treatment and follow up.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN D

(EXPRESSED ON 4 DECEMBER 2002)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

The principal physiological function of vitamin D in all vertebrates including humans is to maintain serum calcium and phosphorus concentrations in a range that support cellular processes, neuromuscular function, and bone ossification. Vitamin D accomplishes this goal by enhancing the efficiency of the small intestine to absorb dietary calcium and phosphorous, and by mobilising calcium and phosphorus from the bone (Holick, 1999; Holick *et al*, 1998).

The last couple of decades it has become increasingly apparent that vitamin D also has other important functions in tissues not primarily related to mineral metabolism (Brown *et al*, 1999; Holick, 1999). One example is the haematopoietic system, in which vitamin D affects cell differentiation and proliferation including such effects also in cancer cells. Vitamin D furthermore participates in the process of insulin secretion. The active metabolite of vitamin D, $1,25(OH)_2D$, regulate the transcription of a large number of genes through binding to a transcription factor, the vitamin D receptor (VDR).

Blood levels of vitamin D are influenced both by dietary intake and the amount of daylight exposure to the skin. Exposure of the skin to ultraviolet light catalyses the synthesis of vitamin D_3 (cholecalciferol) from 7-dehydrocholesterol. Thus vitamin D is more like a hormone and not strictly a vitamin according to the classical criteria that an essential nutrient is a substance the body cannot synthesise in sufficient quantities itself. Deprived of exposure to sunlight vitamin D becomes an essential nutrient. The effectiveness of exposure to sunlight or ultraviolet light in curing or preventing rickets was shown early in the twentieth century (Holick, 1995).

2. NUTRITIONAL BACKGROUND

2.1. Vitamin D supply

2.1.1. Vitamin D forms in food

Vitamin D comprises two closely related substances of nutritional importance: vitamin D_3 (cholecalciferol), which is the physiological form, and the synthetic analogue vitamin D_2 (ergocalciferol). The two forms only differ by the side chain to the sterol skeleton (Holick, 1999). It has been assumed, based on studies in the 1930s showing no conclusive difference between vitamin D_3 (from cod liver oil) and D_2 in their preventing effect against infantile rickets, that vitamin D_2 for practical purposes could be regarded as equal to vitamin D from cod liver oil. There is no contemporary evidence showing that vitamin D_3 and D_2 are equally efficient in increasing the circulating metabolite proximate to the active form. Indeed, later studies have shown important biological differences in this respect between these forms (Trang *et al*, 1998). (See 2.5.2 for further details).

Vitamin D_3 and vitamin D_2, together with the provitamins they are made from, are all derivatives of sterols, their chemical structure resembles cholesterol, bile acids and the sex hormones. Vitamin D_2 is formed by UV radiation from its precursor ergosterol. Ergosterol is found in plants, especially yeast and fungi. The synthesis of ergocalciferol from ergosterol hardly takes place in nature. Plants are thus a poor source of vitamin D_2. Synthetic vitamin D_2 produced by irradiation of ergosterol used to be the form added to food or given as supplements. During the past two decades, vitamin D_3 has also been used to fortify milk, margarine and other foods worldwide, and although the use of vitamin D_2 in food and supplements still is widely used, its use is less than before. Vitamin D_3 is formed from its precursor 7-dehydrocholesterol, which is found in ample amounts in the skin and fat depots in animals and man. Vitamin D is relatively stable in fat solutions, e.g. is not inactivated by pasteurisation or sterilisation. It oxidises in contact with air and in acid solutions and is inactivated when exposed to sunlight.

2.1.2. Vitamin D from breast milk

The British Food composition tables (Holland *et al*, 1991) use the value 0.4 μg/L vitamin D in human breast milk. The same value is used in the Norwegian food composition tables. However, the literature reports a quite large range of concentrations, varying from 0.1 to 1.2 μg/L. A variety of compounds with vitamin D activity (metabolites) are present in human milk, but 25(OH)D accounts for the majority of the antirachitic activity (Reeve *et al*, 1982; Weisman *et al*, 1982; Ala-Houhala *et al*, 1988a; Hillman, 1990). Human milk even from a vitamin D-sufficient mother provides a marginal amount of total vitamin D activity. The 25(OH)D level was higher in hind- than in foremilk (Ala-Houhala *et al*, 1988a). Vitamin D activity in human milk of unsupplemented mothers was lower in the winter than in the summer. The influence of supplementation with 25 μg ergocalciferol or cholecalciferol or 50 μg cholecalciferol on vitamin D activity in human milk in summer and winter was investigated by Ala-Houhala and co-workers (1988a). They found that supplementation with 50 μg of vitamin D could increase vitamin D activity of milk in the winter to that of unsupplemented mothers in the summer, but the responses varied widely among individuals. Markestad (1983) found a strong correlation between infant and maternal plasma 25(OH)D concentrations both at birth and after 6 weeks in unsupplemented infants born in the winter in the northern areas. The 25(OH)D concentrations in the infants were considerably reduced and reached levels associated with rickets during this period. It appears that sun exposure of the infant is a very important determinant for vitamin D status. Although a study in Caucasians from central USA showed that bone mineralisation was normal in unsupplemented and exclusively breast-fed infants up to 16 weeks (Roberts *et al*, 1981), most studies agree that fully breast-fed infants have a reduced vitamin D status after 6 weeks of age if no supplemental D is given. The general recommendation therefore is that infants should be supplemented with vitamin D.

2.1.3. Vitamin D intake from food

Only a few foods contain vitamin D, i.e. vitamin D3, naturally in quantities that have an impact on the dietary intake: fish liver, fish liver oils, fatty fish and egg yolks. Thus, some countries practice fortification of certain foods with vitamin D, most often milk, margarine and/or butter. The mean intakes in different studies vary with age group, food and supplementation habits and gender. Recent publications from various parts of Europe all show that a substantial part of the population including pre-school children has a vitamin D intake below the recommended dietary intakes (Davies *et al*, 1999; de Jong *et al*, 1999; Koenig and Elmadfa, 2000; Lehtonen-Veromaa *et al*, 1999; Ortega *et al*, 1995; van der Wielen *et al*, 1995). The low intake is confirmed by results from the SENECA study, an investigation of the diet and health of 824 elderly people from 19 towns in 11 countries (Greece, Portugal, Italy, Spain, France, Switzerland, Hungary, Belgium, Netherlands, Denmark and Norway). Thirty-six per cent of the men and 47% of the women had 25(OH)D concentrations below 30 nmol (van der Wielen *et al*, 1995). Surprisingly, lowest mean 25(OD)D concentrations were found in southern European countries; more than 80% of Italian and Greek women had values below 30 nmol compared with 18% in Norway. One factor associated with better vitamin D status was increased fish consumption, but the main reasons for the relatively good vitamin D status in the Scandinavian countries are probably fortification of food and a higher percentage of people taking vitamin D supplements. Cod liver oil was taken regularly by 35% of all men and 34% of all women in Norway in 1997, and the percentage was higher among the elderly (Norkost, 1997). A much lower prevalence of vitamin D deficiency was found in the French general adult population; of 1191 adults 11% was below 30 nmol 25(OH)D in serum (Chapuy *et al*, 1997; Guinot *et al*, 2000). They found a correlation to latitude and skin exposure, as 24% of those with low exposure was deficient.

The estimated mean dietary vitamin D intakes in several European countries are given in Table 1.

Table 1. The daily intakes of vitamin D (μg/day)

Country	Type of survey	n	Method	Supplements*	Mean	97.5%
Austria[a]	Individual	2488	24h recall	Not defined	4.0	22.2
Germany[b]	Individual (M) Individual (F)	854 1134	7-day dietary record	- -	4.0 3.1	16.8 11.9
UK[c]	Individual (M) Individual (F) Individual (M) Individual (F)	1087 1110 1087 1110	7-day weighed inventory	- - + +	3.4 (2.9) 2.5 (2.2) 3.8 (3.0) 3.1 (2.3)	9.9 6.9 12.7 12.6
Italy[d]	Household	2734	7-day record	+	3.0	8.4
Netherlands[e]	Household	5958	2-day record	-	3.7	8.9
Norway[f]	Individual (M) Individual (M) Individual (F) Individual (F)	1298 - 1374 -	Semiquantitative FFQ last year, 180 food items	- + - +	5.8 11.2 4.0 10.3	13.0 37.6 10.3 33.3
Ireland[g]	Individual (M) Individual (F)	662 717	7-day estimated food record	+ +	3.7 3.7	13.5 14.9

* + data included supplements; - data excluded supplements.
[a] Elmadfa *et al* (1998). [b] Heseker *et al* (1994) - values are the median.
[c] Gregory *et al* (1990) - values are the mean with the median in parentheses.
[d] Turrini (1996). [e] Hulshof and Kruizinga (1999). [f] Norkost (1997).
[g] IUNA (2001).

2.2. Metabolism of vitamin D

2.2.1. Vitamin D activation

Both forms of vitamin D (vitamin D_3, cholecalciferol, and vitamin D_2, ergocalciferol) are inactive. Major metabolic steps involved in the metabolism of vitamin D_2, mono and dihydroxylated forms, are similar to those of vitamin D_3. Vitamin D without a subscript represents either D_2 or D_3 or both and requires two obligate hydroxylations to form the active hormone, 1,25-dihydroxyvitamin D (1,25$(OH)_2$D). The first step of activation takes place by hydroxylation at position C-25, mainly in the liver. The role of other tissues is uncertain. The product, 25-hydroxyvitamin D (25(OH)D), is transported to the kidneys, where 1α-hydroxylation takes place. The resulting product, 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) , is the active metabolite. 1,25$(OH)_2$D is transported bound to vitamin D-binding protein (DBP). DBP is synthesised in the liver and circulates in plasma at concentrations 20 times higher than the total amount of vitamin D metabolites. The role of the large molar excess of DBP is uncertain. Free 1,25$(OH)_2$D is in equilibrium with the bound form. It is only free 1,25$(OH)_2$D, i.e. 0.5% of the total amount of plasma 1,25$(OH)_2$D, which is hormonally active. The binding to DBP increases the half-life of 1,25$(OH)_2$D and makes the hormone available to the cells (Brown *et al*, 1999). The concentration of DBP is increased during pregnancy and by oestrogen treatment. It also increases in infants after birth.

The 25-hydroxylation of vitamin D is poorly regulated, i.e. the capacity of the 25-hydroxylase in the liver is high. The levels of 25(OH)D increase in proportion to vitamin D intake, and for this reason, plasma 25(OH)D levels are commonly used as indicator of vitamin D status. The half-life of 25(OH)D in circulation is approximately 1-2 months (Vieth, 1999). Steady state in plasma 25(OH)D concentration would, according to the half-life, not be reached before 4 months after a change in the intake. With concentration-dependent kinetics this could, however, vary. The proportion of 25(OH)D to vitamin D intake cannot be determined before steady state is reached.

The serum level of 25(OH)D usually reflects both 25$(OH)D_3$ and 25$(OH)D_2$. The ratio of these two hydroxylated derivatives depends on the relative amounts of vitamins D_2 and D_3 present in the diet and endogenously synthesised vitamin D_3 (Holick *et al*, 1998).

In contrast, the production of 1,25$(OH)_2$D is tightly regulated, both by feedback of the 1,25$(OH)_2$D, through calcium and phosphate levels in the blood and with the help of parathyroid hormone (PTH). This is illustrated by experiments showing that when large doses of vitamin D are given to animals, the serum concentrations of 25(OH)D will increase proportionally, while the concentration of 1,25$(OH)_2$D remains normal. Both the suppression of the kidney 1α-hydroxylase activity and induction of the 24-

hydroxylase activity are VDR-mediated. Experiments with rats have shown that tissue specific down-regulation of renal VDR by calcium restriction blocks $1,25(OH)_2D_3$-dependent suppression of renal 1α-hydroxylase or stimulation of renal 24-hydroxylase (Brown *et al*, 1999; Beckman and DeLuca, 2002).

2.2.2. Catabolism of vitamin D

The major catabolic enzyme is the 24-hydroxylase, a mitochondrial enzyme, and both 25(OH)D and $1,25(OH)_2D$ are inactivated via this pathway. Further oxidation to the ketone, oxidation at C-23(S) and C-26, and subsequent oxidative cleavage of the side chain are associated with progressive loss of biological activity. Also additional pathways have been described (Brown *et al*, 1999).

In contrast to the limited distribution of the vitamin D-activating enzymes, 24-hydroxylase is ubiquitously present in vitamin D target tissues. This enzyme is highly inducible by $1,25(OH)_2D$ providing a regulatory mechanism at the cellular level for attenuating the response of the active compound when abnormally high.

2.3. Functions of vitamin D

The principal function of vitamin D ($1,25(OH)_2D$) in the body is to maintain intracellular and extracellular calcium concentrations within a physiologically acceptable range. The vitamin accomplishes this goal through the action of $1,25(OH)_2D$ on regulating calcium and phosphorus metabolism in the intestine and bone.

2.3.1. Vitamin D receptor (VDR)

The main mechanism of action of vitamin D is the interaction of $1,25(OH)_2D$ with the nuclear vitamin D receptor (Brown *et al*, 1999). VDR belongs to the super family of steroid nuclear receptors. Following ligand binding, VDR heterodimerises with retinoid X receptor (RXR) and acts as a ligand-activated transcription factor by binding to genomic vitamin D responsive elements (VDRE) in vitamin D-regulated genes. These include more than 50 other genes important for mineral homeostasis, vitamin D metabolism, energy metabolism, cell differentiation and proliferation, extracellular matrix proteins, oncogenes, growth factors, signal transduction proteins and peptide hormones. Genes can be both up-regulated or down-regulated, but the exact mechanism is unclear. Among genes down-regulated are PTH, osteocalcin, protein-kinase A inhibitors and interleukin-2 genes.

Several genetic polymorphisms of VDR have been identified, the exact role of these has not been clarified, but most variants do not affect the protein structure (Brown *et al*, 1999). In a study on the efficacy of vitamin D supplementation on bone mineral density of the femoral neck in elderly women it was found that those having one or two *VDR* alleles without the BsmI restriction site responded better than those with a genotype in which this restriction site was absent (Graafmans *et al*, 1997).

The cellular response to 1,25(OH)D is mainly regulated by changing the cellular amount of VDR. Treatment with 1,25(OH)D increases the receptor level presumably due to stabilisation of the receptor. Some growth factors increase, as IGF-I, while others, such as fibroblast growth factor and mitogens, decrease VDR expression. Activation of protein-kinase C and prednisone treatment inhibit VDR expression whereas oestrogen, retinoic acid and PTH increase VDR expression. VDR expression is also dependent on cell type, and its condition, proliferating or differentiating (Kveiborg *et al*, 1999). VDR can also be regulated at the stage of degradation. VDR interacts directly with SUG1, a component of the proteasome complex important for proteolysis. VDR activity might also be modulated by phosphorylation of serine at different positions (Brown *et al*, 1999). VDR knockout mice have been produced. The homozygous mouse ($VDR^{-/-}$) shows no sign of defect until end of weaning. Then they fail to thrive and die within 15 weeks from birth. They suffer from hypokalaemia, defective fur and females have defects in reproductive organs. Furthermore, bone formation and growth are inhibited and the level of 1,25(OH)D is increased indicating a role of VDR in regulation of vitamin D hydroxylation. In some respects the VDR knockout mice show some phenotypic similarities with the disease vitamin D resistant-rachitis type 2, which is seen in children with inherited mutations in VDR (Yoshizawa *et al*, 1997; Kveiborg *et al*, 1999).

2.3.2. Vitamin D and calcium homeostasis

The most critical role of $1,25(OH)_2D$ in mineral homeostasis is to enhance the efficiency of the small intestine to absorb dietary calcium. This was clearly demonstrated in the VDR null mouse (Yoshizawa *et al*, 1997). Calcium absorption from the intestine is dependent on the amount of calcium in the diet and on physiological requirements, and is adaptable. When dietary calcium concentrations are low, almost all calcium is absorbed. The same happens in pregnancy and during lactation. $1,25(OH)_2D$ also

promotes the intestinal absorption of phosphate. However a significant phosphate absorption also occurs in 1,25$(OH)_2$D-deficient states (Brown *et al*, 1999).

1,25$(OH)_2$D is essential for development and maintenance of a mineralised skeleton. Deficiency results in rickets during growth and osteomalacia in adults. 1,25$(OH)_2$D induces bone formation by regulation of matrix proteins important for bone formation, such as osteocalcin, osteopontine, alkaline phosphatase, matrix-gla- protein and collagen, as well as mineral apposition. The bone forming osteoblasts express VDR and it appears that 1,25$(OH)_2$D inhibits osteoblast proliferation through VDR-dependent signal pathway, and promotes their differentiation (Kveiborg *et al*, 1999). Vitamin D does not appear to be absolutely essential for the ossification process, but enhances this through increasing serum levels of calcium and phosphate. It has been suggested that not only 1,25$(OH)_2$D is involved in bone mineralisation, but also 24,25$(OH)_2$D may be required (Brown *et al*, 1999).

1,25$(OH)_2$D enhances the mobilisation of calcium and phosphorus stores from bone at times of calcium deprivation. 1,25$(OH)_2$D induces stem cell monocytes to become mature osteoclasts. It appears though that this effect is not direct, but is mediated via osteoblasts that secrete a factor promoting osteoclast differentiation (Kveiborg *et al*, 1999). 1,25$(OH)_2$D regulate calcium homeostasis in close co-operation with PTH, which is the principal hormone regulating extracellular ionised calcium from minute to minute. PTH stimulates 1,25$(OH)_2$D synthesis and 1,25$(OH)_2$D suppresses the synthesis and secretion of PTH and controls parathyroid growth through negative gene regulation. Studies in the VDR null mouse suggest that VDR is not essential, but works in co-operation with calcium and phosphate (Brown *et al*, 1999).

The most important effects of 1,25$(OH)_2$D in the kidney is suppression of 1α-hydroxylase activity and induction of 24-hydroxylase activity. 1,25$(OH)_2$D increases renal calcium reabsorption and calbinding expression, and it accelerates PTH dependent calcium transport in the distal tubule, which has the highest level of VDR. The enhancing effect of 1,25$(OH)_2$D on renal phosphate absorption might be an indirect action via PTH suppression (Brown *et al*, 1999).

2.3.3. Other effects of vitamin D

Synthesis and cellular receptors for 1,25$(OH)_2$D have been found not only in the intestine, kidney and bone but also in many other tissues, suggesting that 1,25$(OH)_2$D is fundamental to the regulation of gene expression in many cell types in addition to its probable role in intracellular calcium regulation (Brown *et al*, 1999; Zehnder *et al*, 2002a and b). Further local production and action of 1,25$(OH)_2$D, particularly after inflammatory activation of 1α-hydroxylase activity by, for example, cytokines in endothelial cells, could indicate an important autocrine/paracrine mechanism in peripheral tissues (Zehnder *et al*, 2002b). Addition of 1,25$(OH)_2D_3$ or 25$(OH)D_3$ decreased proliferation of human endothelial cells and the adhesion of monocytic cells to these cells (Zehnder *et al*, 2002b). In the skin, 1,25$(OH)_2$D plays an important role by inhibiting proliferation and stimulating differentiation of keratinocytes and vitamin D analogues are used in the treatment of psoriasis. In the immune system, 1,25$(OH)_2$D modulates synthesis of interleukins and cytokines. Besides stimulating monocytes and macrophages, 1,25$(OH)_2$D functions as an immunosuppressive agent by decreasing the rate of proliferation and the activity of both T- and B cells and inducing suppressor T cells (Brown *et al*, 1999). In haematopoietic tissue, vitamin D deficiency causes anaemia and decreased cellularity of bone marrow. 1,25$(OH)_2$D also inhibits proliferation and promotes differentiation of a number of leukaemia cell lines. Also normal myeloid precursor cells mature in the presence of 1,25$(OH)_2$D. In addition, VDR is expressed in many other tissues, such as muscle and nervous tissue, liver, intestine, reproductive organs, pancreas, pituitary, thyroid gland and lung, where 1,25$(OH)_2$D apparently has important functions in regulation of cell proliferation and differentiation (Brown *et al*, 1999; Holick, 1999). In animal experiments and also in epidemiological studies, vitamin D appears to be a protective factor in colon carcinogenesis.

2.3.4. 25(OH)D and the vitamin D receptor

At very high levels it appears that 25(OH)D also has a direct effect on the vitamin D receptor.
However, another possible action of 25(OH)D might also operate (Vieth, 1990). When excess vitamin D is consumed there is increased and uncontrolled formation of 25(OH)D, which is secreted from the liver into blood. The specific vitamin D-binding sites on the circulating vitamin D-binding protein, which normally is less than 5% saturated, become mainly occupied with 25(OH)D. Because 1,25$(OH)_2$D has a lower affinity for this protein than 25(OH)D, the functional hormone is displaced and circulates either in the unbound form or in loose association with plasma albumin. Hence the availability of 1,25$(OH)_2$D to its intracellular receptors is greatly increased by the swamping of vitamin D-binding protein with 25(OH)D. Furthermore, because the intracellular receptors have a much higher affinity for 1,25$(OH)_2$D than does

vitamin D-binding protein, they will readily take up this displaced 1,25$(OH)_2$D from extracellular fluid. Although the production of 1,25$(OH)_2$D may not be increased in hypervitaminosis D, its supply to sites of action will, in this way, be greatly raised.

2.3.5. Non-vitamin D receptor-mediated effects

In addition to VDR-mediated effects, 1,25$(OH)_2$D apparently also elicits rapid cellular responses by interacting with specific cell surface receptors giving rise to rapid changes in phosphoinositide metabolism and increases in intracellular calcium levels, stimulating intestinal calcium and phosphate fluxes. The receptor has only partially been characterised and the role of non-genomic actions of 1,25$(OH)_2$D in most cells remains unclear.

2.4. Biomarkers

2.4.1. Biomarkers of vitamin D intake

Plasma derivatives of vitamin D_2 (25$(OH)D_2$) are of exogenous origin only, while derivatives of vitamin D_3 (25$(OH)D_3$) may arise from either diet or skin. Different studies get very different correlations between vitamin D dietary intake and serum levels of 25(OH)D, and the reason is obvious: the amount of 25(OH)D originating from sun exposure will confound all attempts to use 25(OH)D as a biomarker of dietary intake alone. Thus 25(OH)D can only be used as a biomarker of vitamin D intake in people whose sunshine exposure has been low. Furthermore not all studies on vitamin D supplementation have determined 25(OH)D when in a steady state condition.

It should be noted that 25(OH)D denotes both the D_2 and D_3 metabolites and it is not always reported in publications if total 25(OH)D has been measured or either one. Furthermore there may be a variation between laboratories of more that 30%, making comparisons of exact values from different studies, particularly old ones, difficult (Lips *et al*, 1999).

2.4.2. Biomarkers of vitamin D status and activity

There is now consensus that serum 25(OH)D concentration is a good marker of internal vitamin D status. In patients with hypervitaminosis D, serum 25(OH)D levels are 2-15 times higher than those of normal controls (Hughes *et al*, 1976; Vieth, 1999). Hypercalcaemia frequently coexists with high levels of 25(OH)D and is a useful marker of hypervitaminosis D. In addition PTH will be suppressed.

Plasma levels of 1,25$(OH)_2$D, and particularly free 1,25$(OH)_2$D, is a measure of vitamin D hormone activity, but because of its tight regulation it does not reflect very well vitamin D nutritional status.

2.4.3. Reference serum levels of metabolites

Most diagnostic laboratories consider the upper reference level of 25(OH)D in serum to be about 150 nmol/L (Holick, 1999), i.e. 130-150 nmol/L. The reference levels for 1,25 $(OH)_2$D is 50-145 pmol/L and for 24,25$(OH)_2$D is 2-10 nmol/L. The reference values of 25(OH)D in infants, 130-150 nmol/L, are similar or close to those of adults (Markestad, 1984). PTH is raised in vitamin D deficiency and should also be determined to establish this diagnosis.

2.5. Endogenous synthesis in the skin of vitamin D and nutritional requirement

Exposure of the skin to solar ultraviolet B with energies between 290 and 315 nm catalyses the conversion of 7-dehydrocholesterol to previtamin D_3 (precholecalciferol), which spontaneously isomerises to cholecalciferol (Holick, 1995). Upon prolonged UV exposure a regulation mechanism is operating in that both precholecalciferol and cholecalciferol can be photolysed to inert compounds. Hence, sunlight alone apparently cannot cause overt toxicity due to overproduction of vitamin D. Even though the skin phototype in a study from France (Guinot *et al*, 2000) did not influence vitamin D status, other studies indicate that the degree of pigmentation of the skin also has an impact on the amount of vitamin D synthesised as melanin absorbs UV B photons: the darker the skin, the less is produced. Skin thickness decreases linearly with age from the age of 20 years and there is a marked decrease in the precursor 7-dehydrocholesterol in the skin and less vitamin D production. When healthy young and old men were compared after exposure to UV light, young men had nearly 4 times more circulating 25(OH)D in serum than the old individuals (Need *et al*, 1993; Holick, 1995). The concentrations of 25(OH)D in serum tend to decrease with age.

The vitamin D requirement for healthy adults has never been defined precisely. Because vitamin D is produced in the skin upon exposure to sunlight, humans, with the possible exception for elderly, do not

have any requirement for vitamin D when sufficient sunlight is available. However, vitamin D becomes an important nutritional factor in the absence of sunlight and in the elderly. It is well known that a substantial part of the European population is exposed to sub-optimal levels of sunlight, especially during the winter months. In addition to geographical and seasonal factors, exposure to sunlight is dependent on modern life style such as clothing and indoor life (McKenna, 1992).

There is now a consensus that serum 25(OH)D concentration is the correct functional indicator of vitamin D status, which is also used as a basis for the nutritional recommendations (FNB, 1999; Vieth *et al*, 2001). A level of 25(OH)D below 27.5 nmol/L is considered to be consistent with vitamin D deficiency in infants, neonates and young children (Specker *et al*, 1992). This value should be met to prevent rickets and severe osteomalacia in these groups (FNB, 1999). Little information is available about the level of 25(OH)D needed to maintain normal calcium metabolism and peak bone mass in adolescents and middle aged adults. For elderly there is increasing evidence of a greater requirement of vitamin D to maximise bone mineralisation. Less certain and more controversial is the optimal serum concentration of 25(OH)D. Moderate vitamin D malnutrition is based on the now well documented inverse relationship between serum concentrations of 25(OH)D and PTH (Vieth *et al*, 2001). A serum concentration of 25(OH)D <40-50 nmol/L is considered by several authors to be insufficient, particularly in the elderly with bone loss, and many regard serum 25(OH)D concentrations above 75-100 nmol/L to be desirable, concentrations at which PTH is suppressed to a minimum in its relation to 25(OH)D (Chapuy *et al*, 1997; Chel *et al*, 1998; Dawson-Hughes *et al*, 1997; Gallagher *et al*, 1998; Kinyama *et al*, 1998; Thomas *et al*, 1998; Vieth, 1999; Vieth *et al*, 2001).

2.5.1. Existing recommendations on vitamin D intake

The necessary intake of vitamin D will depend on the shortfall of exposure to effective UV radiation. Most countries have their own recommendations for vitamin D intake, recognising that there may be insufficient sun exposure in larger or smaller groups of the population. Term infants are born with a store of vitamin D reflecting the mother's vitamin D status. These stores provide the infant with sufficient vitamin D for 4-6 weeks. The vitamin D content of mothers' milk from women living in industrialised societies is not considered sufficient to maintain adequate vitamin D status in the child. Thus, many countries recommend 10 μg vitamin D/day to infants from 4 weeks onwards. The same amount is recommended for pregnant and lactating women. The current allowance of vitamin D recommended by most European countries is 5 μg/day (200 IU) for adults and 10 μg vitamin D per day for everyone older than 60-65 years. The separate European countries often have more detailed recommendations than the general ones mentioned here, and the recommended values vary somewhat (Trichopoulou and Vassilakou, 1990). The Population Reference Intake (PRI) recommended by the Committee (SCF, 1993) are as follows: 6-11 months 10-25 μg; 1-3 years 10 μg; 4-10 years 0-10 μg; 11-17 years 0-15 μg; 18-64 years 0-10 μg; ≥65 years 10 μg; pregnancy 10 μg; lactation 10 μg.

2.5.2. Differences in metabolism and bioefficiency of different forms of vitamin D and effect of vehicle

Based on studies in the 1930s showing no conclusive difference between vitamin D_3 (from cod liver oil) and D_2 in their preventing effect against infantile rickets, vitamin D_2, for practical purposes, has been regarded as equal to vitamin D_3 from cod liver oil (Trang *et al*, 1998). However, in several non-human species vitamin D_3 and D_2 show differences in their ability to increase 25(OH)D (Marx *et al*, 1989). Also in the pig and birds vitamin D_3 is far more effective than D_2, whereas the opposite is the case in rats (Horst *et al*, 1982). Although very early studies did not show differences in antirachitic activity between vitamin D_2 and D_3, more recent studies (Tjellesen *et al*, 1985; Hartwell *et al*, 1987 and 1989), including a study by Tjellesen *et al* (1986) in premenopausal women, did show a greater efficacy with vitamin D_3 in humans. This issue was recently addressed in a larger study by Trang *et al* (1998). They were able to show that vitamin D_3 was 1.7 times as efficient as D_2, when given in equimolar amounts during 14 days to healthy volunteers, to raise the serum level of total 25(OH)D. Particularly, higher basal level of 25(OH)D supplementation with high levels of vitamin D_2 was inefficient. Studies have also shown that vitamin D_2 supplementation can suppress endogenously formed $25(OH)D_3$ and also $1,25(OH)_2D_3$ (Tjellesen *et al*, 1986; Hartwell *et al*, 1989; Harris *et al*, 1999).

Studies during the last decade have revealed that the differences in the side chain between vitamin D_3 and D_2 result in differences in hydroxylated products particularly when large doses are administered (Mawer *et al*, 1998). Direct 24-hydroxylation of vitamin D_2 in the liver is of particular relevance since routing of vitamin D_2 to $24(OH)D_2$ would lead to further inactivation or via the kidney to the biologically active $1\alpha,24(OH)_2D_2$. Interestingly however, $1\alpha,24(OH)_2D_2$, which is a significant metabolite at high doses of vitamin D_2, binds strongly to VDR and possesses potent antiproliferative activity in combination with

low calcaemic activity (Jones *et al*, 1996; Knutson *et al*, 1997; Mawer *et al*, 1998). 1α,24(OH)$_2$D$_2$ can also form from 1α(OH)D$_2$, which is less toxic than 1α(OH)D$_3$ (Knutson *et al*, 1997; Sjoden *et al*, 1985).

The observed reduced ability of vitamin D$_2$ to raise plasma 25(OH)D and the low calcaemic effect of 1α,24(OH)D$_2$ formed at high doses of vitamin D$_2$ including suppressing effect on 25(OH)D$_3$ and 1α,25(OH)$_2$D$_3$ synthesis are most probably the reasons for a lower toxicity of vitamin D$_2$ than of vitamin D$_3$ as the toxic effects are mainly related to the distorted calcium metabolism (see below).

The vehicle used (fat or emulsion) in which vitamin D is administered could influence bioavailability. This was shown as early as in 1935 by Stearn and Jeans (cited in Seelig, 1969). Vitamin D from cod liver oil emulsified in milk is about three times as bioavailable as judged by potency as vitamin D given in cod liver oil or propylene glycol.

3. HAZARD IDENTIFICATION AND CHARACTERISATION

3.1. Mechanisms of toxicity

The toxic effects of vitamin D excess are primarily related to the role of free 1,25(OH)$_2$D in the regulation of plasma calcium (Davies and Adams, 1978; Reichel *et al*, 1989). Excessive production of 1,25(OH)$_2$D or greatly increased plasma 25(OH)D (which may displace 1,25(OH)$_2$ D from DBP) may lead to elevated level of plasma calcium due partly to over-stimulated intestinal absorption and partly to excessive calcium mobilisation from bone (Norman, 1996; Pettifor *et al*, 1995; Vieth, 1990). Hypercalcaemia could also lead to an increased calcium excretion into urine, hypercalciuria. There is also limited evidence that high concentrations of vitamin D directly affect various organ systems such as kidney, bone, the central nervous system and the cardiovascular system (Holmes and Kummerow, 1983).

Hypercalcaemia is defined as a serum calcium above 2.75 mmol/L or ionised calcium above 1.35 mmol/L. Hypercalcaemia associated with hypervitaminosis D gives rise to numerous debilitating effects (Chesney, 1990; Holmes and Kummerow, 1983; Parfitt *et al*, 1982). Specifically this would include loss of tubular concentration function of the kidney with polyuria and hypercalciuria, which would predispose to nephrolithiasis and reduced glomerular filtration rate. Prolonged hypercalcaemia can cause calcification of soft tissues, including kidney, blood vessels, heart and lungs (Allen and Shah, 1992; Moncrief and Chance, 1969; Taylor *et al*, 1972). A 24-hour urinary calcium excretion >10 mmol is considered to indicate hypercalciuria. The mean molar calcium/creatinine ratio in randomly collected urine from non-fasting healthy subjects is approximately 0.40. The relation between this ratio in urine and the 24-hour calcium excretion indicate that 10 mmol Ca/24 hours would correspond to a ratio in urine of about 1.0 in molar calcium/creatinine. Whether a high calcium excretion in a human with serum calcium within reference limits should be regarded as an adverse effect is not clear. In the absence of hypercalcaemia and low urine volume urinary calcium *per se* is a minor contributor to renal stone disease (Vieth *et al*, 2001).

3.2. Genotoxicity

Vitamin D$_3$ was tested in the *Salmonella typhimurium* assay at doses 0.033 to 10 mg/plate in *Salmonella typhimurium* (strains TA1535, TA1537, TA97, TA98 and TA100) in the absence and presence of rat or hamster liver S9. Vitamin D$_3$ was negative in these tests. Doses above 1 mg/plate exhibited slight toxicity (Mortelmans *et al*, 1986).

No studies using other test systems for genotoxicity either *in vitro* or *in vivo* have been identified.

3.3. Acute toxicity

3.3.1. Animal data

The lethal dose in dog is said to be 13 mg/kg body weight. Immediate effects are bloody diarrhoea, anorexia, thirst, polyuria and prostration. In surviving animals calcium is deposited as in chronic hypervitaminosis D (Clare and Clark, 1975).

3.3.2. Human data

3.3.2.1. Effects of single doses

Elderly subjects with a serum calcium <2.75 mmol/L tolerated well a single intramuscular dose of 7,500 μg of vitamin D$_2$ when given once a year for 4 years (Heikinheimo *et al*, 1992 and 1991). Measurements

of serum calcium just after the injection were not reported, excluding detection of possible transient hypercalcaemia. Serum calcium was marginally elevated 2-3 months after the injection. Coles *et al* (1985, cited in Heikinheimo *et al*, 1992) used 10,000 μg vitamin D intramuscularly with no apparent toxic effects.

The safety of vitamin D prophylaxis as "stosstherapie" in infants 1-2 years was investigated by Markestad *et al* (1987). An oral dose of 15,000 μg ergocalciferol (vitamin D_2) was given every 3-5 months. Calcium, phosphorous and vitamin D metabolites were measured before and 2 weeks after each dose. 25(OH)D increased to median concentrations between 240 to 430 nmol/L (ranges: 130-930 nmol/L) (data extracted from figure) and returned to levels below 130 nmol/L before the next dose. All infants had normal serum calcium levels before the first dose, but 14 infants (34%) had calcium levels above 2.80 mmol/L (2.81-3.32 mmol/L), indicating that the vitamin D doses were excessive despite the lack of accumulative increases in 25(OH)D concentrations. In a later study (Misselwitz *et al*, 1990) ten children in the age range 1½ to 14 years, who had received such treatment, were diagnosed to have nephrocalcinosis. At the time of investigation, however, their serum vitamin D status was normal. This would indicate that even recurrent transient episodes of vitamin D excess and hypercalcaemia could lead to irreversible toxic effects as, for example, nephrocalcinosis.

In a study using vitamin D_3 (cholecalciferol) in oral doses of 15, 5 or 2.5 mg every 3 months, Zeghoud *et al* (1994) showed that these doses gave 25(OH)D concentrations of 307±160, 150±55, and 92±42 nmol/L, respectively, two weeks after the first dose. Serum calcium transiently increased 2 weeks after 15 mg, but not after the lower doses. Prolonged vitamin D overload, up to 6 months was seen in 50% of the children given the highest dose.

A single episode of moderately severe hypercalcaemia in infants may arrest growth for several months (Haynes, 1990).

3.4. Reproduction

3.4.1. Animal data

Vitamin D has been found to be teratogenic in animals at 4-15 times the recommended human dose. Offspring from pregnant rabbits treated with such high doses of vitamin D had lesions anatomically similar to those of supravalvular aortic stenosis and offspring not showing such changes show vasculotoxicity similar to that of adults following acute vitamin D toxicity (Stockton and Paller, 1990). The symptoms are most likely due to hypercalcaemia.

Sows received diets containing either 55 or 8.15 μg vitamin D_3 per kg basal ration (equivalent to 3.4 or 0.5 μg/kg body weight) and 6 week-old piglets were examined for coronary arterial lesions. Piglets from sows fed the high vitamin D_3 diet had more degenerated smooth muscle cells than those fed the low dose (Toda *et al*, 1985b).

3.4.2. Humans

During pregnancy 25(OH)D in maternal serum correlates with vitamin D intake, whereas the circulating active metabolite 1,25$(OH)_2$D is elevated mainly due to synthesis in the decidual cells of the placenta. Also the binding protein (DBP) increases. The foetus is entirely dependent upon maternal supply of 25(OH)D, which together with 24,25$(OH)_2$D appears to diffuse easily across the placenta. The relationship between 1,25$(OH)_2$D concentrations in maternal and foetal circulation is more complex as some studies show a good correlation whereas others do not (Salle *et al*, 2000).

There are also reports on 1,25$(OH)_2$D treatment during pregnancy of women suffering from hypoparathryoidism (Salle *et al*, 1981) (dose 0.5-2 μg/day) or insensitivity to 1,25$(OH)_2$D (dose 17-36 μg/day) (Marx *et al*, 1980). In the latter case the mother had extremely high plasma 1,25$(OH)_2$D and normocalcaemia. At parturition the cord serum concentration of 1,25$(OH)_2$D was strongly elevated, 940 pmol/L (normal mean: 47.5 pmol/L) and the child had mild hypercalcaemia the first two days of life. None of the children had other signs of toxicity. This indicates a minor impact of circulating 1,25$(OH)_2$D on calcium levels *in utero*. This is further supported by the fact that supplementary vitamin D (25 μg/day) during the last trimester reduced the fraction of infants displaying growth retardation (Salle *et al*, 2000).

However, maternal hypercalcaemia during pregnancy may increase foetal sensitivity to effects of vitamin D, suppression of parathyroid function or a syndrome of elfin faces, mental retardation, and

congenital supravalvular aortic stenosis. There are, however, no controlled studies in pregnant women indicating at which doses this may occur (Haynes, 1990).

Maternal supplementation of lactating women with 25 and 50 μg vitamin D_2/day during winter time showed that only children of women supplemented with the highest dose normalised the concentration of circulating vitamin D metabolites. Infants who got 10 μg vitamin D/day supplement and were breast-fed by non-supplemented mothers had similar vitamin D status to those of mothers supplemented with the highest dose (Ala-Houhala *et al*, 1986).

3.5. Chronic toxicity

3.5.1. Animal data

Hypervitaminosis D in animals as in humans is associated with hypercalcaemia and adverse effects largely mediated by this condition. The severity of the symptoms and organ manifestations depend on the severity and length of the hypercalcaemia. Soft tissue calcifications are common effects.

Charles River Crl:CD BR rats were given daily doses of 0, 12.5, 25 and 50 μg vitamin D_3/kg body weight from 10 weeks of age (Tischler *et al*, 1999). All doses of vitamin D_3 markedly increased serum calcium and phosphorus levels and calcium excretion into urine. At 4 weeks the rats receiving 12.5 and 25 μg vitamin D_3/kg body weight/day showed occasional foci of kidney tubular calcification while this was more prevalent at the highest dose of 50 μg vitamin D_3/kg body weight. At 26 weeks all kidneys from the highest dose showed mild to moderate nephrocalcinosis, the rats receiving 25 and 12.5 μg vitamin D_3/kg body weight/day showed mild and nearly no calcinosis, respectively.

Groups of two month-old swine were fed dietary vitamin D_3 at doses of 2.5, 7.5, 50, and 100 μg/kg feed (equivalent to 0.15, 0.45, 3 and 6 μg vitamin D/kg body weight, respectively) for four months. Particularly the highest dose group had thickening of the intima of the coronary vessels. Increased levels of lipid containing- and degenerative cells were also seen (Toda *et al*, 1985a)

3.5.2. Symptoms of vitamin D intoxication in humans

The symptoms of hypervitaminosis D are connected with the physiological consequences of hypercalcaemia, which occur once the calcium eliminating capacity of the kidneys is exceeded. The most frequently noted clinical manifestations of hypervitaminosis D are anorexia, weight loss, weakness, fatigue, disorientation, vomiting and constipation (Blank *et al*, 1995). Hypercalcaemia may also lead to growth retardation in children, irritability, asthenia, persisting fever, polyuria and polydipsia, dehydration, hypertension and functional renal insufficiency. Long-term toxicity with persistent hypercalcaemia may cause excess calcium precipitates as extra-skeletal calcium in soft tissues, particularly in the renal parenchyma, urinary tracts, vascular walls, muscles and tendons.

Linden (1974) observed that myocardial infarct patients in Tromsø, Norway, were more likely to consume vitamin D in excess of 30 μg/day than were matched controls, but two subsequent studies (Schmidt-Gayk *et al*, 1977; Vik *et al*, 1979) failed to confirm this.

Further studies are needed to clarify progressive health effects of regular and moderately high amounts of vitamin D over several decades.

3.5.3. Serum 25(OH)D and vitamin D toxicity

Vieth (1999) summarised the dose-response for mean vitamin D intake *versus* final serum 25(OH)D concentration of supplemented groups from 35 reports. Many of the studies involved not more than 4 weeks of supplementation, and according to the half-life for 25(OH)D of 1 to 2 months, one would not assume steady state to be achieved in such a short time. Remarkably, serum level of 25(OH)D concentration is maintained within a narrow range, ≈75-220 nmol/L across vitamin D supplies from 20 μg/day up to 250-500 μg/day. Beyond this level of vitamin D intake, which may be the physiologic limit, there is a classical rise in the dose-response curve associated with toxicity. Apparently there are homeostatic control systems to regulate serum 25(OH)D and to buffer against variability in vitamin D supply. Interestingly, this physiological limit of vitamin D intake is comparable with the amount of vitamin D (250-625 μg/day) estimated to be produced by full-body exposure to sunlight (Stamp, 1975; Holick, 1995).

A patient who had received vitamin D as a single monthly dose of 7,500 μg for several months had a serum 25(OH)D level of about 600 nmol 25(OH)D/L and experienced toxicity (Rizzoli *et al*, 1994). The dose was toxic since the production of 25(OH)D apparently had exceeded the instant capacity of the

homeostatic control system. Exposure to a single large dose of vitamin D resulted in a rapid and high peak in serum 25(OH)D concentration, with concentrations falling progressively thereafter (Davie *et al*, 1982; Weisman *et al*, 1986).

Barger-Lux *et al* (1998) administered 25, 250 or 1250 μg cholecalciferol/day to young healthy men with a mean serum 25(OH)D of 67 nmol/L. After 8 weeks serum 25(OH)D increased by 29, 146 (100-225) and 643 (400-1000) nmol/L for the three dosage groups. Body mass index (BMI) and not weight contributed significantly to the variance in 25(OH)D response. A high BMI would predict less change in 25(OH)D upon supplementation. The treatment time in this study could have been too short to achieve steady state level of 25(OH)D.

Himmelstein and coworkers (1990) supplemented elderly with 50 μg cholecalciferol/day in a double blind study for six weeks. At week 7 the serum concentration of 25(OH)D had reached 80.1±6.7 nmol/L. It cannot be excluded that a plateau had not been reached.

Davie and co-workers (1982) gave 10, 25 and 250 μg vitamin D/day for 2.5 months. The two lowest doses reached a plateau of about 55 nmol 25(OH)D whereas the highest dose, 250 μg/day, reached a serum level of about 120-140 nmol/L. However, it is likely that steady state had not been reached in this case, furthermore the form of vitamin D is uncertain.

When Stamp *et al* (1977) measured 25(OH)D in 128 individuals receiving the same daily amount of vitamin D_2 or D_3 for a period more than 4 months and having achieved steady state, those receiving 45 μg/day were all below 130 nmol 25(OH)D/L, whereas among those receiving 150 μg/day a large fraction had values above 130, but less than 200 nmol/L. The upper 95% confidence limit for the regression line crossed 130 nmol/L at about 60-70 μg/day. (All the data were extracted from the figures).

Vieth and coworkers (2001) supplemented two groups of 33 and 28 healthy volunteers with 25 and 100 μg cholecalciferol daily, respectively, for 1-5 months. At 4 and 5 months the lower dose group had a mean 25(OH)D concentration of about 70 nmol/L (range: 45-120) and in the high dose group the mean was about 100 nmol/L (range: 65-120) (values extracted from figure).

Tjellesen *et al* (1986) supplemented 19 healthy premenopausal women with either 100 μg ergocalciferol or cholecalciferol/day for eight weeks. They also received 0.5 g calcium per day. At eight weeks the total 25(OH)D concentrations in serum were 35.5 (19.7-48.3) and 45.4 (31.0-55.4) nmol/L in the ergocalciferol and cholecalciferol group, respectively. A suppression of $25(OH)D_3$ in serum was observed in the group treated with ergocalciferol resulting in no change in the total 25(OH)D from the pre-treatment status.

3.5.4. Vitamin D intake and hypercalcaemia

Hypercalcaemia is defined as a serum calcium level above 2.75 mmol/L or ionised calcium above 1.35 mmol/L. Normal calcium levels were seen in persons given 50 μg/day of vitamin D for 6 months (Johnson, 1980) and daily intake for 6 weeks of 250 μg by healthy adults did not significantly raise their serum and urine concentrations of calcium (Berlin *et al*, 1986). In individuals with intakes from 1250 μg/day or higher the serum calcium level range was from 2.82 to 4.00 mmol/L (Schwartzman and Franck, 1987; Davies and Adams, 1978; Selby *et al*, 1995; Rizzoli *et al*, 1994; Pettifor *et al*, 1995). Schwartzman and Franck (1987) reviewed cases in which vitamin D was used to treat osteoporosis in middle aged and elderly women. These women had health problems in addition to osteoporosis. An intake of vitamin D between 1250 μg/week and 1250 μg/day for 6 weeks to 5 years was found to be associated with reduced renal function and hypercalcaemia.

Narang *et al* (1984) studied the effect of vitamin D supplementation on serum calcium levels in humans, with and without tuberculosis. Their diet was supplemented with daily vitamin D doses of 10, 20, 30, 60 and 95 μg/day for 3 months. Thirty healthy males and females ranging in age from 21 to 60 years and without tuberculosis were in one study group. Statistically significant increases in serum calcium were observed in these subjects at vitamin D doses of 60 and 95 μg/day. The mean serum calcium concentration in normal controls following administration of 60 μg/day of vitamin D increased from 2.43 to 2.62 mmol/L, a change that did not indicate hypercalcaemia. However, following 95 μg/day, the mean serum calcium level in normal controls increased from 2.46 to 2.83 mmol/L. No information on the nature of the vitamin D preparation, background vitamin D intake or serum 25(OH)D was given.

The results of Narang and co-workers were not supported by Tjellesen *et al* (1986). They monitored serum vitamin D metabolites and calcium in 19 healthy premenopausal women during treatment with 100 μg/day

of vitamin D_2 or vitamin D_3 for 8 weeks. They found that serum calcium increased significantly by a minute amount of 0.05 mmol/L with 100 μg/day of vitamin D_3. The urinary calcium excretion increased slightly with a mean molar calcium/creatinine ratio of 0.518, which is well below hypercalciuric ratio of 1.0.

In a recent study described above Vieth *et al* (2001) supplemented healthy volunteers in groups of 33 and 28 healthy individuals with 25 and 100 μg cholecalciferol, respectively, for 1-5 months. In all subjects serum calcium remained within the reference values for serum calcium and no significant change from baseline values were found. Similarly, on a group basis, there was no significant change from baseline in urinary molar calcium/creatinine ratios. There were more subjects exceeding a ratio of 1.0 in the high dose group than in the low dose group.

3.5.5. Serum 25(OH)D, serum calcium and hypercalcaemia

In patients with rickets, Stamp (1975) demonstrated a parallel increase in serum 25(OH)D and serum calcium during the healing period with ultraviolet light. The treatment did not increase the serum calcium concentration above 2.5 mmol/L, neither did the 25(OH)D concentration increase above 125 nmol/L. Also in patients who had consumed milk excessively fortified with vitamin D, there was a correlation between serum 25(OH)D and serum calcium (Jacobus *et al*, 1992). Serum samples with calcium concentrations larger than 2.75 (hypercalcaemia) were characterised by serum concentrations of 25(OH)D larger than 200 nmol/L.

Adams and Lee (1997) described four normocalcaemic patients with 25(OH)D concentrations at 177±41 (132-222) nmol/L with hypercalciuria and depressed serum PTH. The intake of vitamin D was in the form of supplements of uncertain magnitude. Upon withdrawal of the supplements the patients became normocalciuric and the 25(OH)D concentration returned to normal (<130 nmol/L).

Better *et al* (1980) investigated 45 randomly selected Israeli lifeguards who worked at the beach during August-September and compared this group with a control population matched for age and season. Both groups had similar serum calcium levels, but the lifeguards had a significantly lower serum PTH, a higher serum 25(OH)D level (148±105 *vs* 65±25 nmol/L), and a higher urinary calcium excretion. Eleven lifeguards had nephrolithiasis, a significant higher incidence than in the general population. The lifeguards had slightly lower urinary volume than those of controls did.

3.6. Susceptible groups

3.6.1. Infants

The regulation of 1α-hydroxylase and the normal feedback suppression by $1,25(OH)_2D$ on the kidney enzyme seem to work less well in infants than in adults (Stern *et al*, 1981).

3.6.2. Idiopathic hypercalcaemia of infancy and Williams' syndrome

Idiopathic infantile hypercalcaemia (IIH) and Williams' syndrome are two conditions associated with hypercalcaemia in infancy (Seelig, 1969; McTaggart, 1999; Hockenhull *et al*, 1999; Rodd and Goodyer, 1999). Both conditions occur sporadically, but inheritance has also been described. Williams' syndrome was described in 1961 by Williams and co-workers and is in more than 90% of the cases caused by a microdeletion on chromosome 7 affecting the elastin gene. The Williams' syndrome is a multisystem developmental syndrome with vascular and connective tissue abnormalities, hypercalcaemia, dysmorphic faces and mental retardation. Levels of $1,25(OH)_2D$ are often elevated and followed by excessive intestinal absorption of calcium.

The "mild" or light variant of IIH is a heterogeneous disorder originally described in the 1950s in England during the period of high-dose vitamin D fortification of milk. Lowering the supplementation dramatically decreased the incidence. The relationship to vitamin D metabolism is unclear. Elevated levels of PTH-related protein, as a cause for the disease, has been found in some cases. Generally, the hypercalcaemia disappears after the first year and the prognosis is good. Both IIH and Williams' syndrome are treated with a diet low in vitamin D and calcium and there is hypersensitivity towards vitamin D. Hypercalcaemia might develop with vitamin D intakes as small as 5-10 μg/day.

3.6.3. Patients with sarcoidosis, tuberculosis, lymphomas and infants with subcutaneous fat necrosis

The feedback mechanism of $1,25(OH)_2D$ synthesis seems to operate poorly, if at all, in tissues other than that of the renal tubule. In patients with sarcoidosis, $1,25(OH)_2D$ is believed to be synthesised in macrophages, which in these patients have an increased enzyme capacity, or other cells in the granulomas. Also the

clearance of 1,25$(OH)_2$D may be decreased as well. Contrary to normal in these patients there is a positive correlation between 25(OH)D within reference levels and 1,25$(OH)_2$D in serum. Even normocalcaemic patients with sarcoidosis have unregulated production of 1,25$(OH)_2$D in response to vitamin D. Also exposure to sunlight may increase the level of active metabolite.

In some lymphomas, typically B-cell lymphomas, there is an increased blood level of 1,25$(OH)_2$D, which is probably synthesised by lymphocytes (Narang *et al*, 1984; Bell, 1998).

Excessive endogenous synthesis of 1,25$(OH)_2$D occurs in children with subcutaneous fat necrosis (Rodd and Goodyer, 1999).

Vitamin D deficiency can mask primary hyperparathyroidism and this could account for the occasional cases of hypercalcaemia observed when large groups of elderly people are given vitamin D supplements (Johnson, 1980).

3.7. Interactions

3.7.1. Vitamin A

Earlier work has provided some evidence that vitamin A might antagonise the actions of vitamin D. Recently this was clearly shown in experiments with rats. Twenty-one day-old male Holtzman rats were fed a rachitogenic diet supplemented with 15.5 ng ergocalciferol (D_2)/day every 3 day and retinyl acetate in doses from 0-8621 ng/day. Increasing the level of retinyl acetate caused a progressive decrease in the total amount of ash in the femur and increase in epiphyseal plate. This antagonistic effect of retinyl acetate was also present even at higher vitamin D_2 dosages. In addition retinyl acetate also inhibited vitamin D_2 action in rats fed a normocalcaemic diet (Rohde *et al*, 1999). These experiments show that an antagonism takes place at physiological levels of these two vitamins. At the molecular level vitamin D and A share RXR as a common partner for the receptor: the vitamin D receptor heterodimerises with RXR whereas RXR alone or together with RAR function as a mediator of the biological effects of retinoic acids.

3.7.2. Magnesium

Elevation of plasma magnesium increases the secretion of PTH (Rude *et al*, 1978), which stimulates the synthesis of 1,25$(OH)_2$D. Magnesium deficiency in humans, on the other hand, may result in an impaired PTH secretion followed by hypocalcaemia and a reduced serum concentration of 1,25$(OH)_2$D. This explains why patients with hypoparathyroidism may be resistant to vitamin D therapy unless magnesium is also given (Fatemi *et al*, 1991).

3.7.3. Drugs

Ketoconazole, which inhibits the 24-hydroxylase activity, markedly enhances the potency of 1,25$(OH)_2$D. Enhanced potency of 1,25$(OH)_2$D is also seen in 24-hydroxylase null mice.

Thiazide drugs, which increase the tubular reabsorption of calcium, would enhance the hypercalcemic effect of a high dose of vitamin D.

Glucocorticoids, phenobarbital and phenytoin antagonise the effect of vitamin D on intestinal calcium absorption. These drugs also protect rats against high doses of vitamin D (Haynes, 1990)

4. DOSE-RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

4.1. Critical effects

Due to great uncertainty data from animal studies are considered to be inappropriate for identification of critical endpoints and establishment of a NOAEL or a LOAEL.

The principal critical effect of hypervitaminosis D/vitamin D toxicity is hypercalcaemia. It has, however, been reported that patients with hypervitaminosis D (increased level of 25(OH)D >130 nmol/L), hypercalciuria and a depressed PTH status can be normocalcaemic (Adams and Lee, 1997). Thus, hypercalciuria apparently is an earlier phenomenon than hypercalcaemia which could predispose to kidney stone formation.

4.2. Adults

4.2.1. Establishment of a NOAEL on the basis of hypercalcaemia

In the report by Narang *et al* (1984) a modest hypercalcaemia (level >2.75 mmol/L) was demonstrated at a vitamin D intake of 95 μg/day. The changes in serum calcium following 60 μg vitamin D/day were still within the reference range. There are, however, major drawbacks in this study. There are no data on pre-treatment vitamin D status, no data on sun exposure and no information about the compound given. Neither are there any data on serum 25(OH)D concentrations nor information on the physical status on the healthy participants. Other studies by Tjellesen *et al* (1986) and Vieth *et al* (2001) could not confirm the data of Narang *et al* (1984) as they found no or only a very small increase in serum calcium at an intake of 100 μg vitamin D/day. Increases in the 25(OH)D concentration in serum were seen in both latter studies, but within reference values (<130 nmol/L). The discrepancies between the study of Narang *et al* (1984) and the latter ones could be vitamin D compound given, vitamin D status before supplementation, body weight, body fat or solar exposure.

4.2.2. Using 25(OH)D in serum for the establishment of a NOAEL

An alternative approach is to use serum 25(OH)D levels as basis for the assessment. Adams and Lee (1997) described hypercalciuria and depressed serum PTH in normocalcaemic patients with plasma 25(OH)D concentrations at 132-222 nmol/L. Interestingly, upon withdrawal of the supplements the patients became normocalciuric and the 25(OH)D concentration returned to normal (<130 nmol/L). Hypercalciuria was also observed in Israeli lifeguards with a serum 25(OH)D concentration at 148±105 nmol/L (Better *et al*, 1980).

A NOAEL could also be based on a 25(OH)D concentration in serum of about 150 nmol/L, which is considered the upper reference value, and which has not been reported to be associated with hypercalcaemia or hypercalciuria.

The vitamin D intake associated with exceeding the upper reference value of 25(OH)D in serum would vary greatly in the population. It is, for instance, dependent on the exposure to sunlight and sensitivity to vitamin D. The importance of the chemical form of vitamin D, i.e. vitamin D_2 or D_3 as described above (see 2.5.2) with a lower biological efficiency of vitamin D_2, should be noted. In addition the vehicle used (fat or emulsion) could influence bioavailability. This was shown as early as in 1935 by Stearn and Jeans (cited in Seelig, 1969). Vitamin D from cod liver oil emulsified in milk is about three times as potent as vitamin D given in cod liver oil or propylene glycol. For some individuals an intake of 250 μg vitamin D would not cause an exceed of this value while in others this could occur. The data of Stamp *et al* (1977) (data taken from figure 1 of Stamp *et al*, 1977) indicate that the upper reference value of serum 25(OH)D at 150 nmol/L or 200 nmol/L is exceeded by 5% of the population at an approximate vitamin D intakes of about 80 or 100 μg/day, respectively. These levels of 25(OH)D in serum can be considered NOAELs with respect to increased risks of hypercalciuria and hypercalcaemia, respectively. On the other hand, the study by Tjellesen *et al* (1986) and the recent study of Vieth *et al* (2001) reported that the upper reference serum concentration of 25(OH)D was not exceeded upon supplementation with 100 μg cholecalciferol (vitamin D_3)/day.

4.2.3. Derivation of an UL for adults

Taking into account all the information in the studies above (particularly information on vitamin D_3 provided by Tjellesen *et al*, 1986 and Vieth *et al*, 2001), the risk of hypercalciuria/hypercalcaemia probably starts to increase in some parts of the population at an intake above 100 μg vitamin D/day. The risk of exceeding the upper reference concentration of 25(OH)D in serum will also increase. A dose of 100 μg vitamin D/day and a serum level of 200 nmol 25(OH)D/L are considered a NOAEL. An uncertainty factor of 2 is considered adequate to account for the inter-individual variation. An upper intake level of 50 μg vitamin D/day is considered to offer adequate protection against the risk of hypercalciuria and hypercalcaemia. On this basis the Committee sets an **UL of 50 μg vitamin D/day for adults[1].**

Regarding less frequent intakes of larger doses of vitamin D, this has been discussed above (see section 3.3.2) and no recommendation is given.

1. It should be noted that the intake of vitamin D via food would add to synthesis caused by exposure to sunlight. Depending on the amount of sunlight the risk of adverse effects at an intake at the upper level would increase. It should also be noted that higher doses of vitamin D might be needed, particular in elderly, to achieve optimal serum levels of 25(OH)D for purpose of optimal mineralisation of the skeleton. Such treatment should be under medical surveillance.

No data are available to suggest that other life-stage groups have increased susceptibility to adverse effects of high vitamin D intake. Given the minor impact of circulating vitamin D on calcium levels *in utero* and in breast-fed infants with maternal supplements of 25 and 50 μg vitamin D/day (see section 3.4) there does not seem to be an increased sensitivity during this period. Therefore the UL of 50 μg/day should be considered to apply also to pregnant and lactating women.

4.3. Infants

Most of the studies also documenting duration and magnitude of intake in infants regarding adverse effects of vitamin D such as growth retardation and hypercalcaemia are old studies or case reports.

4.3.1. Establishment of LOAEL/NOAEL on the basis of growth and hypercalcaemia

Jeans and Stearns (1938) found retarded linear growth in 9 infants up to one year of age who received 45-112.5 μg vitamin D/day as supplements in comparison with standard growth curves of infants receiving daily supplements at doses of 8.5 μg or less for a minimum of 6 months. The group receiving high vitamin D supplements showed a retarded linear growth and increased rate of growth was seen when the dose of vitamin D was reduced to 10-15 μg/day. The children received both cod liver oil, a cod liver oil concentrate emulsified in cream and viosterol (vitamin D_2). In another study, Fomon *et al* (1966) compared linear growth in a group of 13 infants daily ingesting 34.5-54.3 μg vitamin D (mean 45 μg) with 11 infants who received 8.8-13.8 μg/day. The infants were enrolled before the age of 9 days and followed at ages 28, 56, 84, 112, 140 and 168 days. No effect on growth was found in this small study. Neither was any hypercalcaemia seen. These studies were performed before vitamin D metabolites in serum could be routinely measured. The authors of the latter study provided some information on the preparation given, Deca-Vi-Sol, but not whether this is vitamin D_2 or D_3.

An epidemic of infantile hypercalcaemia was observed in the UK during 1953 to 1957 when National Dried Milk and other foods including infant food were supplemented with vitamin D. More than 200 cases of hypercalcaemia were reported by June 1955 to 196 consultant paediatricians in the UK. Also other conditions related to vitamin D toxicity increased during this period. In 1957 the vitamin D contents in dried milk, cereals for infants and cod liver oil were reduced: dried milk from 80 to 25 μg/100 g of dry matter, cereals: 8 to 2.5 μg/100 g of dry matter and in cod liver oil from 4 to 2 μg/mL. Following this, the incidence of hypercalcaemia was reduced substantially, but not completely (Seelig, 1969). According to the two surveys conducted by the British Paediatric Association (BPA, 1956 and 1964) there was a reported decline in hypercalcaemia in infants from 7.2 in the 1953-1955 survey to 3 cases per month in the 1960-1961 survey. The estimated total intake of vitamin D in infants for the 75 percentile showed 100 μg/day in the first survey declining to a range of 18 to 34 μg/day in the second survey (BPA, 1956; Bransby *et al*, 1964). The non-specific early symptoms of hypercalcaemia are failure to thrive, hypotonia and susceptibility to infections would suggest that the diagnosed cases might only be the tip of an iceberg (Seelig, 1969).

Among infants of the age of 6 to 20 months with hypercalcaemia, the intake of vitamin D was estimated to be below 50 μg/day in 50 children (Seelig, 1969). In several American reports of individual cases of infantile hypercalcaemia the vitamin D estimated intake was between 20 and 35 μg/day (cited in Seelig: Bongovanni *et al*, 1957, N Eng J Med 257: 951; Schwartz, 1957, J Pediat 51: 461; Snyder, 1958, Am J Diseases Children 96: 376; Garcia *et al*, 1964, N Eng J Med 271: 117; Rashkind *et al*, 1964, J Pediat 58: 390).

Taken together these early data indicate that at least some infants are sensitive to excessive intake of vitamin D and at risk for hypercalcaemia. It cannot be excluded that some of the cases of infantile hypercalcaemia during this period represent cases of Williams' syndrome or idiopathic infantile hypercalcaemia in addition to those of vitamin D intoxication. With regard to the reports of hypercalcaemia cases in particular could be due to other causes not related to vitamin D. Furthermore, none of these publications had any information on 25(OH)D in serum or any other biomarker of vitamin D status as these studies were performed before such measurements were available. And the magnitudes of the daily vitamin D dosages of the survey data and lack of data on sunlight add to the uncertainties. Thus, it is not possible to exactly define a NOAEL or LOAEL. However, based on these data, it cannot be excluded that an increased risk of vitamin D toxicity and hypercalcaemia might be present at exposures below 50 μg/day. The observations on hypercalcaemia in infants in the UK furthermore indicate that the susceptibility among infants, although less expressed, extends beyond the first year of age.

4.3.2. Establishment of LOAEL/NOAEL on the basis of 25(OH)D in serum and hypercalcaemia

The upper reference level for 25(OH)D for infants is similar to that of adults and the approach used for adults by setting the upper level at an oral dose of vitamin D not associated with exceeding the upper reference level (i.e. 130-150 nmol/L) could in theory be done. A problem is that there are very few data on doses of vitamin D above the recommended intake and corresponding concentrations of 25(OH) in serum.

In a study from Germany, Hövels and co-workers (1983) determined 25(OH)D in serum in infants 12, 18 and 24 months of age. Levels of 25(OH)D were determined in infants 12 and 24 months of age receiving 12.5 (n=58 and n=87) and 25 μg vitamin D_3/day (n=34 and n=15), respectively. It is difficult to evaluate the absolute ranges of 25(OH)D reported in this study since they deviate from those usually reported in other studies and no information on the method of determination and reference values was given in the paper. Thus, this study cannot be used for the establishment of a relationship between vitamin D intake and 25(OH)D or a NOAEL.

In a study from Norway by Markestad (1984), 25(OH)D was measured in eight infants 6 weeks of age in the winter and only receiving a formula containing 10 μg vitamin D_3/L (corresponding to about 8-10 μg/day). The concentrations varied from 60 to 125 (mean 90) nmol/L, showing that this amount of vitamin D was enough to give approximately the reference concentration of 25(OH)D. Thirty-seven 6 and 12 month-old infants who had received vitamin D from cod liver oil (vitamin D_3) or a commercial multivitamin supplement (unknown form of vitamin D) in the following doses: 7.5-10 μg/day (n=23), 5 μg/day (n=9) and 2.5 μg/day (n=5) had in the winter a concentration range of less than 20 to 115 nmol 25(OH)D/L in serum. Twenty-two infants 7 to 18 months of age were studied at the end of the summer. They had not received any vitamin D fortified food or supplements for 4 months, and their serum concentration of 25(OH)D ranged from 30 to 164 (mean 85) nmol/L.

In a study from Finland, Ala-Houhala (1985) supplemented breast-fed infants with 0, 10 and 25 μg vitamin D_2/day for 20 weeks, using 14-17 infants and mothers in each group. In the group where the infants were not given vitamin D, the mothers were given 25 μg/day. Two studies were conducted, one starting in January and one starting in July. No signs of hypercalcaemia were reported. The serum level of 25(OH)D increased rapidly in both groups of infants supplemented with 10 and 25 μg/day. The levels obtained in the July-December groups were 15-20% higher than those of the groups starting in January. At 20 weeks of age the 25(OH)D level did not show any sign of reaching a steady state level. In the groups supplemented with 10 μg/day the 25(OH)D levels (mean±SEM) were 83±13 and 98±3 nmol/L in the winter and summer groups, respectively. In the groups supplemented with 25 μg/day the 25(OH)D levels (mean±SEM) were 110±13 and 138±7 nmol/L in the winter and summer groups, respectively. (All data were derived from figures 2, 3 and 5 in Ala-Houhala, 1985). The 25(OH)D levels obtained with a supplementation of 10 μg/day seem to agree well with the data of Markestad (1984).

Hesse and co-workers (reported in an abstract, 1993) examined the effects of prophylactic administration of 10 μg vitamin D_3 tablets in 2707 newborn to 15 month-old infants. Infants were breast-fed or given vitamin D-free formula milks. The treatment was started in the second week of life. 25(OH)D increased from 22.6±13.8 to 83.1±36.1 nmol/L from the second week to the third month and to 93.9±36.6 nmol/L between 4-6 months. Elevated 25(OH)D (>130 nmol/L) were found in the 10.1% and 2.6% of the infants in the age of 0-6 months and 6-12 months, respectively. Importantly, elevated serum calcium (>2.8 mmol/L) was observed in 6.4% of the infants in the first 6 months. One of the 2707 infants had rickets. In an extended study (also reported in an abstract, Hesse *et al*, 1994; Hesse, 1994) it was found that among 3481 infants treated in this way 2.9% of the infants aged 2 weeks to 6 months had 25(OH)D levels above 173 nmol/L (>3 standard deviations) and 0.9% had serum calcium >3.08 mmol/L. On this basis, the authors proposed to reduce the vitamin D supplement to 7.5 and 10 μg D_3/day for the first and second half year of life, respectively, and to the reduce the supplementation of infant formula from 10 to 7 μg D_3/L (Hesse *et al*, 1993 and 1994; Hesse, 1994). It should be noted that this study was carried out in the eastern part of Germany a few years after the German reunification and that it is possible that the practise of "stossprophylaxis" with vitamin D (see section 3.3.2) had not been discontinued completely. However, no information on this was provided. Because of this and since only limited information is available from the abstract, it is inappropriate to use this study in the derivation of the upper level.

Vervel and co-workers (1997) determined serum 25(OH)D in infants 1 to 4 months of age seen as outpatients The infants received 25 μg vitamin D_2/day in addition to infant formula unfortified (n=23) or fortified with vitamin D (n=41). The 25(OH)D levels in serum was 92.5±28 and 72.8±24.3 nmol/L in the summer and winter, respectively. Those fed vitamin D fortified formula in addition had 99±22 nmol/L

in the summer. In a prospective controlled study serum 25(OH)D was determined in healthy neonates born to unsupplemented (n=48) or vitamin D_3 supplemented mothers (n=22) between April and July. The infants were given vitamin D_2 fortified milk, providing either 12.5 or 25 μg vitamin D/day and followed from birth to 3 months of age. At birth, 25(OH)D was 27.4 and 37.8 nmol/L in unsupplemented and supplemented mothers, respectively. After 1 and 3 months there were only marginal differences between the groups receiving 12.5 and 25 μg vitamin D_2 and no differences between the groups from unsupplemented and supplemented mothers, respectively. The values at 3 months were 61.5±12.3 and 61±7 nmol/L, in the groups receiving 12.5 μg vitamin D_2/day, and 75.25±12.5 and 66.25±13 nmol/L, in the groups receiving 25 μg vitamin D_2/day. No values were above 92.5 nmol/L. Serum calcium values at 3 months in the low groups were within 2.42-2.80 mmol/L and in the high groups within 2.46-2.79 mmol/L. The percentage of children with serum calcium above 2.6 mmol/L was not increased in the group receiving 25 μg vitamin D_2/day. The same group (Zeghoud *et al*, 1997) also examined the increase in 25(OH)D depending on the basal 25(OH)D prior to vitamin D_2 supplementation. The increase was greatest in the group with the lowest basal level, <15 nmol/L, and only in this group did 25 μg vitamin D_2/day cause an increase in 25(OH)D greater than that produced by 12.5 μg vitamin D_2/day.

4.3.3. Derivation of an UL for infants

Two endpoints are used in the derivation of the UL, namely hypercalcaemia, which can be considered an adverse effect, and the serum concentration of 25(OH)D greater than the upper reference level. The upper reference level of 130-150 nmol/L is well below the threshold of increased risk for hypercalcaemia, which in adults is above 200 nmol/L.

The studies by Fomon *et al* (1966) recording no effect on growth and serum calcium might indicate a NOAEL of 45 μg/day, however, only a small number of infants (n=13) was studied. The form of vitamin D given in this study is not known. No hypercalcaemia was recorded in the studies of Ala-Houhala (1985) and Vervel and co-workers (1997) supplementing up to 25 μg vitamin D_2/day in addition to breast milk or to formula fortified at least with a level giving 7 μg vitamin D_2/day.

Using the upper reference serum concentration of 25(OH)D (130-150 nmol/L) in deriving an upper level the only data on intake and 25(OH)D concentrations are vitamin D_2 intakes at the upper, middle and the lower range of the PRI. At intakes of 25 μg vitamin D_2/day in addition to breast-feeding the mean level of 25(OH)D (110±13 and 138±7 nmol/L) is close to the upper reference values reported (130-150 nmol/L), particularly when exposed to sunlight in one study (Ala-Houhala, 1985). In another study of non-breastfed infants receiving 25 μg vitamin D_2/day through fortified formula had a slightly lower level of 25(OH)D, i.e. 92.5 nmol and less (Vervel *et al*, 1997).

Systematic studies that can be used to establish a NOAEL have not been performed on infants receiving more than 10 μg vitamin D_3 in addition to breast milk. However, the present intake of vitamin D_3 in infants were calculated according to current recommendations in Germany on supplement (10 to 12.5 μg vitamin D_3/day to all infants) and use of supplemented infant and follow on formula. It was found that during the first 5 months of life vitamin D_3 intakes could be up to 24 μg/day. The calculated daily intake gradually decreases to up to 16.6 μg/day. The current intake apparently is not associated with any problems of vitamin D excess. It should be noted, however, that no systematic studies have been carried out and that mild hypercalcaemia is associated only with mild and unspecific symptoms such as failure to thrive.

Considering hypercalcaemia the small and old study by Fomon *et al* (1966) indicated a NOAEL of 45 μg vitamin D/day for infants. However, two more recent and larger well-controlled studies showed that neither in infants receiving 25 μg vitamin D_2/day in addition to breast milk (Ala-Houhala, 1985) nor in infants receiving 32 μg vitamin D_2/day (Vervel *et al*, 1997) was hypercalcemia observed. In addition, in neither of these studies the resulting serum 25(OH)D concentrations were observed to be above the upper reference level indicating that these are well below the threshold of hypercalcaemia and consequently an uncertainty factor of 1 is considered appropriate. Using the lower value taking into account the higher biological activity and toxicity of vitamin D_3 (see section 2.5.2) and the other information provided above **the Committee sets an UL of 25 μg vitamin D/day for infants 0-24 months of age[2].**

2. It should be noted that the intake of vitamin D via food would add to synthesis caused by exposure to sunlight. Depending on the amount of sunlight the risks of adverse effects at an intake of the upper level would increase.

4.4. Children and adolescents

4.4.1. Derivation of an UL for the age group 2-17 years

In the age group 2-17 years there are no data on high level intake to support the derivation of an upper level. Finnish 9-15 years old girls were supplemented with vitamin D_2 in the winter season for 3 years (Lehtonen-Veromaa *et al*, 2002). Before start 14% and 75% of the group had severe (<20 nmol 25(OH)D/L) and moderate (between 25 and 37.5 nmol 25(OH)D/L) hypovitaminosis D at baseline in winter. The first and second year, in addition to a median dietary intake of 4 μg vitamin D/day, they were supplemented from October to January with 10 μg vitamin D_2/day and the third year with 20 μg vitamin D_2/day. The daily supplementation with 120 to 140 μg vitamin D_2/week increased 25(OH)D to 45.5±17.2 nmol/L at 36 months. Sunlight in the summer was apparently more effective raising mean levels to 62 nmol/L. The effect of supplementation or sunlight was greatest in the group with severe hypovitaminosis D. In this study 20 μg ergocalciferol/day was well tolerated, but the nutritional effect of supplementation was rather weak, 11.7 25(OH)D nmol/L for the group taking 80-140 μg ergocalciferol/week. This is probably due to the general low efficiency of vitamin D_2, about 1.7 times less efficient (Trang *et al*, 1998) than vitamin D_3 as discussed in section 2.5.2. There are no studies on supplementation with high levels of vitamin D_3 in children and adolescents. Apparently, the response to 10 μg vitamin D_2/day on 25(OH)D was much greater in a group of prepubertal Finnish children (Ala-Houhala *et al*, 1988b). It seems that susceptibility towards vitamin D changes with age. Using a cautious approach taking into consideration a lower weight in children up to 10 years the Committee sets the upper levels as follows:

UL of 25 μg vitamin D/day for children from 2 up to and including 10 years of age[3]

UL of 50 μg/day for adolescents 11-17 years of age[3]

4.5. Summary of upper levels for vitamin D

Age (years)	Tolerable Upper Intake Level (UL) for vitamin D (μg/day)[3]
0-2	25
3-10	25
11-17	50
Adults+	50

+ The UL for adults does also apply to pregnant and lactating women.

5. RISK CHARACTERISATION

5.1. Adults

An UL of 50 μg vitamin D/day is above 5 times the intake (PRI) recommended by the SCF (1993) of 0-10 μg and 10 μg vitamin D/day for adults below and above 65 years of age, respectively. The mean and 95 percentile intakes without supplements in several European countries are about 10-13 times and about 5 times less than the UL, respectively. In Norway the 95 percentile intake with supplements is about 1.5 times less than the UL. The 97.5 percentile values with supplements are 8.4, 12.7, 14.3 and 22.16 μg/day in Italy, UK, Ireland and Austria, respectively. The 97.5 percentile values without supplements are 8.4, 8.9 and 14.1 μg/day in the UK, The Netherlands and Germany, respectively. These values are well below the UL.

5.2. Infants

Recent evaluations in several European countries and the USA recommended an intake for infants of 10 μg vitamin D/day to ensure that the population gets enough vitamin D. The recommended intake (PRI) given by the SCF (1993) is 10-25 μg vitamin D/day for infants 6-12 months of age and 10 μg vitamin D/day for infants 12 to 36 months of age. No recommendation is given for the period 0-6

3. It should be noted that the intake of vitamin D via food would add to synthesis caused by exposure to sunlight. Depending on the amount of sunlight the risk of adverse effects at an intake at the upper level would increase. It should also be noted that higher doses of vitamin D might be needed, particular in elderly, to achieve optimal serum levels of 25(OH)D for purpose of optimal mineralisation of the skeleton. Such treatment should be under medical surveillance.

months. Some infants in this age group may reach an intake up to 22-24 μg vitamin D_3/day according to calculations based on current levels of vitamin D_3 in infant formula in Germany (1-2 μg vitamin D_3/100 Kcal) in addition to recommended supplementation (10-12.5 μg vitamin D_3/day). The UL is similar to the upper end of the present PRI of 10-25 μg/day for 6-12 months (SCF, 1993).

It should be kept in mind that hypercalcaemia due to vitamin D excess is uncommon in hospitals. In this matter there is a dilemma because on the one hand it is important to prevent excess intake, on the other hand it is equally important to secure infants an adequate vitamin D intake to avoid serious deficiency problems such as rickets. This disease has been reported in European countries particularly among infants and small children of immigrants (Brunvand and Nordshus, 1996; Uldall *et al*, 1984).

5.3. Children and adolescents

An UL of 25 μg vitamin D/day is 2.5 times the upper range of recommended intakes for children 2-10 years of 0-10 μg vitamin D/day (SCF, 1993). The mean intake without supplements in European countries is about 5 times less than the UL. In Germany the 97.5 percentile intakes without supplements for the age groups 4-6 years and 7-9 years were 7.5 and 8.7 μg/day, respectively.

An UL of 50 μg vitamin D/day is 3.3 times the recommended intake for young individuals (11-17 years) of 0-15 μg vitamin D/day (SCF, 1993). Values without supplements from Germany on 97.5 percentile intakes for the age groups of 10-12 years, 13-14 years and 15-18 years were 9.0, 11.1 and 11.1 μg/day, respectively. These values are about 4-5 times less than the UL.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF ZINC

(EXPRESSED ON 5 MARCH 2002)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Zinc has an atomic weight of 65.37 and is classified as a group IIB post-transition metal. In biological systems, zinc exists as Zn^{2+} and is present in all tissue and fluids in the body. Total body content of zinc is between 2 and 4 g and plasma concentration is between 11 and 18 µM (approximately 0.1% of total body content). Urinary zinc excretion is between 300 to 700 µg/day. Zinc is also present in foods and supplements as salts of the divalent cation. Under European legislation the following salts of zinc: acetate, chloride, citrate, gluconate, lactate, oxide, carbonate and sulphate are included in the list of substances that can be used in the manufacture of foods for particular nutritional uses and in food supplements (the legal measure on food supplements is expected to be adopted in the immediate future). Zinc content in the most common single nutrient supplements on the market is 30 mg per capsule, range 15-50 mg and in the most common multiple nutrient supplements is 10-15 mg, range 2-20 mg.

2. NUTRITIONAL BACKGROUND

2.1. Function

Zinc is essential for growth and development, testicular maturation, neurological function, wound healing and immunocompetence. Over 300 zinc enzymes have been discovered covering all six classes of enzymes and in different species of all phyla (Christianson, 1991; Coleman, 1992; Vallee and Auld, 1990). Zinc has structural, regulatory or catalytic roles in many enzymes (Vallee and Galdes, 1984; Hambridge *et al*, 1986). Additionally, it maintains the configuration of a number of non-enzymatic proteins such as pre-secretory granules of insulin, some mammalian gene transcription proteins (Struhl, 1989) and thymulin. Well known zinc containing enzymes include superoxide dismutase, alkaline phosphatase and alcohol dehydrogenase.

2.2. Homeostasis

Absorption of zinc takes place in the small intestine and appears to be a carrier-mediated transport process which is not saturated under normal physiological conditions. At high intakes, zinc is also absorbed through a non-saturable process or passive diffusion (Sandström, 1992). Absorption of dietary zinc ranges from 15 to 60%. Mechanisms for the transport of zinc across the intestinal wall, its export into plasma and its uptake into other tissues are uncertain. Once in plasma, zinc is carried by a number of proteins that include albumin, transferrin and caeruloplasmin. Most of the absorbed zinc is excreted in the bile and eventually lost in the faeces. There appears to be no specific zinc “store” in the body.

Tissue content and activity of zinc-dependent processes are maintained over a wide range of dietary zinc intakes. When zinc intake is increased, the fractional absorption decreases and intestinal excretion increases while urinary losses remain fairly constant. Endogenous faecal zinc losses may increase several fold to maintain zinc homeostasis with high intakes (Coppen and Davies, 1987). At very low zinc intakes, absorption can increase to between 59-84% and faecal and urinary losses decrease accordingly (Baer and King, 1984; Johnson *et al*, 1993; Wada *et al*, 1985). When these primary homeostatic mechanisms are not sufficient to handle large dietary excesses of zinc, the excess zinc is lost via the hair (Jackson, 1989). The kinetics of zinc absorption and elimination follow a two-component model. The initial rapid phase has a half-life in humans of 12.5 days and the slower pool turns over with a half-life of approximately 300 days (Hambridge *et al*, 1986).

2.3. Bioavailability

Interactions with a number of dietary factors influence zinc uptake. Ligands, such as phytate, form insoluble complexes with zinc and prevent absorption. Calcium increases binding of zinc by phytate (Oberleas *et al*, 1966). Larger doses of calcium can decrease net zinc absorption (Spencer *et al*, 1984; Wood and Zheng, 1995). High iron content in the diet decreases zinc absorption. Earlier reports indicated that folic acid can also inhibit zinc retention and metabolism (Milne, 1989; Milne *et al*, 1984; Milne *et al*, 1990), but more recent evidence indicates that folic acid does not adversely affect zinc status (Kauwell *et al*, 1995). Copper and zinc compete for absorption but it appears unlikely that modestly increased intakes of copper interfere with zinc absorption. Histidine, methionine and cysteine are thought to facilitate zinc absorption (these amino acids remove zinc from the zinc-calcium-phytate complexes) (Mills, 1985).

2.4. Dietary and other sources

Good food sources of zinc include red meat, whole wheat, raisins, unrefined cereals (high content, low bioavailability) and fortified cereals. Milk, fruit and vegetables are low in zinc (Sandstead and Smith Jr, 1996).

Concentrations of zinc in tap water may be elevated as a result of dissolution of pipes and contaminated wells may lead to high exposure. Drinking water quality standards for European countries provide a zinc content not more than 5 mg/L (Anon, 1971). Exceeding this value may result in an astringent effect, opalescent appearance and a fine granular sediment. The World Health Organisation recommends that concentrations should not exceed 3 mg zinc/L (WHO, 1993).

Other sources of zinc, excluding dietary intakes, include inhalation of zinc metal or oxide fumes in industrial settings and storage of food and drink in galvanised containers.

2.5. Recommended Dietary Allowances

The European Population Reference Intake (PRI) for zinc (SCF, 1993) for adult males and females is 9.5 mg/day and 7.0 mg/day, respectively. In the UK, the Reference Nutrient Intake (RNI) for zinc is the same as the European PRI and was established in 1991 (Department of Health, 1991). Estimated Average Requirements (EAR) are 7.3 mg/day and 5.5 mg/day for males and females, respectively. In the US, new guidelines recommend daily intakes of 11 mg/day and 8 mg/day for men and women respectively (Institute of Medicine, Food and Nutrition Board, 2001).

2.6. Typical intakes

Mean intakes in Europe (excluding supplements) are 13 mg/day for males and 9 mg/day for females (Van Dokkum, 1995). The estimated mean dietary zinc intakes in several EU countries are given in Table 1. Zinc intakes from vegetarian diets have been shown to be similar to non-vegetarian diets (Hunt *et al*, 1998). However, the dietary requirement for zinc may be as much as 50% greater for vegetarians.

Table 1. Mean and 97.5 percentile zinc intake (mg/day) from food and supplements

Country	Population	n	Method	Supplements*	Mean	97.5%
Austria[a]	Individual	2488	24h recall	Not defined	11.2	21.9
Germany[b]	Individual (M) Individual (F)	854 1134	7-day dietary record	- -	12.1 9.7	20.5 16.0
UK[c]	Individual (M) Individual (F)	1087 1110	7-day weighed inventory	+ +	11.4 (10.9) 8.4 (8.2)	19.0 13.6
Italy[d]	Household	2734	7-day record	+	11	19.0
Netherlands[e]	Individual (M,F)	5958	2-day record	-	9.4	17.0
Ireland[f]	Individual (M) Individual (F)	662 717	7-day estimated food record	+ +	10.8 7.5	23.5 22.1

* + data included supplements; - data excluded supplements.
[a] Elmadfa *et al* (1998)

[b] Heseker *et al* (1994) - median values.
[c] Gregory (1990) - values are the mean with the median in parentheses
[d] Turrini (1996).
[e] Hulshof and Kruizinga (1999).
[f] IUNA (2001).

2.7. Zinc deficiency

Clinically defined zinc deficiency in humans is rare. Zinc deficiency, however, has been observed in patients on total parenteral nutrition, patients taking the chelating agent penicillamine and in acrodermatitis enteropathica, a genetic disease resulting in zinc deficiency. The main clinical manifestations of zinc deficiency are growth retardation, delay in sexual maturation, diarrhoea, increased susceptibility to infections, dermatitis, the appearance of behavioural change and alopecia. Symptoms of mild/marginal zinc deficiency include delayed wound healing, impaired resistance to infection and reduced growth rate (Walsh *et al*, 1994; WHO, 1996).

3. HAZARD IDENTIFICATION

3.1. Toxic effects in farm animals

For review see Lantzsch and Schenkel (1978). Normal zinc concentrations in major feeds and foods ranges from 20 to 80 mg/kg on a dry matter basis (Lantzsch and Schenkel, 1978). Growth rate is affected by zinc intakes, approximating 3.6 g/kg in feed on a dry matter basis, in fillies (Willoughby *et al*, 1972). In drinking water, intakes of zinc at a concentration of 8 mg/L were reported to be toxic for dairy cows (Pickup *et al*, 1954) whilst in a trial feeding dairy cows zinc in food up to 1.279 g/kg feed no toxic effects were observed (Archibald, 1944). In sheep, toxicosis has been observed at intakes of 1 g/kg feed where zinc was in the form of zinc oxide. First cases of death occurred at 3 g/kg zinc in feed (Ott *et al*, 1966). Pigs are also affected by zinc toxicity. Symptoms of non-specific arthritis, including stiffness and lameness were reported in pigs at intakes of zinc above 2 g/kg dry matter. Death occurred frequently within three weeks of treatment (Brink *et al*, 1959; Grimmett and McIntosh, 1936).

3.2. Toxic effects and mechanisms in laboratory animals and *in vitro* studies

In rats, dietary zinc intakes up to 1 g/kg body weight have been well tolerated (Kulwich *et al*, 1953; Sutton and Nelson, 1937; Whanger and Weswig, 1971), but dietary zinc intakes above 2 g/kg body weight have usually led to death (Sadasivan, 1951; Smith and Larson, 1946). Studies in laboratory animals (Sandstead, 1982; 1995) have demonstrated that elevated levels of dietary zinc can have a negative effect upon copper balance and, in part, could explain the induction of a microcytic, hypochromic anaemia in rats after ingestion of large amounts of zinc (for review, see Lantzsch and Schenkel, 1978). The mechanism whereby high zinc intakes antagonises copper status has been clarified (Cousins, 1985). High zinc intakes increase the synthesis of metallothionein in intestinal mucosal cells. Metallothionein avidly binds copper and when mucosal cells are rich in this protein, little copper is able to traverse the cells into the body. Studies in rats have shown that high levels of zinc supplementation (0.5-2 g/kg body weight) can affect iron storage and encourage depletion, interfere with iron uptake in the liver and cause anaemia as a result of higher iron turnover (Walsh *et al*, 1994). Conversely, zinc (10^{-4}M) has been shown to alleviate the toxic effects of cadmium in mice and rabbits (Chiba and KiKuchi, 1984).

Zinc is not teratogenic except when high doses (20 mg/kg body weight) are injected intraperitoneally to mice during pregnancy (Chang *et al*, 1977). Similarly, zinc does not exhibit reproductive toxicity in rats until very high concentrations of 1 g/kg body weight given during pregnancy and which caused a significant reduction in foetal growth, birth weight and still births (Cox *et al*, 1969; Heller and Burke, 1927; Schlicker and Cox, 1968). A total failure of reproduction occurred in rats on zinc intakes of 2 g/kg body weight (Sutton and Nelson, 1937). A published report (MAFF, 1998) reviews zinc toxicity in experimental animals.

3.2.1. Genotoxicity

3.2.1.1. In vitro

Zinc was negative in the majority of tests for induction of gene mutations in bacterial or mammalian cells. In particular, zinc sulphate and zinc acetate were not mutagenic in *Salmonella typhimurium* (Marzin and Vo, 1985; Gocke *et al*, 1981; Thompson *et al*, 1989); zinc 2,4-pentanedione was mutagenic in

Salmonella typhimurium, with and without S9 (Thompson *et al*, 1989). Zinc chloride was not mutagenic in the mouse lymphoma *tK* assay (Amacher and Paillet, 1980); zinc acetate was found positive, with and without S9, both in the mouse lymphoma *tK* assay and in the chromosome aberration assay in Chinese hamster ovary (CHO) cells (Thompson *et al*, 1989). Zinc chloride induced chromosomal aberrations in human lymphocytes (Deknudt and Deminatti, 1978). Zinc acetate and zinc 2,4-pentanedione were negative in the UDS assay in rat hepatocytes. Zinc chloride did not induce cell transformation in Syrian hamster (SHE) cells (Di Paolo and Casto, 1979).

3.2.1.2. In vivo

Zinc sulphate did not induce sex-linked recessive lethal mutations in *Drosophila* (Gocke *et al*, 1981), while zinc chloride induced dominant lethal mutations and sex-linked recessive lethal mutations (Carpenter and Ray, 1969). Zinc sulphate did not induce micronuclei in the mouse (Gocke *et al*, 1981); conflicting results, negative or positive at high doses, were reported for the induction of chromosomal aberrations in the mouse bone marrow by zinc chloride (Deknudt and Gerber, 1979; Vilkina *et al*, 1978; Gupta *et al*, 1991). Zinc chloride did not induce dominant lethal mutations in mice (Vilkina *et al*, 1978).

The weight of evidence from the *in vitro* and *in vivo* genotoxicity tests supports the conclusion that zinc, notwithstanding some positive findings at chromosome levels at elevated doses, has no biologically relevant genotoxicity activity (reviewed by Walsh *et al*, 1994; WHO, 2001).

3.2.2. Carcinogenicity

No adequate experimental studies are available to evaluate the carcinogenic potential of zinc (WHO, 2001).

3.3. Toxic effects in humans

Zinc is not stored in the body and excess intakes result in reduced absorption and increased excretion. Nevertheless, there are documented cases of acute and chronic zinc poisoning.

3.3.1. Acute toxicity

Acute toxicity is infrequent in humans. Brown *et al* (1964) described several cases of food poisoning resulting from storage of food or drink in galvanised containers. Symptoms of acute zinc toxicity include nausea, vomiting, epigastric pain, abdominal cramps and diarrhoea. One study reported symptoms of lethargy and light-headedness (Murphy, 1970). This change in presenting symptoms could be a result of the type of zinc (in this case zinc sulphate) ingested (Bennett *et al,* 1997). Zinc acetate (25-50 mg, three times per day), given to Wilson's disease patients to prevent copper accumulation was reported (Henderson *et al*, 1995) to cause less dyspepsia than equivalent doses of zinc sulphate. Fosmire (1990) estimated that an emetic dose of zinc corresponds to 225-450 mg. An industrial hazard associated with inhalation of zinc oxide fumes is "metal fume fever". Subjects present with malaise, fever, headache, nausea and dryness of mouth and throat.

3.3.2. Chronic and sub-chronic toxicity

Studies of chronic and sub-chronic toxicity of zinc are well documented. Prolonged intakes of zinc supplements ranging from 50 mg/day up to 300 mg/day have been associated with a range of biochemical and physiological changes. These changes include hypocupraemia, leucopaenia, neutropaenia, sideroblastic anaemia, decreased concentrations of plasma copper and decreased activity of the copper containing enzymes, superoxide dismutase and caeruloplasmin, altered lipoprotein metabolism and impaired immune function (Sandstead, 1995). Many of these biochemical and physiological changes are similar to those observed during copper deficiency. Nevertheless, there are problems with hazard identification in that these changes are not specific to copper deficiency and the clinical relevance of some are unknown. Sensitive sub-populations may include subjects with haemochromatosis and/or insulin dependent diabetes. Zinc excess in water may decrease iron absorption (Rossander-Hulten *et al*, 1991). Hepatic zinc concentration is increased in haemochromatosis (Adams *et al*, 1991) and there is some evidence that zinc absorption may be increased (Adams *et al*, 1991; Spencer *et al*, 1988).

3.3.3. Adverse effects

3.3.3.1. Changes in copper balance

Doses of 75 mg/d of zinc have been used for some time as effective treatment for Wilson's disease. Negative copper balance can be induced in these patients with doses as low as 75 mg/day, provided that the dose is given as 3 x 25 mg of zinc (Brewer *et al*, 1993). Such doses, and those of 100 mg/day

zinc, have been shown to lack toxicity in these patients and have been used effectively to control body copper levels in individuals for 11 years or longer (Najda *et al*, 2001). Increased copper excretion and decreased copper retention (Festa *et al*, 1985; Burke *et al*, 1981) have been demonstrated also during zinc supplementation of healthy subjects. Burke *et al* (1981) observed significantly higher faecal copper excretion and significantly decreased apparent copper retention when 23.3 mg/day compared with 7.8 mg/day of zinc (fed as fortified food; copper intake, 2.33 mg/day) were fed to 5 men and 6 women, aged 56-83 years for a period of 30 days. Supplementation of diet with 18.2 mg/day zinc for 2 weeks (Festa *et al*, 1985) in young male subjects demonstrated increased faecal copper excretion and decreased apparent retention of copper. No significant change in copper balance was found when 14 adolescent girls were fed 13.4 mg/day compared with 7.4 mg/day zinc (Greger *et al*, 1978). Other studies examining alterations in copper excretion after zinc supplementation found no effect of increasing zinc intakes from 8 mg/day up to 24 mg/day for 18 days (Taper *et al*, 1980) or zinc intakes of 19.9 mg/day for 24 days (Colin *et al*, 1983). Taken together, these studies suggest that an intake of zinc of some 9 mg/day or more over recommended dietary allowances can affect balance at least in the short term. Balance studies, however, may not be indicative of homeostasis in the longer term and it may take three weeks or more before copper absorption and retention are stabilised after changes in copper intake (Turnlund *et al*, 1989). More recently, Milne *et al* (2001) found that 21 postmenopausal women fed 53 mg/day of zinc with adequate copper (3 mg/day) for 90 days maintained positive copper balance, whereas a low zinc (3 mg/day) regimen for 90 days produced a negative copper balance.

3.3.3.2. Decreased activity of copper-dependent enzymes

One of the most consistent findings of zinc supplementation studies is the decrease in erythrocyte copper-zinc superoxide dismutase (SOD) activity. Fischer et al (1984) found decreased erythrocyte SOD activity after 6 weeks of supplementation with 50 mg (2 x 25 mg)/day zinc as gluconate in 26 healthy adult men. Samman and Roberts (1988) studied the effects of 150 mg/day zinc as sulphate on healthy young women (n = 26) and men (n = 21) in a double-blind crossover trial lasting 12 weeks. Significant decreases in erythrocyte SOD activity were observed in the females only. Yadrick *et al* (1989) found significant decreases in erythrocyte SOD activity when 18 healthy adult women received 50 mg/day zinc as gluconate for 10 weeks.

Decreases in other putative indices of copper status have been observed in some studies. For example, Prasad *et al* (1993) found decreased serum copper in 44 older adults (who were zinc deficient, with high inflammatory status) given 20 mg/day zinc in an eight week crossover study. Fischer *et al* (1984) and Yadrick *et al* (1989) found no such changes. Similarly, Black *et al* (1988) who gave young men 50 mg/day or 75 mg/day for 12 weeks, found no change in serum copper concentration. Samman and Roberts (1988) found decreased activity of serum caeruloplasmin but no change in serum copper at 150 mg/day for 12 weeks. Bonham *et al* (2002b) found that zinc supplementation for 14 weeks at 40 mg/day zinc (diet and supplement) did not affect erythrocyte SOD activity and caeruloplasmin concentration or activity in 19 healthy men, whose intake was estimated at 1.2 mg/day copper, compared with placebo controls (n=19). Given the differences in the responses of these putative indices of copper status to the same or similar zinc supplementation regimens, it is possible that the observed decreased erythrocyte SOD activity is not directly related to decreased copper status.

The recent findings of Milne *et al* (2001) question the adverse physiological significance of the decreased erythrocyte SOD activity observed in a number of zinc supplementation studies.

These workers found that intake of zinc at 53 mg/day did not induce changes indicative of decreased copper status or function in 21 post-menopausal women fed low dietary copper (1 mg/day) for 90 days in a metabolic unit. Results suggested that inadequate intake of 3 mg/day zinc was a nutritional stress of copper metabolism and status. These women were in positive copper balance only when the diet provided 53 mg/day zinc and 3 mg/day copper (see section 3.2.3.1). Irrespective of dietary copper intake, high dietary (53 mg/day) zinc compared with low dietary (3 mg/day) zinc, decreased erythrocyte SOD activity but the largest fall in erythrocyte SOD activity was when the women were fed low dietary zinc and copper. Another putative index of copper status, platelet cytochrome c oxidase activity on a platelet number basis, was significantly lower during low dietary than during high dietary zinc intake. Findings with respect to another copper protein, caeruloplasmin, indicate that a moderately deficient (3 mg/day) intake of zinc is more detrimental to copper metabolism and function than a moderately high (53 mg/day) intake. When the women were fed high dietary zinc whole blood glutathione concentration and erythrocyte glutathione peroxidase activity were reduced in comparison with low dietary zinc.

In conclusion, decreased erythrocyte SOD activity, although the most consistent biochemical finding from studies measuring the influence of zinc on putative indices of copper status (Fischer *et al*, 1984;

Samman and Roberts, 1988; Yadrick *et al*, 1989; Milne *et al*, 2001), is not accompanied by adverse effects and is not considered to be a marker of decreased copper status. The physiological relevance of lowered erythrocyte SOD activity in these studies, therefore, is unclear.

3.3.3.3. Lipoprotein and cholesterol metabolism

Two studies have found that zinc supplementation at doses of 50 mg/day and 75 mg/day for 12 weeks (Black et al, 1988) and 160 mg/day for 6 weeks (Hooper *et al*, 1980) decreased high density lipoprotein (HDL) concentrations in male subjects. In contrast, Samman and Roberts (1988) observed no decrease in HDL concentrations in males with zinc supplementation doses of 150 mg/day for 12 weeks, but rather found some indication of decreased low density lipoprotein (LDL) in females. Similarly, Freeland-Graves *et al* (1982) observed no consistent change in HDL cholesterol in women after eight weeks at 100 mg/day zinc. Two more recent studies have also found no adverse changes in lipoprotein metabolism. Bonham *et al* (2002a) found no change in HDL, LDL or triglycerides in healthy men after 14 weeks of zinc intakes at 40 mg/day whereas Milne *et al* (2001) found that total and LDL cholesterol concentration decreased with 53 mg zinc supplementation for 90 days. Lower doses (20 mg/day zinc) in elderly subjects have shown no effect on lipoprotein metabolism (Boukaïba *et al*, 1993). Collectively, these data indicate no consistent adverse effects of zinc supplementation giving total intakes in the range 40-160 mg/day zinc on lipoprotein and cholesterol metabolism.

3.3.3.4. Changes in haemoglobin and blood profile

Very high intakes of zinc over long periods have resulted in anaemia and changes in red and white blood cells indicative of copper deficiency. Patients with sickle cell anaemia (Prasad *et al*, 1978) and coeliac disease (Porter *et al*, 1977) treated with 150 mg/day zinc for 23 months and 10 months respectively developed clinical signs of copper deficiency characterized by hypocupraemia, anaemia, neutropaenia and leucopaenia. These complications could be corrected by copper supplementation or cessation of zinc supplementation. Zinc supplementation at 50 mg/day zinc for 10 weeks decreased haematocrit but had no effect on haemoglobin (Yadrick *et al*, 1989) whereas doses of 150 mg/day for 12 weeks had no affect on haematocrit (Samman and Roberts, 1988). Inclusion of iron (50 mg/day) supplements ameliorated the effects on iron status in the former study. Zinc intake at 40 mg/day had no effect on full blood profile data and flow cytometric analyses of lymphocyte subsets (Bonham *et al*, 2002b). No consistent adverse effects on blood profiles, therefore, have been observed at intakes of zinc below 60 mg/day.

3.3.3.5. Reproductive effects

Dietary supplementation with zinc at 20 mg/day did not result in adverse effects of pregnancy progress or outcome in healthy pregnant women in a number of large, controlled trials (Hunt *et al*, 1984; Kynast and Saling, 1986; Mahomed *et al*, 1989). Similarly, supplementation with zinc at 30 mg/day did not result in any adverse outcomes in a double blind trial involving low income pregnant adolescents (n=268 at delivery) thought to have low zinc status (Cherry *et al*, 1989). In a smaller study, Jameson (1976) gave zinc supplements of 90 mg/day to seven pregnant women with low serum zinc concentrations and found no adverse effects. Moreover, in a follow-up study by the same author (Jameson, 1982), 133 women with low serum zinc concentrations were randomly assigned to either zinc supplementation at 45 mg/day or no supplementation and no adverse effects were reported. These data indicate that zinc supplementation at doses of 20-90 mg/day produce no adverse effects on pregnancy outcome.

3.3.3.6. Other adverse effects

Excessive intake of zinc (300 mg/day) for six weeks can impair immune responses, ie. reduction in lymphocyte stimulation response to phytohaemaglutinin as well as chemotaxis and phagocytosis of bacteria by polynuclear leucocytes (Chandra, 1984).

Although individuals with insulin-dependent diabetes mellitus have chronic hyperzincuria, they do not appear to be zinc deficient (Cunningham *et al*, 1994). Supplementing such individuals (n=7) with 50 mg/day zinc was reported to cause an elevation of HbA_{1C} in each of the seven subjects (Cunningham *et al*, 1994) but the clinical significance of this observation is unclear, given the short duration of the study and the absence of any change in blood glucose concentrations.

Zinc supplementation at 53 mg/day for 90 days can increase bone-specific alkaline phosphatase (a possible indicator of bone formation) in 25 post-menopausal women (Davis *et al*, 2000).

4. DOSE-RESPONSE ASSESSMENT

The available data clearly show that zinc can cause adverse effects in humans and in domestic and laboratory animals. In humans, the most prominent effects of acute zinc toxicity are gastrointestinal disturbances.

Chronic zinc toxicity, undoubtedly, is associated with symptoms of copper deficiency. These overt adverse effects (e.g. anaemia, neutropaenia, impaired immune responses) are evident only after feeding zinc in the form of dietary supplements in excess of 150 mg/day for long periods. It is much more difficult to identify the critical effect of zinc excess at intakes below 100-150 mg/day. Short-term balance studies would indicate adverse effects on copper retention at intakes as low as 18.2 mg/day zinc (Festa *et al*, 1985). Recent longer-term balance studies, however, indicate that positive copper balance can be maintained at 53 mg/day zinc in post-menopausal women for 90 days provided copper intakes are adequate to high (3 mg/day). High dietary zinc, however, did not exacerbate the non-positive copper balance in the women fed low (1 mg/day) dietary copper nor did the higher (3 mg/day) copper diet induce positive copper balance in the women fed low (3 mg/day) dietary zinc (Milne *et al*, 2001). The occurrence of adverse (lower HDL, higher LDL cholesterol) effects on lipoprotein metabolism is inconsistent at zinc intakes below 100 mg/day.

In conclusion, clear adverse effects on copper balance and an array of measures of copper status or lipoprotein metabolism cannot be detected at 53 mg/day zinc, when copper intakes are adequate at 3 mg/day (Davis *et al*, 2000; Milne *et al*, 2001), nor on copper status, lipoprotein metabolism, blood profile and circulating levels of peripheral blood leucocytes and lymphocyte subsets at 40 mg/day zinc (Bonham *et al*, 2002a). Collectively, these data indicate that a NOAEL for zinc is around 50 mg/day.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

5.1. Adults

A NOAEL of 50 mg/day is based on the absence of any adverse effects on a wide range of relevant indicators of copper status (as the critical endpoint) in the studies of Davis *et al* (2000), Milne *et al* (2001), Bonham *et al* (2002a, 2002b). Subjects were 25 and 21 healthy post-menopausal women in the study of Davis *et al* (2000) and Milne *et al* (2001) and 19 healthy young men in the studies of Bonham *et al* (2002a, 2002b). Duration of supplementation was for 90 days in the studies of Davis *et al* (2000) and Milne *et al* (2001) and for 14 weeks in the studies of Bonham *et al* (2002a, 2002b). Total zinc and copper intakes were tightly controlled in the metabolic studies of Davis *et al* (2000) and Milne *et al* (2001) in which the zinc intake was 53 mg/day. Total zinc intake in the studies of Bonham *et al* (2002a, 2002b) was 30 mg/day from supplements on top of 10 mg calculated from dietary intake estimates (total 40 mg/day). An UF of 2 is applied owing to the small number of subjects included in relatively short-term studies but acknowledging the rigidly controlled metabolic experimental conditions employed. An UL of 25 mg/day is recommended.

5.2. Pregnancy and lactation

Available data indicate that pregnant women do not have increased susceptibility to zinc supplementation. Therefore the UL of 25 mg zinc per day applies also to pregnant and lactating women.

5.3. Children and adolescents

There are no data on adverse effects of zinc intakes on children and adolescents. On the other hand, there are no data to indicate that children or adolescents are more susceptible to adverse effects of zinc. Therefore, in the absence of adequate data the Committee chooses to extrapolate the UL from adults to children on a surface area (body weight$^{0.75}$) basis. The reference weights derived by the Scientific Committee on Food (SCF, 1993) are used as a basis for the calculations of surface area and UL.

Age (years)	Tolerable Upper Intake Level (UL) for Zinc (mg per day)
1-3	7
4-6	10
7-10	13
11-14	18
15-17	22

6. RISK CHARACTERISATION

The available studies show that the mean zinc intakes of adults and children in EU countries are below the UL. The 97.5 percentile of total zinc intakes for all age groups are close to the ULs, which, in the view of the Committee, are not a matter of concern.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF COPPER

(EXPRESSED ON 5 MARCH 2003)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Copper is a transition metal with an atomic mass of 63.54. Three oxidation states of copper exist; cuprous (Cu^{+}), cupric (Cu^{2+}) and Cu^{0} (Uauy *et al,* 1998). In biological systems, copper primarily exists as Cu^{2+} with minute quantities of Cu^{+} being found in solution.

2. NUTRITIONAL BACKGROUND

2.1. Function

It is well established that the trace element copper is essential for life. Copper in living organisms, including humans, forms an essential component of many enzymes (cuproenzymes) and proteins. The biochemical role for copper is primarily catalytic, with many copper metalloenzymes acting as oxidases to achieve the reduction of molecular oxygen, for example cytochrome-C-oxidase and superoxide dismutase. Studies have also shown that copper is required for infant growth, host defence mechanisms, bone strength, red and white cell maturation, iron transport, cholesterol and glucose metabolism (Uauy *et al,* 1998). Copper plays additional roles that are less well understood and may be in part non-enzymatic, such as in angiogenesis, nerve myelination and endorphin action (Linder and Hazegh-Azam, 1996).

2.2. Homeostasis

As an essential trace element, copper is third in abundance in the human body after iron and zinc and it is estimated that the adult human body contains between 50-150 mg (Turnlund, 1994). At physiological pH, there is little or no free copper in solution and “free” copper content is currently not measureable. The current best estimate of free Cu^{2+} content of human plasma is approximately $2x10^{-16}$ M (Linder, 2001). Total copper concentrations in most tissues are approximately $5x10^{-5}$ M total copper (Prohaska, 1990). Copper absorption occurs primarily in the small intestine with a small amount absorbed in the stomach. Absorption is probably by a saturable, active transport mechanism at lower levels of dietary copper; at high levels of dietary copper, passive diffusion plays a role (Turnlund, 1994). The majority of copper is transported to the liver where it is incorporated into newly synthesised caeruloplasmin, metallothionein or cuproproteins. The major excretory route of copper is in the bile.

The percentage absorption of dietary copper is strongly influenced by the amount of copper ingested (with the percentage absorption decreasing with increasing intakes (Turnlund *et al*, 1989; Turnlund, 1988). Turnlund *et al* (1989) used stable-isotope methodology to study copper absorption in adults. Diets were labelled extrinsically with ^{65}Cu and copper absorption was dependent on the amount of copper in the diet. On a low copper (0.78 mg/day) diet, copper absorption was 55.6%, whereas it was 36.3% from the same diet with copper added to give total dietary intake of 1.68 mg/day and only 12.4% when copper was added to produce dietary intakes of 7.53 mg/day. A theoretical maximum absorptive capacity of 63-67% has been estimated from aggregate results of human copper absorption studies at various copper daily intakes (Wapnir, 1998). With typical diets in developed societies, however, the average copper absorption is in the 30-40% range (Wapnir, 1998).

Copper turnover is low when copper intake is low and high when intake is high. The regulation of excretion appears to be more important than the regulation of absorption in determining copper reabsorption following biliary excretion and faecal copper losses reflect dietary copper intakes. When dietary intake changes, balance over a broad range of copper intakes (0.8-5.5 mg/day) can be achieved (Turnlund, 1998).

A series of studies demonstrated that a 10-fold increase in dietary copper resulted in only twice as much copper being absorbed (Turnlund, 1991). Although subjects were in positive copper balance for the first six days, when intakes were increased from 0.8 to 7.5 mg/day, average retention decreased linearly during the next 18 days until it was negative during the last six days of the trial (Turnlund *et al*, 1989). The authors indicated that the negative balance must represent endogenous excretion of excess copper retained during the first six days of the trial. Indices of copper status, as a result of the body's regulation of copper, are resistant to change except under extreme dietary conditions. Turnlund *et al* (1990) showed that when dietary intakes increased from 0.8 mg/day to 7.5 mg/day (for 24 days), putative indices of status, including plasma copper, erythrocyte superoxide dismutase (SOD), caeruloplasmin and urinary copper excretion were not significantly different. When dietary intake is very high, regulation is challenged. At first, balance is positive but later (approximately three weeks) it becomes negative as the excess copper retained early after a change in diet is eliminated (Turnlund *et al*, 1989). Therefore, any intervention studies should be at least of this duration. It has been estimated that copper excretion by infants, might be as low as 50 µg Cu/kg body weight (Aggett, 1999).

2.3. Bioavailability

Dietary interactions of copper with sucrose or fructose (Schofield *et al,* 1990; O'Dell, 1993), animal proteins, S-amino acids (Brown and Strain, 1990; Strain and Lynch, 1990; Fields *et al*, 1993), histidine (Harvey *et al*, 1981), and ferrous iron (Yu *et al*, 1994; Wapnir *et al*, 1993) may inhibit copper absorption to varying degrees in animal models. Ascorbic acid supplements (1500 mg/day) (Finley and Cerklewski, 1983), molybdenum (Underwood, 1977) and other dietary factors, specifically high intakes of calcium and/or phosphorus (Snedecker *et al*, 1982) and cadmium (Underwood, 1977), may also inhibit copper absorption in diets containing high amounts of these factors. The interaction between zinc and copper is well documented in humans (Fischer *et al*, 1984). High levels of dietary zinc have been shown to adversely influence copper absorption and bioavailability (Turnlund, 1988). A decrease in serum/plasma copper (Festa *et al*, 1985) and reduction in concentration of the copper containing enzyme Cu,Zn SOD (Yadrick *et al*, 1989) can be induced by high intakes of dietary zinc.

2.4. Dietary and other sources

The highest contents of copper in foods are in organ meats, seafood, nuts and seeds (Pennington *et al,* 1995; Strain, 1994a). Other good sources of copper are whole bran cereals and whole grain products.

Copper piping used for water distribution can add 0.1mg/day to intakes in hard water areas but 10x this amount in acid and soft water conditions (Ralph and Arthur, 2000). The current EU standard is 2 mg/L for the maximum concentration of copper in drinking water (EU Directive 98/83).

Other sources of copper, excluding dietary intakes, include emissions from mines, smelters and foundries. Environmental copper can also arise from the burning of coal for power generation from municipal waste incinerators.

2.5. Recommended Dietary Allowances

An EU population reference intake (PRI) of 1.1 mg/day for adults was established in 1992 (SCF, 1993). In the UK, a reference nutrient intake (RNI) of 1.2 mg copper/day has been set for adults (Department of Health, 1991). Insufficient data exist, however, to set lower RNI (LRNI) and estimated average requirements (EAR) for different age groups and sexes. In the United States, new guidelines for recommended intakes have been recently published (FNB, 2001). It has been recommended that adult males and females should consume a dietary intake of 0.9 mg copper/day. Previous to this, an estimated safe and adequate dietary intake was proposed of 1.5-3 mg/day.

2.6. Typical intakes

Mean dietary copper intakes from food of adults in different European countries have been estimated with a range of 1.0-2.3 mg/day for males and 0.9-1.8 mg/day for females (Van Dokkum, 1995). The estimated dietary copper intakes in several European countries are given in Table 1. Gibson (1994) compiled several studies and found that copper intakes in adults were approximately 1-1.5 mg/day from omnivore diets. Vegetarian diets provided greater dietary intakes of copper, approximately 2.1-3.9 mg/day.

The main sources of copper in diets in Great Britain were cereals and cereal products, vegetables and potatoes. Copper is not usually used to fortify foods and copper from this source makes a negligible contribution to total copper intakes. Similarly, a very small proportion of consumers take dietary supplements containing copper, but for those few who did, median intakes from supplements were 0.1-0.5 mg/day (Church, personal communication).

2.7. Copper deficiency

Although clinically defined copper deficiency in humans is rare, it has been observed under a variety of different clinical conditions including in patients on long term total parenteral nutrition (TPN) (Dunlap *et al,* 1974), premature infants, neonates, and previously malnourished children (Paterson and Burns, 1988; Manser *et al*, 1980). Symptoms of severe copper deficiency are similar to those seen in experimental animals and include anaemia, neutropaenia (Williams, 1983) and bone abnormalities (Danks, 1988), while less frequent signs are hypopigmentation (Danks, 1988), impaired growth (Castillo-Duran and Uauy, 1988), increased incidence of infections (Castillo-Duran *et al*, 1983), alterations of phagocytic capacity of neutrophils (Heresi *et al*, 1985) and abnormalities of glucose (Klevay *et al*, 1986) and cholesterol metabolism (Reiser *et al*, 1987).

Table 1. Daily intakes of copper from food in EU countries (mg/day)

Country	Type of survey	n	Method	Supplements*	Mean	97.5%
Austria[a]	Individual	2488	24h recall	Not defined	2.0	4.2
Germany[b]	Individual (M) Individual (F)	854 1134	7-day dietary record	- -	2.2 1.8	4.0 3.3
UK[c]	Individual (M) Individual (F) Individual (M) Individual (F)	1087 1110 1087 1110	7-day weighed inventory	- - + +	1.6 (1.5) 1.2 (1.1) 1.6 (1.5) 1.2 (1.1)	3.5 2.8 3.5 2.8
Italy[d]	Household	2734	7-day record	+	1.4	2.8
Netherlands[e]	Individual (M, F)	5958	2-day record	-	1.1	1.2
Ireland[f]	Individual (M) Individual (F)	662 717	7-day estimated food record	+ +	1.5 1.2	3.1 2.7

* + data included supplements; - data excluded supplements.
[a] Elmadfa *et al (1998).*
[b] Heseker *et al (1994) (VERA Study) - median values.*
[c] Gregory *et al (1990) - values are the mean with the median in parentheses.*
[d] Turrini (1996).
[e] Hulshof and Kruizinga (1999).
[f] IUNA (2001).

3. HAZARD IDENTIFICATION

3.1. Toxic effects in laboratory animals

Tolerance to high intakes of copper varies greatly from one species to another (Underwood, 1971; Osterberg, 1980). Sheep are most sensitive to copper poisoning and cases of chronic copper poisoning have been reported in lambs fed diets containing 170 mg Cu/kg dry weight (Süveges *et al*, 1971). Rats, however, have a higher tolerance to copper excess (Underwood, 1971; Osterberg, 1980). Because of species differences and the effects of zinc, iron and molybdenum in the diet, the minimum toxic copper level varies. Most rat strains are relatively tolerant of copper, but at intakes exceeding 100 mg/kg body weight, growth is impaired and extensive necrosis of hepatocytes develops (Haywood, 1980). The susceptibility to copper excess is also influenced by the chemical form. In rats, cupric chloride and cupric carbonate are more toxic than cupric nitrate, cupric acetate and cuprous oxide (JECFA, 1982). Manifestations of copper toxicity include weakness, tremors, anorexia and jaundice. As tissue copper levels increase, haemolytic crisis may ensue producing liver, kidney and brain damage.

3.2. Mechanisms of toxicity

Mechanisms of toxicity have been reviewed by Britton (1996). Evidence to date suggests that the hepatic mitochondrion is an important target in hepatic copper toxicity and that oxidant damage to the liver may be involved in the pathogenesis of copper-induced injury. In humans, chronic copper toxicity has its most pronounced effects on liver function whilst acute effects of copper toxicity are primarily observed in the gastrointestinal tract, as a local intestinal irritation effect.

3.2.1. Genotoxicity

3.2.1.1. In vitro

As reported by WHO (1998) the genotoxicity of copper compounds has not been extensively studied. Studies with copper (II) sulphate indicated that it was not mutagenic in strains TA98, TA100 and TA102 of *Salmonella typhimurium* with and without exogenous metabolic activation (Moriya *et al*, 1983; Marzin and Phi, 1985). Furthermore copper (II) sulphate was found to be negative in the SOS Chromotest in *Escherichia coli* PQ37 (Olivier and Marzin, 1987) and in the rec-assay with *Bacillus subtilis* H17 and M45 (Matsui, 1980), both in the absence of a metabolic activation system. Conversely, copper (II) sulphate did induce a significant increase of unscheduled DNA synthesis (UDS) in cultured rat hepatocytes in a dose range between 7.9 and 78.5 µmol/L (Denizau and Marion, 1989). Copper (II) chloride showed no evidence of mutagenic activity in *S. typhimurium* strains TA98, TA102, TA1535, and TA1537 with and without metabolic activation (Wong, 1988) and was negative in the rec-assay *with B. subtilis* H17 and M45 (Nishioka, 1975; Kanematsu *et al*, 1980). Copper (II) 8-hydroxyquinoline was weakly mutagenic in strain TA100 *of S. typhimurium* only after metabolic activation, and negative in four other strains of Salmonella and in *E. coli* WP2 hcr (Moriya *et al*, 1983). Negative results were previously reported in strains TA98, TA100, TA1535 and TA1537, with and without metabolic activation, but the maximum concentration tested was very low (Räsänen *et al*, 1977). Copper (II) nitrate produced dose-related increases in the mutation frequency (resistance to 8-azaguanine) and in the frequency of sister chromatid exchanges in cultured V79 Chinese hamster cells. The authors reported an increase in the molecular weight of DNA, which was attributed to binding of the copper ions to the DNA (Sideris *et al*, 1988).

3.2.1.2. In vivo

A single i.p. injection of copper (II) sulphate pentahydrate in mice induced a dose-related increase in the incidence of chromatid-type chromosome aberrations in the bone marrow 6 h after dosing between 0.28 and 1.7 mg Cu/kg body weight (Agarwal *et al*, 1990). Only at the highest dose tested were chromosomal breaks significantly increased. No induction of micronuclei was found in mice given a single injection of copper (II) sulphate pentahydrate at 1.7, 3.4 and 5.1 mg Cu/kg body weight (Tinwell and Ashby, 1990). In a study carried out without a positive control, Bhunya and Pati (1987) reported a significant dose-related increase in the incidence of micronuclei in the bone marrow of mice after two injections at doses between 1.3 and 5 mg Cu/kg body weight per injection.

To summarise, the conflict in experimental data do not allow an adequate evaluation of the genotoxic potential of copper and copper compounds *in vivo*.

3.2.2. Carcinogenicity

Studies on the carcinogenicity of copper compounds in rats and mice have given no indication of carcinogenic potential; however, the available studies present strong limitations in the experimental protocols (small group sizes, limited extent of histopathological examination, inadequate reports) and do not allow the evaluation of the carcinogenic potential of copper compounds with any degree of certainty. These studies are summarized in IPCS (1999), Table 11.

According to the IARC evaluation (1987), copper (II) 8-hydroxyquinoline has been allocated in Group 3 “Not classifiable as to their carcinogenicity to humans”, based on inadequate evidence in experimental animals.

3.2.3. Reproductive toxicity

There is some evidence to indicate an effect of exposure to copper compounds on animal reproduction. In some studies in rats, chronic oral exposure to 27-120 mg/kg bw per day of copper resulted in altered weight and/or histology of the testes, seminal vesicles, uterus or ovaries, albeit the results were not consistent. Other studies have demonstrated that exposure to copper compounds during gestation induced embryo/foetotoxic effects at doses of 12 mg of copper/kg body weight and above (IPCS, 1999).

3.3. Toxic effects in humans

Effective homeostatic controls are in place to reduce absorption and increase excretion, if excess copper is ingested. Nevertheless, there are documented cases of acute and chronic copper poisoning.

3.3.1. Acute Toxicity

Acute toxicity is infrequent in humans and is usually a consequence of contamination of food stuffs or beverages from copper containing vessels or dispensers. Acute symptoms include salivation, epigastric pain, nausea, vomiting and diarrhoea (Olivares and Uauy, 1996). Copper ions have an irritant effect on mucosal membranes and daily intakes ranging from 2 to 32mg in drinking water have been reported to cause symptoms of general gastric irritation (US EPA, 1987). Two studies (Pizarro *et al*, 1999; Donohue, 1997) have identified the threshold for acute gastrointestinal effects from copper in water at about 4.8 mg/day (based on a level of 3 mg copper/L in the water and a mean intake of 1.6 L of water/day). A recent combined international trial determined a NOAEL and LOAEL for effects of nausea in healthy individuals who drank distilled water containing copper as the sulphate salt. An acute NOAEL and LOAEL of 4 mg and 6 mg copper/L, respectively were determined (Araya *et al*, 2001). Preliminary unpublished data from the same research groups indicate that in a further study as volume increased, the effect of Cu-induced nausea decreased; and as copper dose increased the incidence of nausea increased. An acute NOAEL for nausea in females (more sensitive than males) was confirmed at 4 mg copper/L of bottled water (Araya *et al*, 2003).

Fatalities from acute copper sulphate poisoning have been reported. An 11 year old female died within hours of accidentally ingesting a solution of copper sulphate (Gulliver, 1991). The postmortem blood sample was found to contain 66 µg/mL copper. In India, copper sulphate poisoning has been used as a method of suicide. Of 48 cases of copper poisoning examined, 12 were fatal and ingested doses ranged from 1 g to 100 g copper dissolved in water (Chuttani *et al*, 1965). All cases were characterised by metallic taste, nausea and vomiting. Of the 12 fatal cases, seven apparently died from shock and hypotension or from subsequent renal damage with coma and uraemia. WHO have concluded that the fatal oral dose of copper salts is about 200 mg/kg body weight (WHO, 1993).

3.3.2. Chronic Toxicity

In humans, toxicity to chronic doses of copper has been less extensively studied. There are data to suggest that chronic copper exposure may cause diarrhoea in children (Stenhammar, 1979), gastrointestinal irritation from tap water (Schafer and Schumann, 1991) and acute liver failure (O'Donohue *et al*, 1993). Scheinberg and Sternlieb (1994) retrospectively examined populations of 0-5 year olds in three towns in Massachusetts from the period 1969-1991. Copper content of the drinking water was 8 mg copper/L. Exposure covering 64,124 child-years did not reveal a single death from any form of paediatric liver disease and no gastrointestinal problems were reported.

3.3.2.1. Carcinogenicity and genotoxicity

Serum copper, caeruloplasmin and other copper binding components (such as transcuprein) are reported to be increased in cancer patients (Campbell *et al*, 1981, Zowczak *et al*, 2001; Borella *et al*, 1997). However, elevated serum copper or caeruloplasmin in cancer does not necessarily imply increased body copper status as caeruloplasmin is an acute phase protein (DiSilvestro, 1990; Arnaud, 1994; Strain, 1994b) Copper induced DNA lesions have been shown in the liver of patients with Wilson's disease (Carmichael *et al*, 1995). Organ dysfunction rather than cancer, however, is usually considered the cause of death from Wilson's disease in humans (Linder, 2001). Evidence linking copper toxicity to cancer is, therefore, unsubstantiated at present.

3.3.2.2. Coronary heart disease (CHD)

High copper levels have been cited as a possible risk factor for heart disease (Ferns *et al*, 1997) and elevated serum caeruloplasmin levels have been observed in patients suffering from cardiovascular disorders (Reunanen *et al*, 1992; Manttari *et al*, 1994) However, serum copper and caeruloplasmin levels are increased as part of the acute-phase response in inflammatory conditions, such as CHD (DiSilvestro, 1990). Indeed, elevation of caeruloplasmin in CHD patients has been shown to be associated with the inflammatory process and not with pro-oxidant activity of caeruloplasmin (Klipstein-Grobusch *et al*, 1999). Furthermore, there is no evidence of higher rates of CHD in Wilson's disease, a genetic disease associated with copper loading. There is no current evidence to link copper excess with CHD.

3.3.2.3. Neurological disease

Copper has been described as having a critical role in neurological diseases and there is speculation that copper-induced production of hydroxy radicals may contribute to the neurodegeneration in Alzheimer's disease (Multhaup *et al*, 1996). Recent studies have also implicated copper in the pathogenesis of neuronal injury in prion-mediated encephalopathies (for review see Waggoner *et al*, 1999); however, evidence is weak, largely speculative and there is no indication that any effects are related to copper status.

3.3.2.4. Effects of copper supplementation

A subject given supplementary copper (30 mg/day) for two years followed by 60 mg/day for an unspecified period developed acute liver failure (O'Donohue *et al*, 1993). This level of chronic supplementation is extremely rare. Pratt *et al* (1985) saw no evidence of liver damage or gastrointestinal effects in seven subjects given 10 mg/day supplementary copper as copper gluconate for 12 weeks. Turley *et al* (2000) saw no effects of six week supplementation with 6 mg/day copper as copper amino acid chelates on LDL oxidizability in 24 healthy male and female subjects (FOODCUE project). Additional results from the FOODCUE project indicate no effect of copper supplementation (3 and 6 mg/day giving total copper intakes of approximately 4 and 7 mg/day respectively) on liver function and mononuclear leucocyte DNA damage as assessed by the comet assay (O'Connor *et al*, 2003). Moreover, a protective effect of supplementation with 6 mg copper (total intake approximately 7 mg/day copper) on erythrocyte oxidizability was observed in middle-aged subjects (Rock *et al*, 2000). Urinary pyridinoline and deoxypyridinoline (markers of bone resorption) were significantly increased after six weeks of a low copper diet (0.69 mg/day) compared with a medium copper diet (1.6 mg/day), and significantly decreased on a high copper diet (6.0 mg/day) compared with the low copper diet (Baker *et al*, 1998).

3.3.3. Reproductive toxicity

In humans, there appears to be no evidence related to oral copper intakes and reproductive toxicity.

3.4. Sensitive subpopulations

3.4.1. Wilson's Disease

Wilson's disease is an autosomal recessive disease of copper storage caused by numerous (over 100 recognised) mutations in the ATPase gene for copper transport. Copper accumulates in the liver, the brain and the cornea of the eye. There appears to be a defect in the catabolism and excretion of caeruloplasmin copper into the bile. Presenting symptoms include hepatic, neurological, and ophthalmological involvement; low serum concentrations of copper and caeruloplasmin and an increase in urinary copper excretion (Tanner *et al*, 1983). The worldwide incidence of Wilson's disease is 1 in 30,000 and the corresponding prevalence of the heterozygous and asymptomatic carrier of a mutated ATPase gene is 1 in 90 (Scheinberg and Sternlieb, 1996). If the disease goes untreated, copper accumulation in the liver and brain results in hepatitis, haemolytic crisis and hepatic failure may ensue.

3.4.2. Indian Childhood Cirrhosis

Indian childhood cirrhosis (ICC) is a fatal disease of infants in India associated with massive levels of copper accumulation in the liver (Portmann *et al*, 1978, Tanner *et al*, 1979). Clinically, ICC differs from Wilson's disease with earlier onset of hepatic abnormalities, normal or high serum concentrations and distinctive hepatic histology (Pandit and Bhave, 1996). Occurrence of ICC has been attributed to the practice of boiling and storing milk in copper and brass vessels (Bhave *et al*, 1987). However, there also appears to be an element of genetic predisposition in many cases of ICC (Agarwal *et al*, 1979).

3.4.3. Childhood Idiopathic Copper Toxicosis (ICT)

Idiopathic copper toxicosis has been attributed to high levels of copper (up to 6.8 mg/L) in drinking water (Müller *et al*, 1996) and there are approximately 30 proven cases in published reports (Müller *et al*, 1998). In a multicentre retrospective study across 16 paediatric centres in Germany for the years 1982-1994, 103 cases of histologically confirmed early childhood cirrhosis could be identified (Dieter *et al*, 1999). Excessive copper intake from copper plumbing/acid well water was the probable or sole causative environmental factor to trigger the cirrhosis in at most five of the cases. In the cases of confirmed copper associated early childhood cirrhosis, copper concentrations in private well waters were around 10 mg/L or more. Another 138 cases termed Tyrollean infantile cirrhosis have been identified in the Tyrol (western Austria) and have been associated with high dietary copper concentrations (Müller *et al*, 1996).

4. DOSE RESPONSE ASSESSMENT

The available data clearly show that copper can cause adverse effects in humans and in domestic and laboratory animals. Liver damage is observed almost exclusively in patients with Wilson's disease and children with ICC and ICT. Acute copper toxicity in drinking water appears to have a threshold of approximately 6 mg/L (Araya *et al, 2001).*

The occurrence of either acute or chronic copper toxicity in humans, however, is rare and tends to be confined to certain subpopulations, such as populations with high copper concentrations in drinking water, populations that utilise copper vessels (e.g. for boiling and storing milk) and those individuals who have a hereditary predisposition to a disease of copper toxicity. There appears, to date, to be little convincing evidence that excess copper is associated with the development of cancer in humans and data are inadequate to assess the reproductive developmental effects of copper excess in humans. Preliminary links between copper intakes and CHD are also inconclusive.

On the basis of the information presented in this report, a critical endpoint from which to derive an upper level (UL) is liver damage. Liver damage is selected because it is perhaps a more reliable indicator of a long-term chronic ingestion of copper. Although gastrointestinal effects of copper toxicity are better documented in humans than liver complications, gastrointestinal effects are more representative of acute copper poisoning. The aim of the UL is to identify safety of maximal copper intakes over a longer period of time.

A case study by O'Donohue *et al* (1993) observed acute liver failure in a subject taking 30 mg copper a day for two years followed by 60 mg/day for an unspecified period. The retrospective study of Scheinberg and Sternlieb (1994) demonstrated no effect of high concentrations of copper in drinking water (8 mg/L) on the incidence of death from any form of liver disease in children (0-5 years) from three Massachusett towns over a 23 year period. No adverse effects were observed in a double blind study of 12 weeks supplementation with 10 mg copper/day as copper gluconate supplement in seven healthy men (Pratt *et al*, 1985). A further seven healthy men took placebo for the duration of the trial. Results indicated no effect of copper supplementation on liver function. In the FOODCUE project, no adverse effects on measures of oxidative damage and liver function in 24 healthy male and female subjects given supplements of 6 mg/day copper (approximately 7 mg/day total copper intake) as copper amino acid chelates for six weeks in a cross-over design were found (Turley *et al*, 2000; O'Connor *et al*, 2003).

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

5.1. Adults

A NOAEL of 10 mg/day is based on the absence of any adverse effects on liver function (as the critical endpoint) in the study of Pratt *et al* (1985). This was a supplementation study of seven healthy adults with 10 mg/day copper for 12 weeks. In the FOODCUE trial, where copper intakes were around 7 mg/day, no observed adverse effect of copper supplementation on liver function in 24 healthy males and females was observed (O'Connor *et al*, 2003). In the study by Turnlund *et al* (1991) homeostatic data indicated that a 10-fold increase in dietary copper resulted in the absorption of only twice as much copper and that indices of copper status, as a result of the body's regulation of copper, are resistant to change except under extreme dietary conditions. For example, Turnlund *et al* (1990) showed that when dietary intakes increased from 0.8 mg/day to 7.5 mg/day (for 24 days), putative indices of status, including plasma copper, erythrocyte SOD, caeruloplasmin and urinary copper excretion were not significantly different. In the light of this evidence, the Committee decided that an UF of 2 is adequate to allow for potential variability within the normal population. An UL of 5 mg/day is derived.

5.2. Pregnancy and lactation

The upper level of 5 mg/day is not applicable during pregnancy or lactation because of inadequate data relating to this critical life stage.

5.3. Children and adolescents

Liver damage in children appears to be restricted to children with a predisposition for enhanced copper toxicity. Extrapolating adult UL values for children based on relative body weight (using reference weights) result in the values given in the table. These values are consistent with data from the studies of Scheinberg and Sternlieb (1994) and Dieter *et al* (1999) as discussed above.

Age (years)	Tolerable Upper Intake Level (UL) for Copper (mg per day)
1-3	1
4-6	2
7-10	3
11-14	4
15-17	4

6. RISK CHARACTERISATION

The available studies show that the mean copper intakes of adults and children in EU countries are below the UL. The 97.5 percentile of total copper intakes for all age groups are close to the ULs, which, in the view of the Committee, are not a matter of concern.

The Committee notes that the additional copper intakes from drinking water may be appreciable and may need to be taken into account.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF CALCIUM

(EXPRESSED ON 4 APRIL 2003)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Calcium (Ca) belongs to group II of the third period of the Periodic Table of Elements. It has an atomic weight of 40.08; its atomic number is 20, its valency is 2. It is the fifth most abundant element in the human body. The calcium content of the human body is 25 to 30 g at birth (0.8% of the body weight) and between 900 and 1300 g in adult men (up to 1.7% of body weight) (Weaver *et al*, 1996). Over 99% of the total calcium of the body is located in the bones, where it accounts for 39% of the total body bone mineral content (Weaver, 2001), and in the teeth, mostly as hydroxyapatite. Bone mineral provides structure and strength to the body and, very important, a reservoir of calcium that helps to maintain a constant concentration of blood calcium. Less than 1% of total body calcium is found in soft tissues (~7 g) and body fluids (~1 g). Calcium in the extracellular fluid and the blood are kept constant at 2.5 mmol/L (10 mg/dL) (between 2.25 and 2.75 mmol/L) via cell surface calcium-sensing receptors in parathyroid, kidney, intestine, lung, brain, skin, bone marrow, osteoblasts and other organs. Calcium is present in blood in three different forms: as free Ca^{2+} ions, bound to protein (about 45%), and complexed to citrate, phosphate, sulphate and carbonate (about 10%). Ionised calcium is kept within narrow limits (Worth *et al*, 1981) by the action of three hormones, parathyroid hormone, 1,25-dihydroxycholecalciferol, and calcitonin. Extracellular calcium serves as a source for the skeleton and participates in blood clotting and intercellular adhesion. Intracellular calcium varies widely between tissues and is predominantly bound to intracellular membrane structures of the nucleus, mitochondria, endoplasmatic reticulum or contained in special storage vesicles. Free Ca^{2+} is only 0.1 µmol/L in the cytosol, which is 10,000 times lower than in the extracellular fluid (1 mmol/L). Intracellular calcium rises in response to stimuli interacting with the cell surface receptor. The increase of intracellular calcium comes from influx of extracellular calcium or from release of intracellular calcium stores. This activates specific responses like hormone or neurotransmitter release, muscle contraction, cellular differentiation and many others.

2. NUTRITIONAL BACKGROUND

2.1. Food sources

Calcium must be ingested with the diet in sufficient amounts to allow for calcium deposition during bone growth and modeling and to compensate for obligatory intestinal, faecal and dermal losses during the life-time.

Foods vary widely in calcium content. The best sources are milk (120 mg/100 g) and milk products (up to 1100 mg/100 g), from which about 32% is absorbable (Weaver, 2001). In European diets about 45 to 70% of the dietary calcium intake is provided by dairy products (Guéguen and Pointillart, 2000; IUNA, 2001). Some plants are good sources of well-absorbable calcium, e.g. brassica, almonds, dried apricots. However, some vegetables contain considerable amounts of calcium, which is poorly absorbed because of a high content in oxalate (rhubarb, spinach) and which forms sparingly soluble calcium oxalate. Drinking water and mineral waters (>150 mg calcium/L) can also be good sources of absorbable calcium.

In the European Union the following calcium compounds are permitted as source of calcium in foods for particular nutritional uses and in food supplements: carbonate, chloride, citrates, gluconate, glycerophosphate, lactate, orthophosphates, hydroxide and oxide.

Within populations and population groups dietary calcium intakes show a great variability related to varying dietary habits. It appears from nutrition surveys that calcium intake is below actual recommended intakes in high percentages of the population.

2.2. Dietary intake

Dietary calcium intakes from various European countries are given in Table 1.

Table 1. Mean and 97.5 percentile calcium intakes (mg/day) from food and supplements

Country	Type of survey	n	Method	Supplements*	Mean	97.5%
Austria[a]	Individual	4972	7-day weighed 3-day weighed 24h-recall	Not defined	834	1678
Germany[b]	Individual (M) Individual (F)	2006	7-day record	-	753 683	1731 1421
Ireland[c]	Individual (M) Individual (F)	1339	7-day record	+	949 742	1657 1340
Netherlands[d]	Individual (M, F)	5958	2-day record	-	944	1970
UK[e]	Individual (M) Individual (F)	1087 1110	7-day weighed	+	940 730	1607 1317

* + data included supplements; - data excluded supplements.
[a] Koenig and Elmadfa, 2000.
[b] Heseker *et al*, 1994.
[c] IUNA, 2001.
[d] Hulshoff and Kruizinga, 1999.
[e] Gregory *et al*, 1990.

Men consume in absolute amounts about 10% more calcium than women. In Germany the highest calcium intake was observed in young men between 15 to 24 years: 2100 mg/day without supplements (Heseker *et al*, 1994). A longitudinal observational study (*DONALD study*), which started in 1985 and follows children from the age of 3 months to 18 years (sample size 400 to 500 subjects) showed that the mean calcium intake values in these healthy children were below the recommended intake values beyond the age of 3 years. Less than 10% of adolescents (13 to 18 years) consumed more than the recommended calcium intake (Alexy and Kersting, 1999). In this group calcium from fortified food amounted to maximal 5% of the total daily intake between 2 and 14 years of age (Sichert-Hellert *et al*, 2001).

Total calcium intake of men and women who consumed calcium supplements more than once per week was significantly higher (men: 1275-1394; women: 1146-1221 mg/day) than in those never taking calcium supplements (men: 1190-1242; women: 1081-1117 mg/day) (Mensink and Ströbel, 1999).

2.3. Absorption and regulation of absorption

Calcium must be in a soluble form or bound to soluble organic molecules to be absorbable. However, undissociated low-molecular-weight salts of calcium can also be absorbed independent of vitamin D by paracellular routes or pinocytosis. Depending on solubility, chemical form and on other factors of the food between 10 to 40% of dietary calcium is absorbed. The bulk of unabsorbed calcium is complexed to bile acids, free fatty acids, oxalic acid and excreted with the faeces (Heaney, 2002a). Lactose in the food, vitamin D, inulin, fructooligosaccharides and some casein phosphopeptides increase absorption, the latter by preventing precipitation of calcium by phosphates. Most calcium salts used in fortified foods or dietary supplements are absorbed to a similar extent as calcium from dairy foods. The absorbability of calcium citrate malate is higher (Weaver, 2001). Phytates, and especially oxalate inhibit calcium absorption. Fibre consumed without phytates does not have a negative influence. A combined high intake of predominantly insoluble fibre and phytate in the form of wheat bran over four weeks had no adverse effects on bone turnover markers in 19 healthy young women. The observed decrease in urinary calcium excretion sufficiently compensated for the reduced net absorption of dietary calcium without changing calcium retention (Zitterman *et al*, 1999). Both the protein and the sodium content of diets have a negative effect on calcium retention by increasing urinary calcium losses. The effect of

higher protein intakes on increased urinary calcium losses appears only to result in negative effects on bone status if the calcium intake is inadequate (Heaney, 2002a).

There are two kinds of calcium transport in the intestine:

a) Active transport in the duodenum and upper jejunum is saturable and regulated by dietary intake and the needs of the body. Active transport involves three stages, namely entry across the brush border of the enterocyte via calcium channels and membrane-binding transport proteins, diffusion across the cytoplasm attached to calcium binding protein calbindin-D9K, and secretion across the basolateral membrane into the extracellular fluid against an electrochemical gradient either in exchange for sodium or via a calcium pump, a Ca-ATPase activated by calbindin, calcium and calmodulin. Active transport is negatively correlated with dietary calcium intake. This control is mediated via parathyroid hormone and $1,25(OH)_2D$. The renal production of $1,25(OH)_2D$ is stimulated by increased parathyroid hormone secretion in response to a decrease in Ca^{2+} in blood and it stimulates the expression of the gene encoding calbindin, thereby enhancing calcium absorption in the intestine. Both parathyroid hormone and $1,25(OH)_2D$ also increase renal reabsorption of calcium and bone resorption.

b) Passive diffusion down an electrochemical gradient together with water, sodium and glucose via intercellular junctions or spaces occurs in all parts of the gut and is predominantly dependent on the calcium concentration in the gut lumen. This process is independent of vitamin D and age (Bronner, 1992). Passive diffusion requires that calcium is kept in solution, which can be enhanced by casein phosphopeptides (Mykkänen *et al*, 1980), by chelating with some amino acids (lysine and arginine) (Bronner, 1987), and by high doses of lactose (50 g/day) (Pansu *et al*, 1979). Increases in the osmolarity of the luminal contents of the intestine stimulate passive diffusion. Except in premature infants passive calcium absorption accounts for not more than 8 to 23% of the total calcium absorbed (McCormick, 2002).

Fractional calcium absorption, is highest (about 60%) in breastfed infants (Abrams *et al*, 1996). Net calcium absorption, defined as intake minus faecal excretion in percent of intake, is lower in infants fed cows' milk formula, decreases in young childhood, shows a rise in puberty, decreases to 15 to 20% in young adults (Matkovic, 1991; Miller *et al*, 1988; Peacock, 1991) and declines gradually thereafter (Heaney *et al*, 1989). Calcium absorption is increased in pregnant and lactating women compared to non-pregnant women (Moser-Veillon *et al*, 2001).

Calcium absorption is under genetic control. The FF genotype for the Fok 1 polymorphism, a C → T transition in the vitamin D receptor translation initiation site, was related to increased calcium absorption in 72 children 7 to 12 years of age and it was associated with greater bone mineral density (Ames *et al*, 1999a), but these findings were not confirmed in another study with 99 girls 16.9 ± 1.2 years old (Lorentzon *et al*, 2001).

2.4. Calcium losses

The majority of absorbed calcium is stored in the skeleton. Excess absorbed calcium is excreted in urine, faeces, and sweat. Calcium balance is positive in healthy children, adolescents and young adults before bone growth and modeling cease, provided that they have an adequate calcium intake.

Renal calcium excretion is the result of glomerular filtration (about 8 to 10 g calcium per day in adults) and tubular reabsorption (normal over 98% of the filtered load), which is primarily passive in the proximal tubules and for 20% active in the distal part of the convoluted tubules and connecting tubules. Active transport is under the control of parathyroid hormone, calcitonin and $1,25(OH)_2D$ (Hoenderop *et al*, 2002). Average 24-hour excretion of calcium is 40 mg in young children, 80 mg in prepubertal children and reaches about 150-200 mg in adults. It is not strongly related to dietary calcium intake (Charles *et al*, 1991; Matkovic, 1991) in healthy persons. Calcium excretion is increased in hyperparathyroidism and decreased in untreated osteomalacia.

Urinary calcium excretion is increased by dietary sodium intake (30 to 40 mg of calcium excreted per each two grams of dietary sodium) (Matkovic *et al*, 1995), by caffeine (Massey and Whiting, 1993) and in chronic metabolic acidosis (Bushinsky, 2001). Calcium excretion rises with excess dietary protein intake (by 0.5 mg for each gram of dietary protein, when intake was above 47 g/day) (Walker and Linkswiler, 1972; Whiting *et al*, 1998). This effect can be offset by simultaneous phosphorus intake (Guéguen and Pointillart, 2000).

Increased calcium excretion is also observed in idiopathic (hypocalcaemic) hypercalciuria, a genetic disorder of heterogenous pathogenesis (absorptive, renal or dietary) observed in 2.2 to 6.4% of children and adults (Kruse *et al*, 1984; Moore *et al*, 1978) and which is the most frequent risk factor for nephrolithiasis. Idiopathic hypercalciuria could be the result of a defective renal epithelial Ca^{2+}-channel leading to decreased active renal reabsorption of calcium or to an increased intestinal activity of the epithelial Ca^{2+}-channel with augmented intestinal calcium absorption (Hoenderop *et al*, 2002). Hypercalciuric stone formers are more sensitive to dietary sodium chloride than individuals without stones with respect to calcium excretion (Massey and Whiting, 1995) and than normocalciuric stone formers (Burtis *et al*, 1994). Sodium restriction and/or protein restriction with a normal calcium intake reduces or normalises calcium excretion in hypercalciuric stone formers, whereas calcium restriction does not (Borghi *et al*, 2002).

Calcium losses via the skin are between 4 and 96 mg/day in normal individuals, calculated from combined calcium balance and kinetic studies with ^{47}Ca in 11 subjects (Charles *et al*, 1991). The authors consider the minimal obligatory loss to be 3 to 40 mg calcium per day. The amount rises with increasing serum calcium levels.

Calcium is also secreted throughout the gastrointestinal tract, where about 85% is available for reabsorption with the same absorption efficiency as dietary calcium. Faecal secretory calcium loss has been estimated to be 80 to 224 mg/day in normal individuals.

2.5. Calcium requirement and dietary reference values

Adequate dietary calcium intakes are determined by information from balance studies, from fractional estimates of required intakes to compensate for urinary, faecal and dermal calcium losses and more recently from the intakes necessary to achieve “maximal calcium retention” for bone mineral deposition, while taking into account calcium absorption, and also from studies on bone mineral density and bone mineral content development during life (Heaney, 2002b). From the analysis of pooled calcium balances performed in 519 individuals between birth and 30 years of age intakes of calcium were identified above which an increase in calcium intake did not further increase calcium retention (Matkovic and Heaney, 1992).

Different scientific bodies have applied different models to different data in order to derive dietary reference intake values. The population reference intakes defined by the Committee in 1992 are based on a factorial approach (compensation of obligatory calcium losses and accounting for absorption efficiency in adults and addition of desirable calcium retention corrected for absorption for children and adolescents) without considering measurements of bone mineral accretion under different calcium intakes (Kanis, 1991; SCF, 1993). The PRI (in mg calcium per day) is 400 for infants in the second half of the first year and for children up to age 3 years, 450 for children between 4 and 6 years, 550 for children between 7 and 10 years, 1000 and 800 for male and female adolescents between 11 and 17 years and 700 in adults and pregnant women. For lactating women it is 1200 mg per day. More recent reports include the attainment of peak bone mass during childhood, adolescence and young adulthood in their calculations (IOM, 1997; D-A-CH, 2000; AFSSA, 2001). The adequate intakes (AI) (IOM, 1997) and recommended daily intakes (RDA) (D-A-CH, 2000; AFSSA, 2001) thus derived are generally higher than the PRI. They are between 500 and 800 mg calcium per day for children up to the age of 7 years, 1200 to 1300 mg per day for older children and adolescents and 900 to 1200 mg calcium per day for adults. Pregnant and lactating women below the age of 18 years should receive between 1200 and 1300 mg calcium per day.

Some authors have suggested higher recommend intake values for calcium because of observed beneficial effects of calcium intakes of 1200 mg to 2000 mg per day on the risk of colon cancer, kidney stones, obesity and hypertension (Fujita and Palmieri, 2000; Heaney, 2002b). But there is no consensus on this.

2.6. Calcium deficiency

Calcium deficiency can result from low dietary intake, low absorption or excessive losses. A decrease in ionised calcium in the extracellular fluid stimulates the secretion of parathyroid hormone to mobilise calcium from bone and maintain the pre-set serum calcium level. Parathyroid hormone also increases the intracellular calcium concentration in many types of cells - cardiomyocytes, blood cells, adipocytes, hepatocytes and pancreatic endocrine cells as well as in osteoblasts and renal tubular cells. An increase in intracellular calcium sets off a large number of reactions involving the permeability of the plasma membrane, signaling pathways, including activation and deactivation of enzymes, cyclic-nucleotide formation and break-down, cytoskeletal rearrangement, and gene transcription (Saimi and Kung, 2002;

Carafoli, 2002). The pathophysiologic changes and disorders resulting from this have been named *calcium paradox disease* (Fujita and Palmieri, 2000). They include hypertension and arteriosclerosis, Alzheimer's disease, muscular dystrophy, diabetes mellitus and malignancies. The role of parathyroid hormone secretion and its effects in the relevant target cells has been demonstrated in *in-vitro* models, animal experiments and/or epidemiological studies (Fujita and Palmieri, 2000).

3. HAZARD IDENTIFICATION

Calcium levels in the body are under control of genetic and hormonal factors. Therefore an excessive accumulation of calcium in blood or tissue solely through excessive calcium consumption should not occur in the absence of diseases such as bone cancer, hyperthyroidism, and hyperparathyroidism or in the absence of excessive vitamin D intake. Adverse effects which have been reported due to high calcium intakes include the so-called milk-alkali syndrome, the formation of kidney stones in persons with a propensity for nephrolithiasis, hypercalciuria and for hyperabsorption of calcium, and interference with the absorption of other minerals (Whiting and Wood, 1997).

3.1. Adverse effects in animals

3.1.1. Acute toxicity

The LD_{50} for calcium gluconate in rats is 10 g/kg body weight, corresponding to 930 mg calcium/kg (Sarabia *et al*, 1999).

3.1.2. Short- and medium-term studies and reproductive studies

Greger *et al* (1987) tested the bioavailability of different calcium sources (milk, dibasic calcium phosphate, oyster shell, calcium carbonate, calcium lactate, calcium amino acid chelate and dolomite) in rats fed diets with similar calcium contents (approximately 0.5%). Apparent calcium absorption was comparable with all calcium compounds. Dibasic calcium phosphate caused increased kidney size and more than 20-fold higher calcium content in kidneys.

Ten dogs (weight 14 to 25 kg) supplemented for two weeks with 100 mg calcium gluconate and 250 µg vitamin D/kg/day developed severe hypercalcaemia and hypomagnesaemia, polyuria, hyperexcretion of calcium, sodium and magnesium, hypotension, a decrease in the heart stroke volume and increased total peripheral arterial resistance (Zawada *et al*, 1986).

Growing pigs fed *ad libitum* with diets which differed in the calcium-phosphorus ratio (1:1, 2:1, 3:1) and calcium content (0.3% up to 2.7%) but without extra vitamin K showed coagulation disorders. All pigs in the 2.7%-calcium group died between three and four weeks from internal haemorrhage (Hall *et al*, 1985).

Pregnant rats fed diets differing in calcium content (0.01%; 0.6%; 1%) and calcium:phosphorus ratios (1.3; 0.02; 2.4) produced comparable litter numbers. However pregnant rats on the high-calcium diet decreased their food consumption excessively near term and lost weight and the weight of the foetuses were reduced. The calcium balance in the high-calcium rats was markedly negative in the days just before term. Their foetuses had a lower body calcium content than those of the calcium-free and normal-calcium diet groups (Lai *et al*, 1984).

Rats fed diets with 1.5, 2 and 2.5 times higher than normal (0.5%) calcium contents (as calcium carbonate), starting after mating and continued during twenty days of gestation showed no dose related changes in maternal clinical findings, average numbers of implantation, foetal resorption and viable foetuses, neither were there adverse effects on foetal length and weight nor signs of foetotoxicity or teratogenicity. However, there were dose-related increases of the femoral calcium content as well as of the phosphorus, magnesium and zinc content of the liver in non-pregnant control rats on the same diets. There were dose-related decreases of the iron and copper contents in kidneys of non-pregnant animals and of iron in the liver of pregnant rats. Foetuses showed dose-related decreases in the whole body content of phosphorus, magnesium, iron and copper (Shackelford *et al*, 1993 and 1994).

Richards and Greig (1952) tested the effect of four different calcium levels (0.3%, 0.5%, 0.7% and 1.1%) in four different diets on reproductive performance in mice and on survival and organ pathology in litters. All diets with a calcium (carbonate) content of 1.1% resulted in decreased number and total weight of litters and increased both the number and proportion of litter deaths. Young mice born to mothers on high-calcium diets showed pale speckled livers, enlarged hearts and small thymus when

killed at age 21 days. Increased heart weights were negatively correlated with haemoglobin levels. Addition of iron to high-calcium diets diminished heart enlargement.

While studying the effects of diets with low (0.2%) and high (4%) calcium contents in rats over 31 weeks on lead toxicity (lead supplied in drinking water) it was observed that the high calcium diet resulted in higher blood pressure, in kidney and bladder stones, slower growth and death in half of the animals. In a similar experiment, feeding rats low (0.1%), normal (0.5%) and high (2.5%) calcium diets over one year, caused dose-related decreases of the iron content of the femur and testis, magnesium in plasma and femur, the zinc and calcium contents of the femur and the calcium content of the kidneys (Bogden *et al*, 1992).

High intakes of dietary calcium (1.5% in 50 days pregnant ewes have caused disturbed bone formation from cartilage (osteochondrosis) and an increase in thyroid C cells (calcitonin producing) in the foetuses compared to the foetuses of ewes fed normal feeds (0.59% calcium) (Corbellini *et al*, 1991).

3.2. Adverse effects in humans

3.2.1. Intervention studies

Intervention studies with supplemental calcium, predominantly in the form of calcium salts but also with milk products or with elemental calcium from chicken egg-shell powder, which have been performed in children, pregnant and lactating women and elderly men and women, have exposed subjects to total calcium intakes of up to 3000 mg/day for up to 4 years. Annex I lists some relevant studies.

Children between 6 and 14 years of age received up to 1900 mg calcium/day for one to 3 years to study the effect on bone status (Johnston *et al*, 1992; Lloyd *et al*, 1993; Chan *et al*, 1995; Bonjour *et al*, 1997 and 2001). The recommended dietary calcium intake for that age is 550 to 1200 mg/day.

Elderly men and women between 50 and 85 years of age have received calcium in amounts between 1300 and 3000 mg/day for 6 months to four years to study the effect of supplemental calcium on bone metabolism and bone loss (Kochersberger *et al*, 1991; Reid *et al*, 1993; Elders *et al*, 1994; Riggs *et al*, 1996; Heaney *et al*, 1999; Peacock *et al*, 2000; Dawson-Hughes and Harris, 2002; Schaafsma *et al*, 2002). The recommended dietary calcium intake for that age range is 1200 mg/day.

Pregnant women have received calcium in amounts between 2000 and 3000 mg/day, starting between 13 and 23 weeks of gestation, to study the effects on hypertensive disorders of pregnancy, preeclampsia, preterm delivery, adverse perinatal outcomes, and foetal bone mineralisation (Villar and Repke, 1990; Belizán *et al*, 1991; Bucher *et al*, 1996; Levine *et al*, 1997; Koo *et al*, 1999). The recommended intake for pregnant women is between 700 and 1300 mg/day.

A calcium intake of 2000 mg/day was tested over 4 years in patients aged more than 60 years with colorectal adenomas to determine if there was an influence of calcium on the recurrence rate of adenomas (Baron *et al*, 1999).

Calcium in amounts between 1300 and 2300 mg/day was given to healthy men and women and to lactating women with durations between 12 weeks and one year to study the effects on iron, zinc and magnesium status (Sokoll and Dawson-Hughes, 1992; Yan *et al*, 1996; Minihane and Fairweather-Tait, 1998; Kalkwarf and Harrast, 1998). Recommended dietary calcium intakes for this population range between 800 and 1300 mg/day.

Adverse effects of calcium supplementation observed in these studies are given in the relevant sections below.

3.2.2. Hypercalcaemia and renal insufficiency (milk-alkali syndrome)

The milk-alkali syndrome is named after the adverse effects observed in consequence of the combined therapeutic application of calcium-rich milk and absorbable antacids (mostly sodium bicarbonate or calcium carbonate) for peptic ulcers. It results eventually in metabolic alkalosis and hypercalcemia, probably as a result of increased calcium retention by alkali, and leads to the usual consequences of hypercalcemia, i.e. loss of appetite, weight loss, nausea, constipation, polyuria, polydipsia, hyposthenuria, dehydration, renal failure, nephrocalcinosis and nephrolithiasis, apathy, confusion, lethargy and coma in variable combination and severity. Onset can be insidious or acute within days or weeks after starting very high calcium and alkali intakes. It can be reversible or fatal (Orwoll, 1982; Abreo *et al*, 1993).

The original therapeutic regimen (Sippy, 1915) included calcium intakes of 20 g/day from both milk and for example calcium carbonate. With changes in the therapy of peptic ulcers the frequency of the milk-alkali syndrome has declined. Whiting and Wood (1997) identified 29 reported cases of clinically adverse effects of high calcium intakes or combined high intakes of calcium and alkali in a review of the literature between 1980 and 1994. The youngest patient in their list was 29 years old with most cases over 50 years old. One third of the cases consumed both alkali and calcium (between 2.0 and 16.5 g/day of supplementary calcium) and symptomatology appeared to be precipitated by an increase of their calcium intake while consuming antacids, sometimes for many years. One third, however, developed symptoms of milk-alkali syndrome as a result of high calcium carbonate intakes alone (between 2 to 10.8 g additional calcium per day from several months to 30 years). About 40% of the listed cases were patients with associated promoting factors, such as the use of thiazide diuretics (which decrease renal calcium excretion), pre-existing renal failure, dehydration, or alkalosis. In these cases supplemental calcium intake was between 2 and 16 g/day. One study estimated that 12% of patients hospitalised in one hospital because of hypercalcemia were the result of excessive calcium carbonate consumption (Beall and Scofield, 1995). Annex II contains some details of the case reports evaluated by Whiting and Wood (1997) with additional cases added reported both before 1980 and after 1994. This compilation of 82 patients reported in the literature between 1965 and 2001, ranging in age from 24 to 95 years, shows that the milk-alkali syndrome occurs predominantly in patients with complaints of the stomach, oesophagus or duodenum (55 of 82), who ingested high amounts of milk (43 of 82) corresponding to more than 0.9 to 6.8 g calcium per day, and/or calcium supplements (76 of 82) containing between 1 and 23 g of calcium/day, or ingesting only calcium supplements (37 of 82). Thirty five high-milk consumers took calcium supplements. Eight of the high-milk consumers did not take calcium supplements, but consumed sodium bicarbonate regularly. Eleven calcium supplement-only users also took sodium bicarbonate. In 33 cases the use of “antacids” is reported, both absorbable or unabsorbable or unspecified. The reported range of total calcium intake was between 0.4 and 23 g/day. In many cases, however, calcium intake was inadequately documented.

All calcium supplements consisted of calcium carbonate The duration of a high calcium intake is reported to be between 3 days and 30 years. One case with a latency of 3 days only for the development of the milk-alkali syndrome was a 40 years old female patient who received 4.8 g calcium as carbonate for peptic ulcer prevention after a cardiac transplant, in addition to prednisone. She developed hypercalcaemia and transient renal failure (Kapsner *et al*, 1986). The same authors report that 65 of 297 cardiac surgery patients on the same peptic ulcer prevention regime developed hypercalcaemia, accompanied by renal failure in 37 of these 65 patients within one week to 6 months. The therapeutic regimen in these cases consisted of calcium carbonate (1.3 to 4.6 g calcium/day) plus prednisone.

McMillan and Freeman (1965) randomised 40 patients with gastric or duodenal ulcers to receive either 11.2 g calcium as carbonate or a non-absorbable antacid in addition to milk every 2 hours, corresponding to 1.8 g per day calcium over 7 days. They observed a significant rise in serum calcium in the group on calcium carbonate only, from a mean of 2.45 mmol/L to a mean of 2.8 mmol/L on day 3, with 5 patients reaching values above 3 mmol/L. In the same group serum creatinine rose significantly, as did serum phosphorus and carbon dioxide content. No significant changes of these parameters were observed in the group treated with non-absorbable antacid.

A dose of 3.2 g of calcium per day given as the carbonate over 6 days under clinical conditions provoked hypercalcaemia (3 mmol/L) and hypercalciuria, a rise in serum phosphorus and 24 hours later in creatinine and a decrease in the glomerular filtration rate in a patient with recurrent severe hypercalcaemia due to 15 years of consumption of calcium carbonate in high amounts (Smit and Bijvoet, 1986).

Lin *et al* (1996) describe the development of the milk-alkali syndrome in a 70 year old woman after 4 weeks of osteoporosis treatment with 3.75 g calcium (as carbonate) and 0.5 µg calcitriol per day. Of interest is also the report of Wu *et al* (1996) on two men, who had chewed betel nut covered by a calcium carbonate containing paste (estimated amount of calcium 2.5 and 3.5 g) over 30 years and who demonstrated hypercalcaemia and persistent renal insufficiency.

The two cases in whom milk and calcium carbonate were ingested for some weeks for relief of pregnancy-associated gastric discomfort and emesis and one case of bulimia/anorexia with recent use of milk and calcium carbonate can be classified as cases of milk-alkali syndrome provoked by dehydration and alkalosis (Ullian and Linas, 1988; Kleinman *et al*, 1991; Muldowney and Mazbar, 1996). The malformations of the stillborn foetus which was born after 37 weeks of pregnancy to a mother with milk-alkali syndrome in the 23rd week of gestation were not attributed to this disorder because the foetus revealed no signs of

tissue calcification (Ullian and Linas, 1988). In many case reports, however, it is not clear if the symptoms described signify the manifestation of the milk-alkali-syndrome or are part of preexisting and predisposing disorders.

Milk-alkali syndrome was not observed in the course of intervention studies which involved between 11 and 2295 individuals (children, pregnant and pre- or perimenopausal women, elderly people) and lasted between 12 weeks to 4 years. The studies tested the effects of calcium supplements (500 to 2000 mg/day, given as milk or milk extracts, citrate, carbonate, citrate malate, gluconate or egg-shell powder) on bone metabolism, on hypertensive pregnancy complications, on recurrence of colorectal adenomas and on iron, zinc and magnesium status. However, in the *Calcium for Pre-eclampsia Trial*, in the course of which 2295 women pregnant for 13 to 21 weeks were supplemented daily with 2000 mg calcium as the carbonate, women with a known risk for nephrolithiasis and with elevated levels of serum calcium and creatinine were excluded (Levine *et al*, 1997).

Elders *et al* (1994) observed a mean increase of serum creatinine of 1.2 µmol/L in 64 perimenopausal women taking 2000 mg calcium (as lactogluconate and carbonate) as daily supplement in addition to a calcium intake of 1000 mg from the diet over 2 years. One case of hypercalcaemia was reported among 119 postmenopausal women supplemented during 4 years with 1600 mg calcium (citrate) per day (Riggs *et al*, 1996). The constipation which has occasionally been reported in studies on calcium supplementation (750 to 1200 mg calcium/ day during 4 years and 6 months, respectively, can be a consequence of hypercalcaemia, however this was not looked for (Peacock *et al*, 2000; Kochersberger *et al*, 1991).

3.2.3. Kidney stones

Kidney stones affect between 8 to 15% of the population in Europe (Pak, 1998). About 80% of kidney stones are composed of calcium oxalate or a mixture of calcium phosphate and calcium oxalate. Stones form only in urine that is supersaturated. Hypercalciuria (more than 4 mg/kg body weight/day) is the most common abnormality in patients with calcium containing stones. Thirty to 50% of patients with kidney stones and hypercalciuria have idiopathic hypercalciuria that is not secondary to causes like primary hyperparathyroidism, hyperthyroidism, malignancy, renal tubular acidosis, vitamin D intoxication, immobilisation and Paget's disease. The hypercalciuria may be either renal (increased calcium/creatinine quotient in the urine at all times) or hyperabsorptive (increased calcium/creatinine quotient after calcium load) (Pak *et al*, 1975).

Dietary calcium is not the determining factor in kidney stone formation (Goldfarb, 1994) but higher intakes of oxalate, protein and vegetable fibre may play a role (Massey *et al*, 1993). In a population-based study, which involved 1309 women aged 20 to 92 years, no relationship between renal stone formation (n=44) and high-oxalate food, vitamin C, protein, fibre, or alcohol consumption could be demonstrated. Neither was there a positive association between the amount of dietary calcium and the fluoride content of drinking water and kidney stones. Women with stones ingested on average 250 mg less calcium per day than women without stones (840 versus 1070 mg), but calcium supplements appeared to have no protective effect on kidney stone formation (Sowers *et al*, 1998).

In two prospective observational studies with 45,619 men (aged between 40 and 75 years) followed over 5 years (*The Health Professionals Follow-up Study*) and 91,731 women (aged between 34 and 59 years) followed over 12 years (*Nurses' Health Study*), without kidney stones at the beginning of the observation period, it appeared that total calcium intakes above 1050 mg/day in men and above 1100 mg/day in women decreased the risk of kidney stone formation by approximately 35%. The mean calcium intake of stone formers was significantly lower than in those remaining free of stones, after adjustment for age, body mass index, intake of animal protein, alcohol, sodium, sucrose, fluid, and supplemental calcium (Curhan *et al*, 1993 and 1997). The relative risk for stone formation was significantly lower in women in the highest quintile of dietary calcium intake (median 1303 mg) compared with women in the lowest quintile. Dietary vitamin D intake was 5.5 in the lowest and 9 µg/day in the highest quintile. Similar findings were reported for men. In the women's study the intake of calcium supplements in daily amounts between 1 and 100 mg increased the risk for stone formation by 20% compared to women who did not take supplemental calcium. There was no further increase in the relative risk for stone formation by higher intakes of supplemental calcium (Curhan *et al*, 1997). In both studies a reduction of the risk of stone formation was observed with increasing intakes of dairy products (rich in phosphorus) and an increase of the risk with increasing sodium and sucrose intakes.

From the studies of Sowers *et al* (1998) and Curhan *et al* (1997) it can be concluded that a calcium intake in the range of the most recent dietary recommendations does not promote kidney stone formation on a population basis.

Dietary calcium reduces dietary oxalate absorption, whereas calcium restriction increases intestinal oxalate absorption and renal oxalate excretion. In a recent randomised trial over 5 years with 120 men with recurrent calcium oxalate stones and idiopathic hypercalciuria a diet normal in calcium (1200 mg/day) [30 mmol] and low in sodium [50 mmol] and normal in protein (15% of energy intake, 60% animal protein) reduced the risk of stone recurrence by 50% and decreased oxalate excretion more than a calcium restricted diet (400 mg [10 mmol]/day) (Borghi *et al*, 2002).

Both calcium and sodium intake were positively associated with hypercalciuria in patients with kidney stones and a regression equation was developed to predict the effect of dietary calcium on urinary excretion of both. From this equation the calcium intake that would be associated with hypercalciuria can be calculated to be 2243 mg/day for men and 1422 mg/day for women, assuming a sodium excretion of 100 mmol/day and defining hypercalciuria as >300 mg calcium/day for men and >250 mg/day for women (Burtis *et al*, 1994).

One short report has been published on the occurrence of pure calcium carbonate gallstones in a two year old girl whose mother had taken calcium carbonate and vitamin D in unknown quantities during the last four months of pregnancy because of leg cramps (Powell, 1985).

Although intervention studies with supplemental calcium have not been performed to study the risk for kidney stone formation, no increased incidence can be deduced from those studies listed in Annex I with approximately 5000 subjects, who received between 500 and 2000 mg calcium as supplement in addition to 300 to 1800 mg of calcium from the diet (total intakes between 1300 and 3000 mg calcium/day during three months to four years. Women with an increased risk for nephrolithiasis were excluded from the big *Calcium for Pre-eclampsia trial* (Levine *et al*, 1997). In a group of 124 women on calcium supplements (total daily intake 1400 mg/day) one patient with kidney stones was reported (Peacock *et al*, 2000). Riggs *et al* (1996) observed hypercalciuria (>350 mg/day) in 44 of 119 postmenopausal women taking calcium supplements (~1600 mg) for four years, and in seven of 117 women without supplements. One woman in the supplemented group developed mild hypercalcaemia. Three of 50 infants who received a calcium-rich formula from age 3 months onwards (1700 to 1560 mg calcium per day) developed hypercalciuria (Dalton *et al*, 1997).

3.2.4. Interactions between calcium and dietary minerals

High calcium diets and supplements can affect the bioavailability of other essential minerals, iron, zinc, magnesium and phosphorus.

3.2.4.1. Iron

Calcium inhibits the absorption of both iron salts and heme-iron (Hallberg *et al*, 1991) in a dose-dependent manner. A dose of 300 mg of calcium chloride added to a meal inhibited iron absorption maximally. An inhibitory effect was also seen with a variety of calcium sources both from supplements and food (Whiting and Wood, 1997). The absorption of non-heme iron (15 mg/day) was 16% with a low-calcium diet (<320 mg/day), but it decreased to <5% with the addition of 400 mg calcium (carbonate) to three daily meals (Minihane and Fairweather-Tait, 1998).

Long-term intervention studies on the effect of calcium supplementation on iron status failed to show reductions in indicators of iron status, unless the habitual calcium intake was very low. Calcium supplements had no effect on iron status in infants fed iron-fortified formula, in lactating women, adolescent girls and adult men and women (Lynch, 2000).

Three month old infants (n=103) who received either a calcium/phosphorus enriched formula (calcium intake from formula after 4 months 1700 mg, after 9 months 1560 mg/day) or a standard formula (calcium intake from formula 400 mg and 350 mg/day, respectively) showed no differences in serum ferritin, total iron-binding capacity, erythrocyte protoporphyrin or haematocrit during the remainder of the first year of life. Both formulae provided the same high amount of iron (12.8 mg/L) (Dalton *et al*, 1997).

Eleven children between 3 and 5 years of age receiving for 5 weeks each a low-calcium (502 mg/day) and high-calcium (1180 mg/day) diet providing 9 and 9.7 mg iron per day were tested for iron incorporation into red blood cells and calcium absorption and retention with ^{44}C and ^{58}Fe given orally with meals and ^{46}Ca given intravenously. There was no significant difference of iron incorporation into

red blood cells 14 days after dosing with the low-calcium (6.9%) compared to the high calcium diet (7.9% of administered dose), while calcium absorption (36.2%, 181 mg/day versus 23.7%, 277 mg calcium/day on the low- versus the high-calcium diet) and net calcium retention (74 mg/day versus 124 mg/day) differed significantly (Ames *et al*, 1999b).

Supplementation with 1000 mg calcium (carbonate) over five weeks did not affect serum ferritin levels in sixty women consuming diets low in calcium (280 mg/day) (Yan *et al*, 1996). There were no differences in serum ferritin levels in 158 women who received either 500 mg calcium (carbonate) twice daily with meals or placebo during months 6 to 12 postpartum (Kalkwarf and Harrast, 1998). Intake of 500 mg calcium (citrate-malate) supplements twice daily by 354 girls aged 8-13 years during four years did not result in differences in serum ferritin values, haemoglobin concentration or erythrocyte indices compared to a placebo group. The basal dietary calcium intake in these girls was between 798 and 878 mg/day, the iron intake 12.1 to 14.3 mg/day (Ilich-Ernst *et al*, 1998).

Seventy-five premenopausal women taking 500 mg calcium (carbonate) twice daily with meals during 12 weeks showed no effect on plasma ferritin, serum iron, total iron-binding capacity, transferrin saturation, haemoglobin level or haematocrit compared to a control group. Their dietary calcium intake was 600 mg/day (Sokoll and Dawson-Hughes, 1992).

Eleven iron-replete adults, aged 18 to 69 years, who received for six months daily calcium supplements of 1200 mg (as carbonate) in addition to dietary calcium of 1100 mg/day did not show changes in haemoglobin, haematocrit, zinc protoporphyrin and plasma ferritin (Minihane and Fairweather-Tait, 1998).

Seven of nine cross-sectional studies in various countries in adults, young adults and infants showed a small negative correlation between iron status and consumption of dairy products. It was calculated that for every 100 mg/day increase of calcium intake in girls, serum ferritin would be reduced by a factor of 1.6%, and by a factor of 3.3% in women (van de Vijver *et al*, 1999). A threshold effect for dose could not be detected. However, these findings with dairy products were not reported with other calcium sources, suggesting that another milk constituent could be responsible. It appears that changes in the calcium content of Western diets are not likely to have significant influence on iron absorption (Lynch, 2000) and that supplementation with calcium at the levels found to enhance bone mineral density (1000 to 1200 mg/day) does not affect normal iron status in healthy menstruating females (Bendich, 2001).

3.2.4.2. Zinc

Whereas human studies have shown that added dietary calcium either as salts or milk did not interfere with the intestinal absorption of radiolabeled zinc, there are two studies that report a negative effect on dietary zinc absorption and balance. Stepwise increases in calcium intake from 230 mg to 860 mg to 2000 mg/day in older men decreased fractional net zinc absorption from 24% to 12% to minus 3% on a zinc intake of 14 mg/day. However, there was no effect on zinc excretion and zinc balance (Spencer *et al*, 1984). When postmenopausal women were fed during two periods of 12 days a diet with approximately 1500 mg calcium, half of them were in negative zinc balance despite zinc intakes of 17 mg/day. However the directly inhibitory effect of a calcium supplement (600 mg) on zinc absorption from a meal could be offset by additional zinc (Wood and Zheng, 1997).

Yan *et al* (996) investigated the effect of calcium carbonate supplements (1000 mg/day on five days per week) given throughout one year to 30 lactating women aged 16 to 41 years on zinc status and found no difference compared to a placebo group. Both groups had a low habitual dietary calcium intake of less than 300 mg per day.

Ten healthy men who received calcium phosphate supplements of 600 and 1200 mg daily each for two weeks in addition to a dietary calcium intake of 1800 mg did not develop changes in renal and faecal zinc excretion. However, serum zinc concentrations decreased from 1.1 mg/dL to 0.9 mg/dL (Raschke and Jahreis, 2002)

3.2.4.3. Magnesium

High calcium intakes (2 g/day) can reduce intestinal magnesium absorption and decrease renal magnesium excretion. The combined effect would not result in magnesium depletion in the absence of other risks for magnesium depletion such as diabetes mellitus, malabsorption and alcoholism (Whiting and Wood, 1997). Abrams *et al* (1997) determined magnesium balance (intake 6.4 mg/kg/day or 194 to 321 mg/day) in relation to dietary calcium intake (mean 1310 mg/day) in 25 children between 9 and 14 years of age and found no influence.

Calcium phosphate supplements of 600 and 1200 mg/day for 2 weeks, in addition to dietary calcium intakes of 1800 mg, did not influence magnesium metabolism in 10 healthy men (Raschke and Jahreis, 2002).

The magnesium status of lactating women with a low habitual calcium intake was not influenced by calcium carbonate supplements (1000 mg/day) during one year (Yan *et al*, 1996).

3.2.4.4. Phosphorus

Calcium acetate and calcium carbonate bind phosphate in the intestinal lumen and are given in chronic renal failure (up to 2 g calcium/day) to reduce phosphorus absorption in the intestine. This inhibitory effect on phosphorus absorption can also be demonstrated in healthy humans; 1000 mg calcium doses reduced phosphorus absorption by 58%. In view of the usual high dietary phosphorus intake this effect is without significance (Whiting and Wood, 1997).

3.2.5. Cytogenetic effects

An increase in the number of micronucleated erythrocytes (damaged red blood cell precursors which are normally selectively removed from peripheral blood by the spleen) have been reported in those of splenectomised subjects who regularly used calcium supplements. However, no data on dietary or supplemental calcium intake were given (MacGregor, 1990; Smith *et al*, 1990). These findings do not provide evidence that calcium supplements damage cells.

4. DOSE-RESPONSE ASSESSMENT

4.1. Kidney function

A trend for an increase in serum creatinine (by 1.2 µmol/l) with calcium supplements of 1000 and 2000 mg during 3 years in addition to dietary intakes of around 1000 mg/day (total intake 2000 and 3000 mg/day) was seen in a study involving 130 perimenopausal women (Elders *et al*, 1994). No effects on serum creatinine were reported in 46 women aged between 50 and 70 years who ingested calcium supplements of 1000 mg daily over one year in addition to a dietary calcium intake of 1290 mg/day (Schaafsma *et al*, 2002).

In conclusion, some perimenopausal women with total calcium intakes between 2 and 3 g/day may show a tendency for compromised glomerular function as indicated by increases in serum creatinine. No such effect was observed in another study with women receiving comparable calcium amounts. This finding should be investigated systematically before it is attributed to calcium.

4.2. Milk-alkali syndrome

Manifestation of the milk-alkali syndrome through the combined intake of calcium both from food and especially from supplements and of absorbable alkalinising substances is facilitated by renal insufficiency, alkalosis and dehydration due to vomiting and anorexia and/or the use of thiazide diuretics, which increase renal tubular calcium reabsorption. All reported cases of milk-alkali syndrome in association with the prolonged or acute ingestion of calcium supplements used calcium carbonate as the nutrient source. In these reports the supplemental calcium intakes were reported as between 1.0 and 23 g/day. These patients also differ in their medical history, use and duration of use of drugs and alkali consumption, and their diets. Their dietary calcium intakes are often not known.

The FNB of the IOM (1997) has taken the approximate median of 4.8 g of reported calcium supplements (the same value derives from our extended list) as the LOAEL for total calcium intake, applied an uncertainty factor of 2 and defined an upper level of 2.5 g calcium/day. From the number of reported cases with milk-alkali syndrome and calcium supplement intakes below or equal to 2.5 g/day (11 of 82) in the list in Annex II, this definition of the LOAEL is not appropriate. Seven of these low-supplement users are reported not to have an additional high dietary calcium intake (>0.9 g/day). Only five of these eleven are reported to have ingested additional sodium bicarbonate or other antacids. Moreover, it is questionable if it is justified to derive a LOAEL for the total dietary calcium intake from data on effects of alkalinising substances plus calcium.

The use of calcium carbonate supplements in doses up to 2000 mg/day, and thereby achieving total daily calcium intakes up to more than 3000 mg/day, for preventive purposes in presumably healthy subjects, has not provoked the development of the milk-alkali syndrome, whereas the administration of large amounts (11.2 g calcium/day) of calcium carbonate in addition to large amounts of milk (1.8 g

calcium/day) over 7 days to 20 gastric/duodenal ulcer patients resulted in reversible hypercalcaemia (2.8 mmol/L) in nine patients and renal insufficiency in all. The control group of 20 patients with gastric/duodenal ulcers who received aluminium hydroxide and milk for the same duration did not develop these abnormalities (McMillan and Freeman, 1965).

A patient with a 15-year history of calcium carbonate use (3.3 g/day) had recurrent episodes of severe hypercalcaemia. He was known to have diabetes mellitus, hypothyroidism and renal insufficiency and it is not known if the renal insufficiency was the consequence of recurrent hypercalcaemic episodes or if it was the promoting factor (Smit and Bijvoet, 1986).

Hypercalcaemia occurred in 65 of 297 patients who had undergone major cardiac surgery and who received between 1.3 and 10 g of calcium/day as carbonate (total daily intake 7 to 11 g calcium) for peptic ulcer prevention. It was accompanied by renal failure in 50%, which developed within days of starting the regimen in a few patients, and was completely reversible after stopping calcium carbonate (Kapsner *et al*, 1986).

Cases of milk-alkali syndrome have been reported with long-standing calcium intakes in the range of 2 to 2.5 g/day with chronic high intakes of antacids (Barragry and Counihan, 1975; Gibbs and Lee, 1992) and of low supplemental calcium intakes (1g/day) in addition to unknown dietary intakes plus sodium bicarbonate (Abreo *et al*, 1993). These observations seem to indicate that the harmful calcium dose can be lower than 3 g/day if taken together with alkali.
In conclusion, on the basis of the available evidence, a calcium dose which by itself might cause milk-alkali syndrome cannot be identified.

4.3. Kidney stones

The quantitative relationship between calcium intake, both from the diet and from supplements, and hypercalciuria as a risk factor for nephrolithiasis is far from clear. Also, it is dependent on other dietary factors, especially sodium intake. From epidemiologic studies it appears that dietary calcium intakes in the range of recent recommendations have a favourable effect in the prevention of kidney stone formation and that lower intakes increase the risk (Curhan *et al*, 1993 and 1997; Sowers *et al*, 1998).

The influence of a controlled diet for 3 days (1000 mg calcium, 100 mmol sodium, 32.3 mmol potassium/day and 1 g protein/kg body weight/day) and of an oral calcium tolerance test (1000 mg) on urinary calcium excretion was investigated in 124 patients with hypercalciuria (more than 4 mg/kg/day or more than 300 mg/day in men and more than 250 mg/day in women of calcium excreted) identified from 282 patients with calcium oxalate stones. The strongest correlation was found between urinary calcium and sodium. Calcium excretion was less strongly correlated with calcium intake, sodium intake, phosphorus intake, carbohydrate and protein intake. From the regression equation derived from these investigations (Burtis *et al*, 1994) hypercalciuria in men would be associated with a calcium intake of 2243 mg/day and in women with a calcium intake of 1422 mg/day assuming a moderate sodium excretion of 100 mmol/day. A higher sodium intake (e.g. 150 mmol/day) would result in even lower hypercalciuric calcium intakes, 1685 mg for men and 866 mg/day for women, which are lower than the recommended calcium intake in many countries. The validity of these calculated predictions has never been systematically investigated in hypercalciuric subjects.

From the available data no conclusion is possible on a detrimental calcium dose in individuals with idiopathic hypercalciuria (up to 6% of the population). From the study in patients with kidney stones and idiopathic hypercalciuria it can be deduced that a sodium restricted diet with a normal recommended calcium content of 1200 mg/day does not raise urinary calcium excretion but reduces it (Borghi *et al*, 2002).

Hypercalciuria which is a risk factor for kidney stone formation has been observed in three of 50 infants receiving 1200 mg of supplemental calcium/day (Dalton *et al*, 1997) and in postmenopausal women during 4 years of taking calcium supplements of 1600 mg six times as often as in unsupplemented women (Riggs *et al*, 1996). Different doses have not been systematically tested.

In conclusion, both observational studies on the relationship between total calcium intake and kidney stone incidence and interventional studies with calcium supplements do not allow definition of a calcium intake on a population basis which promotes kidney stone formation. On dietary calcium intakes in the range of the recommended dietary intake the risk of nephrolithiasis is determined by other dietary components and by genetic factors.

In persons with idiopathic hypercalciuria, which is in itself a heterogeneous disorder, the risk of stone formation is not increased with calcium intakes in the range of recommended intakes, when sodium intake is restricted (Borghi *et al*, 2002). Higher dosages have not been tested.

4.4. Interaction with minerals

The studies of acute effects of single calcium supplements at various doses and from various sources on iron and zinc absorption (Spencer *et al*, 1984; Hallberg *et al*, 1991) cannot be converted into general statements on a dose dependent negative effect of total daily dietary calcium intake, because the timing of the supplement and other interfering factors of the diet have to be taken into account.

Observational epidemiological studies on the influence of dietary calcium intake in different populations and age groups on parameters of iron status do not allow the identification of threshold values of calcium intake that lead to reductions in these parameters (Lynch, 2000). Intervention studies with calcium supplements up to 1200 mg/day in addition to dietary intakes between 280 and 1100 mg/day did not show adverse effects on iron status (Lynch, 2000). Negative interactions of calcium intakes in excess of 2000 mg/day that have been reported for iron, phosphorus, magnesium and zinc would be a problem only when these are ingested in inadequate amounts (Whiting and Wood, 1997).

In conclusion, single-dose experiments demonstrate interference of both dietary and supplemental calcium with the absorption of other minerals. This effect is not demonstrable in long-term observational and interventional studies at dietary calcium intakes in the range of recommended intakes and at supplemental calcium of up to 2000 mg/day in adults and up to 1200 mg/day in one study with infants (Dalton *et al*, 1997).

The decrease of serum zinc levels in 10 healthy adults after two weeks of a total calcium intake of 3000 mg/day (Raschke and Jahreis, 2002) is of insufficient power to consider it as a systematic effect. The cross-sectional study in seven countries which shows a dose dependent effect of calcium intake from dairy products on serum ferritin levels in young women did not define a threshold dose of calcium intake (van de Vijver *et al*, 1999).

4.5. Cytogenetic effects

The data are insufficient to allow conclusions to be drawn from the available studies.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

5.1. Adults

The Committee decided to base the derivation of an UL for calcium on the evidence of different interventional studies of long duration in adults, some of which were placebo-controlled and in which total daily calcium intakes of 2500 mg from both diet and supplements were tolerated without adverse effects. Because of the abundance of data the application of an uncertainty factor was considered unnecessary. An UL of 2500 mg of calcium per day for calcium intake from all sources is proposed.

5.2. Pregnancy and lactation

Large placebo controlled intervention studies for preventive purposes with supplemental calcium carbonate of up to 2000 mg calcium in addition to the calcium intake from the diet (>400 mg/day) have been conducted in more than 3000 pregnant women and no adverse effects have been reported. There are no data to suggest an increased susceptibility for lactating women. Therefore, the UL of 2500 mg calcium per day applies also to pregnant and lactating women.

5.3. Children and adolescents

Six percent of 50 infants who received a calcium-enriched formula after the third month of life (1700 to 1560 mg calcium per day after 4 and 9 months, respectively), developed hypercalciuria (Dalton *et al*, 1997). These data are insufficient to define an UL for infants.

No adverse effects of calcium citrate-malate supplements (500 to 1000 mg calcium over 1.5 to 3 years) and of extra dairy foods or foods fortified with milk extracts (700 to 820 mg calcium extra over one year) were reported in 217 children between 6 and 14 and 6.6 and 11 years, respectively in comparison to unsupplemented controls.

These data are considered insufficient to derive an UL for children and adolescents. The Committee decided that it was inappropriate to base the UL for calcium for this age group on the tolerable upper level for adults of 2500 mg calcium/day, with correction for differences in basal metabolic rate using scaling according to body surface area (body weight$^{0.75}$). For calcium deposition in bone during the growth period proportionality to lean body mass cannot be assumed. Therefore, the Committee cannot propose age-dependent ULs for children and adolescents.

6. CHARACTERISATION OF RISK

Data from European populations indicate that the intakes of calcium from all sources in adolescents and adults can be close to the UL in a small percentage of the population, especially in those taking supplements. In the United Kingdom the 97.5 percentile of calcium intake in men 16 to 49 year old is 1600 mg/day (EGVM, 2001). In the Netherlands with a traditionally high consumption of milk products the 95 percentile of calcium intake without supplements is 2100 mg per day in young men between 16 to 22 years old (Hulshof and Kruizinga, 1999). In Germany the mean calcium intake of male subjects between 15 and 24 years old is 2100 mg/day (Heseker *et al*, 1994), but some 10% of adolescents consume more than 2100 mg per day (Alexy and Kersting, 1999).

In Dutch children the 95 percentile of calcium intake in boys and girls between one and 4 years of age is around 1300 mg/day, it is between 1400 and 1700 mg/day in boys and girls 4 to 13 years of age (Hulshof and Kruizinga, 1999). Somewhat lower 97.5 percentile intakes of 1200 to 1500 mg/day have been observed in British children between 1.5 and 14 years of age. The 90 percentile of calcium intake of 750 German children participating in a longitudinal observational study was 800 to 1000 mg/day between age one and 2 years, 700 to 900 mg/day between age 4 to 6 years and 1000 to 1600 mg/day between age 7 to 14 years (Alexy and Kersting, 1999).

These calcium intakes are quite similar to the calcium intakes of 1100 to 1900 mg/day supplied in intervention trials with children between 6 and 14 years of age which studied the effect on bone mineral mass and bone density (Johnston *et al*, 1992; Lloyd *et al*, 1993; Chan *et al*, 1995; Bonjour *et al*, 1997).

In British infants the 97.5th percentile of calcium intake was 1400 mg/day (EGVM, 2001). In German non-breast-fed infants the 90th percentile of calcium intake was 700 to 900 mg/day (Alexy and Kersting, 1999).

Although there are no data to set a numerical UL for children and adolescents no appreciable risk has been identified even with current extreme levels of calcium intake in this age group.

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Annex I. Intervention studies with calcium supplements

No	Authors	Study aim	Study design	Dietary calcium mg/d	Calcium supplement mg/d	Calcium compound	Effects	Adverse effects
1	Johnston *et al*, 1992	Effects of calcium supplementation on BMD in identical twins n = 70 pairs	Randomised double-blind placebo-controlled				n = 20 prebubertal supplemental twins greater increases in BMD at 5 of 6 sites by 2.8 to 5.1% compared to control twin after 3 years There was no benefit in supplemented 23 twins who were in or post puberty	None reported
			control n = 70	908				
			supplement n = 70	894 (total 1612)	1000	citrate malate		
			age 6 to 14 y over 3 years One twin control for the other 45 pairs completed the study.					
2	Lloyd *et al*, 1993	Effect of calcium supplementation on bone acquisition n = 94	Randomised double-blind placebo-controlled				BMD increased by 1.3% more with supplement. Calcium excretion with supplement by 17 mg/24 h higher	None reported
			placebo control n = 48	935	-	citrate malate		
			supplement n = 46 age 11.3 years, 18 months	1020	350			
3	Chan *et al*, 1995	Effect of extra dairy products on bone n = 46	randomised, controlled				BMD increased by 10% more with supplement	None reported
			control n = 24	700				
			supplement n = 22	1400		dairy products		
			age 11 years, 12 months					
4	Bonjour *et al*, 1997	Effect of calcium supplementation on bone mass in prepubertal girls	double blind placebo-controlled age 6.9-9.4 y				None reported mean increment in bone mass greater at 6 sites; more so if spontaneous dietary Ca intake <860 mg/d; greater height 1 year after treatment ended effect persisted	None reported
			placebo n = 67	880		unfortified food		
			supplement n = 77	920	810	milk-extracted fortified foods		
			1 year					
			n = 100 placebo n = 54 supplement n = 55					
	Bonjour *et al*, 2001		n = 16 placebo n = 54 supplement n = 62				3.5 years after treatment ended BMD still higher in supplemented, also BMC and bone area, height	
5	Kochersberger *et al*, 1991	Effects of calcium supplementation on PTH and bone metabolism in elderly	Randomised double-blind placebo-controlled				Decrease in PTH with decrease $1,25\text{-}OHD_3$ Increase in urinary calcium from 120 to 188 mg/d creatinine	Constipation in supplemented
			placebo n = 24	726	-			
			supplement n = 26	708	1200	carbonate		
			66-83 y 6 months					

No	Authors	Study aim	Study design	Dietary calcium mg/d	Calcium supplement mg/d	Calcium compound	Effects	Adverse effects
6	Reid *et al*, 1993	Effect of calcium supplementation on BMD of postmenopausal women	Randomised placebo-controlled				BMD decline less in supplement group Serum PTH lower	None reported
			placebo n = 61	730	-	sucrose		
			supplement n = 61	760	1000	lactate gluconate carbonate		
			age 58 ± 5 2 years					
7	Elders *et al*, 1994	Effect of two doses of calcium supplementation on bone loss in perimenopausal women	Randomised controlled				Rate of bone loss and bone turnover less in supplemented in 1rst year, similar to control in 2nd year. Bone loss after 3 y in early menopausal women 3.2% in control versus 1.6% in supplement groups (lumbar and metacarpal)	Gastrointestinal discomfort → change to calcium citrate n = 35 Mean increase of serum creatine (1.2 µm/L) in supplement II, trend in supplement I
			control n = 84	1065	-	-		
			supplement I n = 66	994	1000	lactogluconate + carbonate		
			supplement II n = 64	1052	2000	lactogluconate + carbonate		
			age 46-55 y 2 years + 1 years					
8	Riggs *et al*, 1996	Effect of calcium supplementation on serum PTH, bone turnover and bone loss in the elderly	Randomised placebo-controlled				Decrease in serum PTH, bone resorption and bone loss (weak effects)	One case of hypercalcaemia in supplement group Hypercalciuria in 51 cases (44 supplemented. 7 unsupplemented)
			control n = 117		-			
			supplement n = 115		1600	citrate		
			menopausal women (66 ± 0.2 y) 4 years					
9	Heaney *et al*, 1999	Effect of dietary calcium supplementation on calcium economy in older adults	controlled intervention				Extra calcium decreased serum PTH by 9% and N-telopeptide in urine by 13%. Urine calcium increased by 21 mg/d. Bone specific alkaline phosphatase fell by 9% in both groups Serum IGF-1 rose by 10% in the milk group	Extra weight gain of 0.7 kg in milk group
			control n = 103	649-779	5-36			
			intervention n = 101	690-801	714-755	milk		
			age 55-85; men and women; 12 weeks					

No	Authors	Study aim	Study design	Dietary calcium mg/d	Calcium supplement mg/d	Calcium compound	Effects	Adverse effects
10	Peacock *et al*, 2000	Effect of calcium supplement or 25-OH-D on bone loss, bone turnover	Randomised double-blind placebo-controlled				Control group lost BMD at total hip, the Suppl. II group did not lose BMD. The 25-OH-D group was intermediate. Lowest fracture rate in group II, highest in group I	Constipation in group II One proband with kidney stone in group II
			placebo n = 129	629	-			
			supplement I n = 124	739	25(OH)D			
			supplement II n = 124	670	750	citrate malate		
			60-74 years over 4 y ♂ and ♀					
11	Dawson-Hughes and Harris, 2002	Influence of calcium plus vitamin D on protein intake effect on BMD in older adults	Randomised placebo-controlled				A higher protein intake had favourable effect on 3-y change in total body BMD in the supplemented group only	None reported
			placebo n = 184	755-940	-	-		
			supplement n = 158	809-855	500 17.5 µg Vit. D	citrate malate		
			Healthy ♂and♀, age > 65 y 3 years					
12	Schaafsma *et al*, 2002	Effect of two calcium supplements on femoral BMD, biochemical markers of bone and calcium metabolism in late postmenopausal women	Randomised double-blind placebo-controlled over 12 m age >50 <70 y				Increase of BMD femoral neck supplement II versus control, Supplement groups showed changes in serum markers of bone resorption and bone formation indicating decreased bone turnover	None reported No changes in serum calcium, phosphate, creatinine
			placebo n = 27		50	skimmed milk powder		
			supplement I n = 24	1294 ± 421	1000	egg-shell powder		
			supplement II n = 22		1000	carbonate		
			supplements provided Vit. D 10 µg Vit. K 80 µg Mg 350 mg					
13	Villar and Repke, 1990	Effect of calcium supplementation on risk of preterm delivery	Randomised double-blind placebo-controlled				Preterm delivery: (<37th week) placebo 21.1%, suppl. 7.4%. Low birth weight: placebo 21.1%, suppl. 9.6%. Duration of labour: placebo 9.9 h, suppl. 12 h suppl. increased gestation by 1.3 weeks birth weight by 189 g	None reported
			placebo n = 95	1200	-	-		
			supplement n = 94	1200	2000 (1500)	carbonate		
			age <17 y 23rd week of gestation					

No	Authors	Study aim	Study design	Dietary calcium mg/d	Calcium supplement mg/d	Calcium compound	Effects	Adverse effects
14	Belizán *et al*, 1991	Effect of calcium supplementation on hypertensive disorders of pregnancy	Randomised double-blind placebo-controlled				Hypertensive disorders Placebo 14.8%, Suppl. 9.8%. OR 0.63 (95% CI 0.44-0.9) Preeclampsia Placebo 3.9%; Suppl. 2.6% OR 0.65 (95% CI 0.35-1.25)	None reported
			placebo n = 588	642 ± 448	lactose			
			supplement n = 579	646 ± 396	4 x 500	carbonate		
			Nulliparous women 20 weeks pregnant					
15	Bucher *et al*, 1996	Effect of calcium supplementation during pregnancy on blood pressure, preeclampsia and adverse outcome metaanalysis	Metaanalysis 1966-1994 14 randomised placebo-controlled studies n = 2459		357-1500-2000		Reduction in systolic blood pressure by 5.4 mm Hg and in diastolic blood pressure by 3.44 mm Hg. Preeclampsia OR 0.38 (95% CI 0.22-0.65) for calcium supplements	
16	Levine *et al*, 1997	Effect of calcium supplementation during pregnancy on preeclampsia hypertension, adverse perinatal outcome	Randomised double-blind placebo-controlled				No significant differences for preeclampsia, hypertension nor in perinatal outcome	None. Women with increased risk for nephrolithiasis, with increased serum calcium and creatinine had been excluded
			placebo n = 2294	~1000	corn-starch	-		
			supplement n = 2295		2 x 1000 (total 2400)	carbonate		
			Nulliparous women 13-21 weeks pregnant age 21 ± 4 y					
17	Koo *et al*, 1999	Effect of maternal calcium supplement during pregnancy on fetal bone mineralisation	Randomised double-blind placebo-controlled Pregnant <22 w				No significant difference in infants in BMC, BMD total body and lumbar spine of infants Increase in BMC in infants of supplemented mothers in lowest quintile of spontaneous dietary calcium intake and with increasing maternal calcium intake of all source	Non reported
			placebo n = 128	1035 (83-3613)	-	-		
			supplement n = 128	1010 (83-3613)	2 x 1000 (mean 1300)	carbohydrate		
18	Baron *et al*, 1999	Effect of calcium supplementation on recurrence of colorectal adenomas	Randomised double-blind placebo-controlled				Recurrence risk ratio for supplement compared to placebo 0.85 (95% CI: 0.74-0.98) after one years; after 4 years 0.81 (95% CI: 0.67-0.99)	No difference between placebo and supplement
			placebo n = 466 completed n = 423	865	-	cellulose/sucrose		
			supplement n = 464 completed n = 409	889	1200	carbohydrate		
			age 61 y over 4 years					

No	Authors	Study aim	Study design	Dietary calcium mg/d	Calcium supplement mg/d	Calcium compound	Effects	Adverse effects
19	Sokoll and Dawson-Hughes, 1992	Effect of calcium supplementation on iron stores in healthy premenopausal women	Randomised controlled				No differences between control and treatment groups in plasma ferritin, serum iron, total iron binding capacity, transferrin saturation, haemoglobin, haematocrit	None reported
			control n = 52	610	-	-		
			supplement n = 57	559	1000	carbonate		
			12 weeks					
20	Yan *et al*, 1996	Effect of calcium supplementation on indices of iron, zinc, magnesium status in lactating women	Randomised double-blind placebo-controlled				No differences in status indices for zinc, iron, magnesium	None reported
			placebo n = 30	290	-	dextrose		
			supplement n = 30	280	1000 5 d/week	carbonate		
			One year 16-41 years					
21	Minihane and Fairweather-Tait, 1998	Effect of calcium supplementation on body iron in healthy adults	Controlled				No effect of calcium (400 mg per meal) supplementation on functional iron indices (Hb, haematocrit, zinc, proto-prophyrin, plasma ferriti	None reported
			control n = 13	980	-	-		
			supplement n = 11	1090	1200	carbonate		
			age 18-69 y over 6 months					
22	Kalkwarf and Harrast, 1998	Effect of calcium supplementation on iron status lactating women	Randomised double-blind placebo-controlled				No effect of calcium on iron status	None reported
			placebo n = 80	680-780		lactose		
			supplement n = 78	680-744	1000	carbonate		
23	Raschke and Jahreis, 2002	Effect of calcium and phosphorus on calcium and mineral metabolism	Intervention of 2 weeks each after 3 weeks control n = 10 healthy men				Increase of faecal calcium excretion and decrease of urinary calcium excretion with supplement II. No change on calcium balance No influence on magnesium metabolism No influence on iron status No influence on renal and faecal zinc excretion	Decrease in serum zinc control 1.1 mg/dl suppl. II 0.9 mg/dl
			basis	1800				
			supplement I	1800	600 (+800 mg P)	phosphate		
			supplement II	1800	1200 (+1600 mg P)			

Annex II. Milk-alkali syndrome (from 1965 to 2001)

No	Author	Patient No.	Male/ Female	Age (years)	Pre-existing disease	Calcium intake Total	Calcium intake Milk	Calcium intake supplements	Calcium salt	Alkalising drugs/antacids	Other drugs	Duration of Ca intake	Provoking factor	Ca in Serum mmol/L	Symptoms Renal failure	(Nephro) calcinosis	Outcome
1	Mc Millan + Freemann, 1965	A17	M	51	peptic ulcer 3 y	n.r.	high 2 g			Alka Seltzer, bicarbonate 2 y	reserpine	chronic 2 y					
							acute 1.8 g	11.2 g	carbonate	Al(OH)		4 d		4.5	+	-	hypertension
2-20				28-64	gastric/duodenal ulcer	n.r.	1.8 g	11.2 g	carbonate		-	7 day-test		8/19>2.8	creatine in serum increased creatinine clearance decreased by 19%	-	normal
														4/19>3.0			
21	Cameron + Spence, 1967		F	40	«heartburn», thirst 8 y	n.r.	< 0.8	6.4	carbonate	+ (Rennie)		11 y	thirst	2.85	+	+	partial renal insufficiency
22	Riley, 1970		M	66	duodenal ulcer	n.r.		3.2	carbonate	Al(OH)	-	30 y	anorexia, thirst	3.75	+	keratopathy	persistent renal insufficiency
										Mg(OH)							
23	Assari + Vennes, 1971		M	52	gastric ulcer	n.r.	4.5-6.8	-	-	sodium bicarbonate	-	2.5 y	salt-losing nephropathy, polyuria	2.8	+	-	persistent renal insufficiency
24	Danells *et al*, 1972	1	F	45	duodenal ulcer	n.r.	>2.4			bismuth	anticholinergica	3 y		4.0	+	+	persistent renal insufficiency
25		2	M	45	duodenal ulcer	n.r.	1.8-2.4	+	carbonate	magnesium trisiliconate	anticholinergica	14 d	vomiting	3.5	+	-	normal
26	Barragry + Counihan, 1975	1	M	35	duodenal ulcer	n.r.	1.8	-	-	60 g/d Soda	-	5 y	polyuria	3.0	+	-	normal
27		2	M	39	epigastric pain, hypertension	n.r.	3.4	+	carbonate	-	-	6 y + 7 y	-	2.3-3.7			chronic renal insufficiency
28	Junor + Catto, 1976	1	M	53	gastric ulcer 20 y	n.r.	large	-	-	sodium bicarbonate	-	20 y	vomiting	n.r.	+	+	normal
29		2	M	40	gastric ulcer 25 g	n.r.	4.5	-	-	sodium bicarbonate	-	25 y	-	n.r.	+	+	persistent renal insufficiency
30		3	M	47	duodenal ulcer >25 y	n.r.	large	-	-	sodium bicarbonate	-	> 25 y	vomiting	n.r.	+	+	persistent renal insufficiency
31	Rochman *et al*, 1977		M	60	duodenal ulcer	n.r.	n.r.	11-23	carbonate	-	-	3 y	vomiting	3.0	+	+	renal acidosis
32	Frame *et al*, 1981		F	51	Münchhausen syndrome	n.r.	n.r.	5-15	carbonate	-	thiazide	months	-	5.0	(+)	-	normal
33	Hart *et al*, 1982		M	65	duodenal ulcer	n.r.	2.3-4.5	+	carbonate	sodium bicarbonate	-	many years	constipation, dehydration	3.5	+	keratopathy	normal
34	Roberts + Tuthill, 1984		M	57	indigestion anxiety	n.r.	n.r.	7.2	carbonate	+	aspirin cimetidine	several months	vomiting	3.4	+	soft tissue calcification	normal

No	Author	Patient No.	Male/ Female	Age (years)	Pre-existing disease	Calcium intake			Calcium salt	Alkalising drugs/antacids	Other drugs	Duration of Ca intake	Provoking factor	Symptoms			Outcome
						Total	Milk	supple-ments						Ca in Serum mmol/L	Renal failure	(Nephro) calcinosis	
35	Schumann + Jones, 1985	1	M	32	gastric ulcer 10 y	n.r.	4.5	-	-	bismuth	cimetidine acetaminophene	6 w	vomiting	3.5	+	-	normal
		2	M	43	epigastric pain, renal failure 14 m	9.8	2.3	+	carbonate	-	-	20 y	-	3.3	+	soft tissue calcification	persistent renal insufficiency
36	Dorsch, 1986		F	52	epigastric pain 8 y, renal failure 6 m	n.r.	n.r.	2.1	carbonate	-	-	8 y	vomiting, weight loss	3.3	+	-	normal
37	Smit + Bijvoet, 1986		M	55	diabetes mellitus, hypothyroidism, renal insufficiency			3.3	carbonate	Rennie +	insulin thyroxine	15 y	dehydration	4.6 recurrent	+	band keratopathy	normal, no diabetes mellitus
38	French *et al*, 1986	1	M	49	gastritis, indiges-tion, hypertension	8	0.8	6.7	carbonate	+	-	years	polyuria, polydipsia 2 w	3.7	+	-	normal
39		2	M	43	hypertension, renal failure	n.r.	n.r.	4.2	carbonate	+	indomethacin thiazide	many years	-	4.0	+	-	normal
		3	F	62	pyelonephritis, analgesic abuse	5	n.r.	4.2	carbonate	+	-	5 y	-	4.0	-	-	normal
		4	F	71	malaise, kidney stones, renal failure	n.r.	n.r.	n.r.	carbonate	+	-	many years	-	3.5	+	-	normal
40	Kapsner *et al*, 1986	1	F	32	cardiac transplantation	>0.6	n.r.	10	carbonate	-	hydrochlo-rothiazide prednison	10 m	vomiting	5.5	+	-	normal
41		2	M	24	cardiac transplantation	>3.8	n.r.	3.2	carbonate	-	furosemide + prednison	10 m	-	6.7	+	-	normal
42		3	M	40	cardiac transplanta-tion, kidney stones	n.r.	n.r.	4.8	carbonate	-	prednison	3 d	-	3.1	+	-	normal
		65/297 cardiac tran-splant patients	52 M 13 F	9-64	peptic ulcer prevention	n.r.	n.r.	1.3-4.8	carbonate	-	prednison	1 w - > 6 m	-	2.7->3.5	37/65	-	normal
43	Kallner + Karlsson, 1987		F	41	anorexia nervosa, bulimia	up to 16		-	-	-	chlorthalidone	12 y	vomiting	4.0 recurrent	+	-	normal
44	Bullimore + Miloszewski, 1987		F	35	achalasia	6	2.3	3	carbonate	Rennies +	-	20 y	nausea, vomiting	4.1	+	-	normal
45	Jenkins *et al*, 1987		M	65	epigastric pain, oesophagitis	n.r.	n.r.	2.4-4.8	carbonate	+	triamterene hydrochlo-rothiazide	1 year, dose increase 2 w	dehydration	5.5	+	-	normal
46	Canning + Slater, 1987		F	61	duodenal ulcer 8 y, renal failure	n.r.	n.r.	1.1	carbonate	sodium bicarbonate	-	8 y	vomiting, dehydration	4.3 recurrent	+	+	persistent renal insufficiency
47	Schaefers, 1987		M	46	alcoholic gastritis	n.r.	n.r.	4.6	carbonate	sodium bicarbonate +	-	many years	vomiting	3.7	+	-	persistent renal insufficiency

No	Author	Patient No.	Male/ Female	Age (years)	Pre-existing disease	Calcium intake			Calcium salt	Alkalising drugs/antacids	Other drugs	Duration of Ca intake	Provoking factor	Symptoms			Outcome
						Total	Milk	supple-ments						Ca in Serum mmol/L	Renal failure	(Nephro) calcinosis	
48	Ullian + Linas, 1988		F	31	hyperemesis of pregnancy	n.r.	large amounts	+	carbonate	-	-	weeks	vomiting 3 d, diarrhea	3.6	+	-	stillborn fetus 37 w with malformation of limbs and ears
49	Gora *et al*, 1989		M	47	hypertension, kidney stones, hypothyroidism	n.r.	n.r.	3-4	carbonate	-	hydrochlo-rothiazide thyroxine	2 y	-	3.4	+	-	normal
50	Kleinman *et al*, 1991		F	31	pregnancy 36 w, nausea	n.r.	1.2	6	carbonate	+	-	2 w	vomiting 3 d	5.6	+	-	normal (child normal 40 w)
51	Gibbs + Lee, 1992		M	51	peptic ulcer	n.r.	2.4	-	-	+ AlOH Mg carbonate sodium bicarbonate	-	years	vomiting, polyuria, polydipsia	3.5	+	keratopathy	normal
52	Nakanishi *et al*, 1992		M	74	cerebral infarction constipation	n.r.	0.4	n.r.	-	magnesium oxide	-	weeks	dehydration	3.6	+	-	n.r.
53	Abreo *et al*, 1993	1	M	60	diabetes mellitus, hypertension	n.r.	n.r.	1	carbonate	sodium bicar-bonate	insulin	15 y	vomiting 1 w	3.8	+	(+)	persistent renal insufficiency
54		2	M	60	dyspepsia	n.r.	2.3	7.2	carbonate	-	-	> 3 m	venous thrombosis	3.3	+	-	normal
55		3	F	54	breast cancer, peptic ulcer	n.r.	n.r.	+	carbonate	-	-	years	-	3.7	+	-	persistent renal insufficiency
56		4	M	53	diabetes mellitus, coronary artery disease	n.r.	> 2.7	6	carbonate	-	-	3 m	nausea 2 m	3.4	+	-	persistent renal insufficiency
57		5	M	65	hypertension	n.r.	> 0.8	3	carbonate	-	reserpine enalapril hydrochlo-rothiazide	several years	vomiting	3.3	+	-	persistent renal insufficiency
58	Newmark + Nugent, 1993		M	55	kidney stones, hypertension, chronic pulmonary disease	n.r.	n.r.	8.4	carbonate	+	-	30 y	vomiting 5 d	4.0	+	-	normal
59	Campbell *et al*, 1994		M	53	epigastric pain		0.6	4.5	carbonate	-	-	months	3 w of thirst, polyuria, vomiting	3.6	+	-	normal
60	Brandwein + Sigman, 1994		M	47	peptic ulcer	5.4	n.r.	2-3	carbonate	-	-	several years	nausea, vomiting 1 w	3.9	+	-	normal
61	Beall + Scofield, 1995	1	F	45	dyspepsia	n.r.	n.r.	2.4	carbonate	+	prednison	1 year	increase of calcium dose 2 w	4.9	+	-	normal
62		2	F	42	peptic ulcer	n.r.	1.2	2.4-4	carbonate	-	-	2 w	vomiting	3.0	-	-	normal
63		3	F	34	fever	n.r.	n.r.	1.6-2.5	carbonate	prednison	prednison	1 m	increase calcium dose	3.0	-	-	normal

No	Author	Patient No.	Male/ Female	Age (years)	Pre-existing disease	Calcium intake Total	Milk	supple-ments	Calcium salt	Alkalising drugs/antacids	Other drugs	Duration of Ca intake	Provoking factor	Symptoms Ca in Serum mmol/L	Renal failure	(Nephro) calcinosis	Outcome
64		4	M	60	n.r	n.r.	n.r.	1.6-4.6	carbonate	n.r.	n.r.	n.r.	n.r.	3.5	n.r.	n.r.	n.r.
65		5	F	58					carbonate					3.5			
66		6	F	53					carbonate					4.9			
67		7	F	95					carbonate					2.8			
68	Duthie *et al*, 1995		M	65	hypertension, diabetes mellitus	n.r.	n.r.	6-12	carbonate	sodium bicarbonate	-	1.5 y	dehydration	4.3	+	soft tissue calcified	persistent renal insufficiency
69	Muldowney + Mazbar, 1996		F	35	bulimia 15 y, chronic vomiting	n.r.	> 0.9	0.8	carbonate	-	-	recently	nausea, constipation	4.0	+	-	normal
70	Wu *et al*, 1996	1	M	55	bladder stones, betelnut chewing	n.r.	n.r.	3.5	carbonate	-	piroxicam	30 y	-	3.4	+	+	persistent renal insufficiency
71		2	M	63	epigastric pain, betelnut chewing	n.r.	n.r.	2.5	carbonate	-	famotidine	> 30 y	-	3.8	+	nephrolithiasis	persistent renal insufficiency
72	Olschewski *et al*, 1996		M	54	duodenal ulcer 10, kidney stone	n.r.	1.2-2.4	2.4	carbonate	+ bismuth	doxepin chlormezanon bezafibrate	4 y	nausea	3.9	+	+	persistent renal insufficiency
73	Lin *et al*, 1996		F	70	osteoporosis	n.r.	n.r.	3.75	carbonate	-	0.5 µg calcitriol	4 w	anorexia, dehydration	4.0	+	soft tissue calcification	normal
74	Fiorino, 1996		M	66	alcoholism	n.r.	n.r.	+	carbonate	+	laxatives	n.r.	anorexia, vomiting 3 w	4.4	+	-	death, multiorgan failure
75	Fitzgibbons + Snoey, 1999		M	39	peptic ulcer, alcoholism	n.r.	n.r.	+	carbonate	sodium bicarbonate	-	n.r.		3.1	+	-	persistent renal insufficiency
76	George + Clark, 2000		F	44	dyspepsia, kidney stones	n.r.	n.r.	2.7	carbonate	-	-	2-3 y	vomiting 2 d	4.0	+	-	normal
77	Vanpee *et al*, 2000		M	64	carcinoma, renal insufficiency 2 y	n.r.	n.r.	2.7	carbonate	-	-	2 w dose increase	vomiting, dehydration 1 w	3.5	+	+	persistent renal insufficiency
78	Grundfast *et al*, 2001		M	59	gastric/duodenal ulcers	n.r.	n.r.	6-10	carbonate	-	+	n.r.	vomiting 2 d, anorexia 2 w	4.1	+	-	n.r.
79	Carroll + Clark, 1983	1	M	50	duodenal ulcer	n.r.	2.3	1	carbonate	sodium bicarbonate	-	10 y	polyuria, polydipsia 6 m	3.2	+	+ soft tissue calcification keratopathy	normal
80		2	M	60	oesophagitis	n.r.	2.3	7	carbonate	-	-	years	n.r.	4.0	+	+ soft tissue calcification	persistent renal insufficiency
81		3	M	60	tongue, carcinoma, pain	n.r.	2.3	0.5	carbonate	-	Vit A 6 mg, Vit E 2 g	2 m	n.r.	3.2	+	+ soft tissue calcification	normal, died from carcinoma
82		4	M	77	dyspepsia	n.r.	n.r.	0.6-2	carbonate	-	-	5 y	anorexia 2 w	4.5	+	-	normal

OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN E

(EXPRESSED ON 4 APRIL 2003)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Vitamin E is the term used to describe a group of related fat-soluble tocochromanols, including eight naturally occurring components, which exhibit antioxidant activity and are nutritionally essential. The two major homologous series of tocochromanols, the tocopherols and tocotrienols, both have vitamin E activity in humans and animals and are synthesised by higher plants and cyanobacteria.

In all homologues, the basic structural unit is a chroman ring system (2-methyl-6-hydroxychroman) with an isoprenoid side chain of 16 C atoms. The compounds, including α-, β-, γ-, and δ-homologues, differ in number and position of the methyl substituents in the chroman ring. Tocopherols differ from their corresponding tocotrienols in having a saturated side chain. The presence of the phenolic hydroxyl group in the tocochromanols is important for their activity as antioxidants. At least one methyl group in the benzene ring is of primary importance. α-Tocopherol with three methyl groups is the most active of all homologues, followed by β-, γ-, and δ-tocopherol. The only forms retained in human plasma are the RRR-α-tocopherol and the 2R-stereoisomers, RSR-, RRS- and RSS-α-tocopherol; the various 2S-stereoisomers (SRR-, SSR-, SRS- and SSS-α-tocopherol) which form part of synthetic *all rac*-α-tocopherol are not maintained in plasma (Traber, 1999). The vitamin E activity is expressed as *RRR*-α-tocopherol equivalents, which accounts for about 90% of the activity in human tissue; the relative potency of α-, β-, γ-, and δ-tocopherol is reported to be approximately 100:50:25:1. The commercially available synthetic form is all rac-α-tocopheryl acetate with the activity of 0.67 x *RRR*-α-tocopherol. For practical purposes, 1 International Unit (I.U.) of vitamin E is referred to as 1 mg of all rac-α-tocopheryl acetate (Schäfer and Elmadfa, 1984; Elmadfa and Leitzmann, 1998).

In the following report the term vitamin E is related to α-tocopherol equivalents.

2. NUTRITIONAL BACKGROUND

2.1. Occurrence in food

The major food sources of vitamin E are vegetable oils, unprocessed cereal grains, and nuts with smaller amounts in fruits and vegetables and meats (mainly the fatty portion).

2.2. Dietary intake of vitamin E

As indicated above, only the *RRR*-α-tocopherol from food and the 2*R*-stereoisomeric forms that occur in supplements and fortified foods are retained in the body because α-tocopherol transfer protein has an affinity only for these isomers . However, most nutrient data bases and survey data do not distinguish between the various tocopherols in food. Consequently, the data are presented as α-tocopherol equivalents which include all eight naturally occurring forms.

Table 1. Estimated intakes of vitamin E (mg TE/day)

Country	Type of survey	n	Method	Supplements*	Mean	97.5%
Austria[a]	Individual	2488	24h recall	Not defined	11.8	30.6
Germany	Individual (M) Individual (F)	854 1134	7-day dietary record	- -	14.6[b] 12.3[b]	33 28
UK[c]	Individual (M) Individual (F) Individual (M) Individual (F)	1087 1110 1087 1110	7-day weighed inventory	- - + +	9.9 (9.3) 7.2 (6.7) 11.7 (9.3) 8.6 (6.8)	19.5 15.2 23.4 20.4
Italy[d]	Household	2734	7-day record	+	11	22
Netherlands[e]	Individual	5958	2-day record	-	12.5	28.1
Ireland[f]	Individual (M) Individual (F)	662 717	7-day estimated food record	+ +	11.2 11.0	28.3 38.3

* + data included supplements; - data excluded supplements.
[a] Elmadfa *et al* (1998).
[b] Heseker *et al* (1994) - values are the median.
[c] Gregory *et al* (1990) - values are the mean with the median in parentheses.
[d] Turrini (1996).
[e] Hulshof and Kruizinga (1999).
[f] IUNA (2001).

2.3. Absorption and metabolism

The bioavailability of vitamin E is related to the efficiency of absorption. Intestinal absorption of lipids and fat-soluble vitamins depends on pancreatic function, biliary secretion to form micelles with the hydrolysed fat, and transfer across intestinal membranes. Nearly all of the vitamin E absorbed across the intestinal mucosa is free tocopherol. *In vivo* and *in vitro* studies suggest that the rate of uptake of vitamin E is controlled by passive diffusion. Absorption of tocopherols is incomplete; the extent of absorption is dependent on intake and varies between 20-80%. The proportion absorbed decreases with increasing amount added to experimental diets; the average absorption is about 40-60% while pharmacological doses of 200 mg and more are absorbed to the extent of <10%.

Cannulation studies indicate that there is no difference in absorption between α-tocopherol and α-tocopheryl acetate at physiological doses. At high levels of intake, (>400 IU/day) a higher degree of absorption was obtained with free tocopherol than tocopheryl esters.

About 90% of the free α-tocopherol is transported via the lymphatic system into the bloodstream, where it is distributed into lipoproteins on passage into the liver. The main systemic transport system of tocopherols is the LDL-fraction (55-65%) followed by the HDL (24-27%) and VLDL (8-18%). There is very close correlation (r=0.925) between the total serum α-tocopherol and that portion carried by LDL.

2.4. Mode of action and nutritional requirements

The basic mode of action of tocopherols in human tissue is to prevent the oxidation of polyunsaturated fatty acids (PUFA) by trapping free radicals and donating hydrogen. It is effective in protecting the integrity of lipid and phospholipid in membranes and thus the requirement for vitamin E and the recommended intake is determined to a large extent by the intake of PUFAs. It has been shown that increasing the PUFA content of a diet low in α-tocopherol equivalents has adverse effects on tocopherol status (Horwitt, 1974; SCF, 1993).

In human metabolism, vitamin E is known to interact with other nutrients which are also involved in the pathways of oxidation processes. Vitamin C, selenium and zinc interact synergistically with vitamin E. Conversely, an iron overload is associated with a lowering of serum vitamin E levels.

Results from animal models and epidemiological studies in humans suggest that vitamin E may protect against cancer. The most consistent associations have been reported for cancers of the lung, oesophagus and colorectum. Three intervention trials showed an inverse rel;ationship between vitamin E intake and cancer risk: LINXIAN study (Blot *et al*, 1993), ATBC study (Heinonen *et al*, 1998) and

Polyprevention study (Greenberg *et al*, 1994). However, in the LINXIAN study, the protective effect for oesophageal and gastric cancer was associated with co-administration of vitamin C, E and selenium and in the Polyprevention study there was no effect on the incidence of colorectal adenomas. The ATBC study did show a protective effect of vitamin E on mortality from prostate cancer.

Although the evidence is stronger for prevention of coronary heart disease, only one of four double-blind, placebo-controlled trials, the Cambridge Heart Antioxidant Study (CHAOS), had a positive result (Stephens *et al*, 1996). Two other trials (the GISSI-Prevenzione Trial and the Heart Outcomes Prevention Evaluation [HOPE] Study) were neutral (GISSI-Prevenzione Investigators 1999 and HOPE study Investigators 2000). In addition, the ATBC Cancer Prevention Study reported no beneficial effects on myocardial infarction rates (ATBC Cancer Prevention Study Group, 1994).

In a recent placebo-controlled trial of the effect of antioxidant vitamin supplementation in 20536 high-risk individuals aged 40-80 years, vitamin E (600 mg) was administered along with vitamin C (250 mg) and beta-carotene (20 mg) daily over a 5 year period. There were no significant differences in all-cause mortality nor deaths due to vascular or non-vascular causes. There were no effects on cancer incidence nor on hospitalisation for an other cause. The study group concluded that in this group, these vitamins were safe but were ineffective in producing significant reduction in 5-year mortality from any cause (Heart Protection Study Collaborative Group, 2002)

A randomised controlled trial was conducted in 1193 healthy volunteers aged 55-80 years to determine whether vitamin E supplementation (500 IU daily) influenced the incidence or rate of progression of age-related maculopathy. After 4 years there was no indication that vitamin E prevented the development of macular degeneration (Taylor *et al*, 2002)

2.5. Vitamin E requirements

The major problem in making recommendations for vitamin E is the dependence on the PUFA intake. Across Europe there are wide variations in PUFA consumption. The intakes are normally distributed but high values are common. Based on the strong relation between vitamin E requirements and PUFA, recommendations have to take into account the different intake of PUFAs in different population groups. Therefore the recommended intakes are given as the ratio mg α-tocopherol equivalents: 0.4 mg x g dietary PUFA. There is no evidence that this level is inadequate for anyone, and it is used by several different countries and organisations (SCF, 1993; Yasuda, 1993; D-A-CH Referenzwerte, 2000). In view of the difficulty in recommending the amount of vitamin E with the optimal effects on human metabolism, the recommendations for vitamin E expressed as α-tocopherol equivalents for adults differ world-wide.

Several double-blind placebo-controlled trials of the efficacy of supplementary vitamin E in preventing or ameliorating CHD are currently in progress but the US Food and Nutrition Board of the National Academy of Science concluded that the evidence presently available does not allow recommendations for higher intakes of vitamin E to be made.

2.6. Nutritional status for vitamin E

Normal plasma vitamin E concentrations in humans range from 12-45 μM (0.5-2 mg/dL). The most important factor influencing the vitamin E plasma concentrations appears to be the content of total lipids. The plasma concentrations alone, however usually do not directly reflect the intake of vitamin E and there is a strong correlation between vitamin E intake and fat intake (Bramley *et al*, 2000).

2.7. Vitamin E deficiency

Vitamin E deficiency in animals is associated with a progressive necrosis of the nervous system and muscle. Chronic marginal deficiency can be generally characterised by an enhanced susceptibility to lipid peroxidation and corresponding lipofuschinosis. In rats this first results in weakening of the basement membranes of the muscle capillaries and a breakdown of endothelial cells. Later, a subendothelial fibrosis arises in combination with fibrous and calcified lesions and necrosis in the media of the aorta. In piglets, vitamin E deficiency leads to a combination of myocardial necrosis with widespread thrombosis of the myocardial circulation.

2.7.1. Sensitive sub-populations

In humans, vitamin E deficiency causes a proliferative vasculopathy in premature, neonatal infants, neuropathological disturbances, cardiomyopathy and haematological disorders in children and adults (for review see Elmadfa and Bosse 1985; Gey, 1993).

As vitamin E is a component of many different foods, a deficiency arising from low dietary intake is improbable. Therefore the ratio of 0.4 mg α-tocopherol equivalents per g dietary polyunsaturated fatty acids expressed as dienoic acid, is valid, provided that the intake does not fall below 4 mg/d for adult men and 3 mg/d for adult women (SCF, 1993; Elmadfa and Leitzmann, 1998).

The sub-populations most likely to have a deficiency of vitamin E are:

- Premature infants and full-term infants of low birth weight (<2500 g);
- Patients with gastrointestinal or hepatic disorders with malabsorption syndromes;
- Subjects with A-β-lipoproteinaemia.

Especially in low-birth-weight infants, iron administration may lead to the development of vitamin E-deficiency anaemia (Melhorn and Gross, 1971; Dallman, 1974), particularly in infants who are also fed a high PUFA formula. These infants are also known to be more susceptible to oxygen injury but without having a high storage capacity and thus have higher vitamin E requirements in ratio to their body weight (D-A-CH Referenzwerte, 2000).

3. HAZARD IDENTIFICATION AND CHARACTERISATION

3.1. Toxicological data in animals

3.1.1. Acute toxicity

Vitamin E has a very low acute oral toxicity. The LD_{50} for α-tocopherol *per se* is greater than 2000 mg/kg body weight in mice, rats (adult and neonate) and rabbits and for the succinate ester it is >7000 mg/kg body weight for young adult rats of both sexes (Krasavage and Terhaar, 1977).

3.1.2. Sub-chronic toxicity

In rats given α-tocopheryl acetate by gavage at doses of 125-2000 mg/kg bw/day, TSH levels were elevated by 30-100%. At a dose of about 500 mg/kg bw/day biochemical indices of hepatotoxicity (serum alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase) were elevated and liver weight was increased. The NOAEL for these effects was 125 mg RRR-α-tocopheryl acetate/kg body weight (Abdo *et al*, 1986).

3.1.3. Chronic toxicity

Two long-term studies of up to 16 months and 2 years duration respectively have been conducted in rats (Yang and Desai, 1977; Wheldon *et al*, 1983). In the second of these studies, the animals received doses of 500, 1000 or 2000 mg dl-α-tocopheryl acetate/kg bw/day. At all dose levels between 15 and 18 weeks the male animals developed spontaneous haemorrhages in the gut, urinary tract, meninges, orbit and at sites of minor injury. This led to some mortality but in survivors the condition was corrected by administration of 10 mg vitamin K_3/kg body weight. The only other treatment-related effect of significance was the presence of vacuolated lipid staining macrophages in the liver.

Vitamin E displayed no evidence of carcinogenicity in either study. However, a NOEL could not be established in the latter study with respect to effects on blood clotting and liver histology (WHO, 1986).

3.1.4. Reproductive toxicity/teratogenicity

The results of reproductive toxicity studies in rats indicated that vitamin E (administered as the water-soluble d-α-tocopherol (polyethylene glycol 1000 succinate) did not have adverse effects on reproductive function at doses of up to 2% of the diet (Krasavage and Terhaar, 1977) and d-α-tocopherol was not teratogenic in mice (Hook *et al*, 1974).

3.1.5. Genotoxicity

No studies designed to investigate the potential genotoxicity of vitamin E *per se* were identified. However, in studies of the modulating effect of vitamin E on the mutagenicity/clastogenicity of other genotoxic compounds, there were no indications of genotoxicity in vitamin E controls.

In investigations of the potential anticlastogenic activity in human lymphocytes *in vitro*, vitamin E did not induce chromosomal damage or sister chromatid exchange (Gebhart *et al* 1985).

In the *Salmonella typhimurium* assay, dl-α-tocopherol caused a significant decrease in point mutations induced by malonaldehyde or beta-propiolactone (Shamberger *et al* 1979).

In a sex-linked recessive lethal mutation assay in Drosophila, alpha-tocopheryl acetate in the nutrient medium at 500 IU/kg did not affect the mutation rate in irradiated males but caused a significant reduction in lethal mutations in subsequent generations bred from unirradiated females (Beckmann *et al* 1982).

3.2. Human studies

There are many reports in the literature dealing with the toxicity of vitamin E in human subjects. It is important to distinguish between these studies in degree and reliability. Some papers report a single observation on one subject, others planned studies with placebos with and without double blinding.

It is noted that inconsistent adverse effects of vitamin E were observed in the uncontrolled studies (Kappus and Diplock, 1992; Diplock, 1995).

One of the reported adverse effects concerns decreased blood coagulation. In a published case report, a prolonged bleeding time was found during chronic warfarin therapy in a man taking 800 mg α-tocopherol equivalents (1200 IU) (Corrigan and Ulfers, 1981). But in a more recent study neither 537mg α-tocopherol equivalents (800 IU) nor 800 mg α-tocopherol equivalents (1,200 IU) were found to influence prothrombin time. None of the test subjects who received vitamin E had a significant change in the bleeding time, so the authors concluded that vitamin E might safely be given to patients who require chronic warfarin therapy (Kim and White, 1996). Studies with healthy humans with vitamin E supplementation have shown that there are no changes in platelet aggregation or adhesion with daily vitamin E intake up to 800 mg α-tocopherol equivalents (1,200 IU) (Farrell and Bieri, 1975; Tsai *et al*, 1978; Steiner, 1991; Steiner, 1993).

It has also been reported, that 604 mg α-tocopherol equivalents (900 IU) per day did not influence the coagulation activity in persons who did not take any anticoagulant drugs (Kitagawa and Mino, 1989). The question of bleeding time was studied by Meydani *et al* (1998) who found no adverse effects, including the bleeding time, after a 4-month daily supplementation with 60, 200 or 800 IU (40, 134 or 537 mg α-tocopherol equivalents) vitamin E. Some other intervention trials were reported to show benefit against heart disease with higher vitamin E doses up to 800 IU = 537 mg α-tocopherol equivalents (Stampfer and Rimm, 1995; Stephens *et al*, 1996) but this observation has not been shown consistently.

The studies considered of most scientific value with adequate controls are presented in Table 2.

Table 2. Studies with oral vitamin E in human subjects with strict controls

Reference	Subjects	Dose/duration	Results
Farrell and Bieri, 1975	N = 28 adults	67-537 mg TE*/d 4 mo-21 yr (mean 2.9 yr) (100-800 IU α-tocopherol)	No evidence of toxicity by clinical chemical blood analysis
Ernst and Matrai, 1985	N = 16 adults	536 mg TE/d 4 wk (800 mg/d all rac-α- tocopheryl-acetate)	No adverse effects by clinical chemical blood analysis
Corrigan, 1982	N = 12 warfarin-treated cardiology patients	67-269 mg TE/d 4 wk (100-400 IU all rac α-tocopherol)	Warfarin effect was intensified

* TE: α-tocopherol equivalents

Regarding controlled double blind studies of vitamin E toxicity in humans, several reports exist that vitamin E has low toxicity and no consistent adverse effects, and these studies are reported in Table 3.

The principal negative effect observed was on prothrombin time or other factors related to blood clotting. In several studies no effects were reported but in others there were effects on blood clotting and it was claimed that high doses of vitamin E only influenced blood clotting in cases of low vitamin K status (Steiner, 1991; Steiner, 1993; Diplock *et al*, 1998). The published reports (Elmadfa, 1985; Kappus and Diplock, 1992; Meydani *et al*, 1998) concluded that vitamin E at high dietary intakes affects blood coagulation if vitamin K status is inadequate. High doses of α-tocopherol affected the vitamin K metabolism by reducing the cyclooxygenase pathway and therefore thromboxane synthesis, thus impairing the thromboxane-dependent blood coagulation and also decreasing the coagulation factor II and VII. It was suggested that high doses (800-1200 α-tocopherol equivalents) should be avoided for two weeks prior to and following surgery (Elmadfa and Bosse, 1985). In a critical comment on the high upper level for vitamin E of 1000 mg/day derived by the US Food and Nutrition Board (Horwitt, 2001) attention was drawn to the observation that the tendency to haemorrhage in aspirin users is increased by vitamin E (Liede *et al,* 1998).

Table 3. Double blind control studies with oral vitamin E in humans

Reference	Subjects	Dose/duration	Results
Anderson *et al*, 1974	N = 38 Angina pectoris patients	2362 mg TE*/d 9 wk (3200 mg RRR-α-tocopheryl succinate)	No adverse effects except some gastrointestinal disturbance (diarrhoea: 3 subj; intestinal spasm)
Bierenbaum *et al*,1985	N = 25 Diabetic subjects	1820 mg TE/d (2000 mg all rac-α-tocopheryl acetate)	No adverse effects by clinical chemical blood analysis (cholesterol, T^3, T^4, blood coagulation)
Gillilian *et al*, 1997	N = 52 Angina pectoris patients	1322 mg TE/d 6 mo (1600 IU RRR-α-tocopheryl succinate)	No adverse effects in cardiac function parameters, urinalysis, blood count, blood chemistry, prothrombin time
Kitagawa and Mino, 1989	N = 19 adults	600mg TE/d 12 wk (600mg α-tocopherol)	No objective or subjective adverse effects
Stampfer *et al*, 1983	N = 30 volunteers	550mg TE/d 16 w (800 IU α-tocopherol)	No group differences
Tsai *et al*, 1978	N = 202 volunteers	441 mg/d TE 4 w (600 IU α-tocopheryl acetate)	Serum T^3 and T^4 lower; no adverse effect
Meydani *et al,* 1998	N = 88 healthy volunteers aged >65 years divided between control and three dose groups (17-19 per group)	60, 200 or 800 IU/d for 4 months	No subjective side effects No effect on GSH peroxidase, superoxide dismutase, immunoglobulin, anti-DNA or thyroglobulin antibodies, body weight, total plasma proteins, albumin, glucose, lipids or lipoprotein profile, total bilirubin, serum liver enzymes, blood count, platelet number, bleeding time, Hb, haematocrit, urinary or serum creatinine

* TE: α-tocopherol equivalents

The effects on blood clotting are not, however, the only adverse effects requiring consideration. Side effects reported in therapeutic use of vitamin E supplements include severe muscular weakness and fatigue induced in adults receiving daily doses of 720 mg α-tocopherol (Cohen, 1973). These side

effects were confirmed in a double-blind study on two healthy male subjects given the same dose of α-tocopherol and the symptoms were associated with a large increase in 24 hr urinary creatinine and elevated serum creatine phosphokinase (Briggs, 1974; Briggs and Briggs, 1974).

When patients with porphyria cutanea tarda were given daily doses of 1.0 g α-tocopherol for 3 months there was a marked increase in 24 hour urinary androgens (androsterone, etiocholanolone plus dehydroepiandrosterone) from 3.5 to 4.6 mg/day while mean 24 hour pregnanediol fell from 2.2 to 0.5 mg/day (Pinelli *et al*, 1972). The authors concluded that the significance of these endocrine changes was uncertain but could be important for patients with endocrine sensitive tumours.
Vitamin E has been reported to cause an increase in iodine uptake by the thyroid and in serum organic iodine at doses of 400-500 mg/day of TE for 4 weeks but this was apparently asymptomatic and not associated with an increase in BMR (Tsai *et al*, 1978).

A group of 52 elderly patients (average age 72 years) showed an average increase in serum cholesterol of 74 mg/dL when given repeated daily doses of 300 mg α-tocopherol (Dahl, 1974). Conversely, no such increase was seen in a small group of healthy men taking 588 mg (800 I.U.) daily (Briggs, 1974).

3.2.1. Epidemiological evidence

There are limited data relating to the effects of vitamin E on morbidity and mortality from chronic diseases.

In the ATBC study (1994) an increase was observed in the numbers of deaths from haemorrhagic stroke among male smokers. Although the number of haemorrhagic stroke cases with 50 mg α-tocopherol was 66 compared to 44 in the control group (total n = 29,133) no statistical significance was published. A more recent analysis of this study indicated that there was an increased risk of subarachnoidal haemorrhage in hypertensive men (RR 2.45; CI 1.08-5.55) and a significantly higher mortality. Gingival bleeding occurred more frequently in subjects who were also taking aspirin (Leppala *et al* 2000 a and b; Liede *et al*, 1998). In two other studies, the Secondary Prevention with Antioxidants of Cardiovascular Disease in endstage renal disease (SPACE) and the Primary Prevention Project (Boaz *et al* 2000; Collaborative Group of the Primary Prevention Project, 2001) there was a non-statistically significant increase in fatal haemorrhages.

4. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

4.1. Adults

The establishment of a NOAEL depends on the interpretation of asymptomatic effects on clinical biochemical parameters reported in some human studies and supported by similar effects in experimental animals. No NOAEL could be established from the chronic toxicity studies in the rat with respect to blood clotting and liver histology. Consequently, in considering food additive use, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) derived an ADI for dl-α-tocopherol of 0.15-2 mg/kg body weight based on clinical experience in humans (WHO, 1986). The SCF reviewed the data on tocopherol extracts, α-, β-, γ- and δ-tocopherol and α-tocopheryl acetate and concluded that their use as antioxidants in food was acceptable and that it was not appropriate to establish an ADI (SCF, 1989).

In considering the derivation of a tolerable upper intake level the present Committee considered that the asymptomatic effects on biochemical indices (urinary steroid hormones, I_2 metabolism), were of doubtful toxicological significance and the reports of fatigue associated with effects on creatine phosphokinase and increased urinary creatine were limited in duration and involved only two subjects. These observations have not been reproduced in other studies. The Committee therefore decided that the critical effect is on blood clotting and that the study by Meydani *et al* (1998) provided the best basis for an evaluation of the tolerable upper intake level. The NOAEL established in this study was 540 mg/day.

Considering the above the Committee concluded that an uncertainty factor of 2 would adequately cover interindividual differences in sensitivity. A larger uncertainty factor was not considered necessary because data from a number of other older but less well controlled studies showed no adverse effects at considerably higher intakes. The UL for vitamin E was therefore established as 270 mg/day for adults and rounded to 300 mg/day.

4.2. Pregnancy and Lactation

The Committee considered that the UL applied also to the women during pregnancy and lactation based on no indication from animal studies of special risk during this period.

4.3. Children and adolescents

There are no data specifically relating to children and adolescents. The UL for children and adolescents is derived by scaling the adult UL on the basis of body surface area (body weight$^{0.75}$).

Age (years)	Tolerable Upper Intake Level (UL) for vitamin E (mg per day)
1-3	100
4-6	120
7-10	160
11-14	220
15-17	260

5. CHARACTERISATION OF RISK

Current estimated intakes from food and supplements, including the 97.5th percentile, in the population are generally well below the UL. However, some users of high dose supplements may exceed the UL.

Oral intakes of high amounts of vitamin E can increase the blood coagulation defects in subjects with vitamin K deficiency caused by malabsorption or due to therapy with anticoagulants. Therefore the UL is not considered to apply to patients receiving anticoagulant drugs or to patients with malabsorption syndromes, nor to other conditions where the synthesis of vitamin K by the gut microflora might be impaired. In addition there is evidence that vitamin E can increase the risk of haemorrhage in individuals taking aspirin.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN K

(EXPRESSED ON 4 APRIL 2003)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

All compounds with vitamin K activity contain a 2-methyl-1,4-naphthoquinone nucleus with a lipophilic side chain at position 3. More than 100 substances with vitamin K activity are known but only three are of physiological importance. Vitamin K_1 (α-phylloquinone), isolated from green plants, has a phytyl group in position 3. Vitamin K_2 (menaquinones), synthesized by bacteria, have an unsaturated multiprenyl group in this position. Of a wide range of menaquinones synthesized by bacteria, those with 7, 8 or 9 isoprenoid groups in the side chain (30 or 35 C-atoms) are most common. Menadione is a synthetic compound without a side chain, the use of which has been discontinued in dietary products and this will not be considered further.

The current consideration of a tolerable upper level for vitamin K concentrates on phylloquinone, the predominant dietary source.

2. NUTRITIONAL BACKGROUND

2.1. Sources and intakes

Vitamin K_1, or phylloquinone, is obtained from the diet whereas vitamins K_2 are also produced by the intestinal microflora (Shearer, 1992 and 1995). The extent to which vitamin K synthesis by intestinal bacteria contributes to vitamin status requires further investigation (Suttie, 1996).

The phylloquinone concentration in most foods is very low (<10 μg/100 g), and the majority of the vitamin is obtained from a few leafy green vegetables and four vegetable oils (soybean, cottonseed, canola and olive) that contain high amounts (Booth *et al*, 1996; Fenton *et al*, 1997). In green vegetables, vitamin K_1 is tightly bound to the thylakoid membrane of chloroplasts from where it is poorly absorbed (5-15% depending on concomitant fat intake) (Gijsbers *et al*, 1996; Schurgers and Vermeer, 2000). The absorption of vitamins K_2, which occur mainly in cheese, curd cheese and natto, is much better and may be almost complete. Thus the nutritional importance of menaquinones is often underestimated.

Reliable measurements of phylloquinone contents in foods are now available, and data from many studies of phylloquinone intake in the United States indicate that the mean intake of younger adults (<45 years) ranges from 60 to 110 μg of phylloquinone/d. In contrast, older adults (>55 years) consume 80 to 210 μg of phylloquinone/d, attributed to their greater vegetable consumption compared to younger age groups (Booth *et al*, 1996). A provisional estimate of the phylloquinone intake in the UK is 68 μg/person/day, based on the food consumption data from the 2000 UK National Food Survey (MAFF, 2001). This figure is similar to earlier estimates for men and women, aged 22-54 years, of 72 μg/day and 64 μg/day respectively (Price *et al*, 1996). A longitudinal study of phylloquinone intakes (Bolton-Smith *et al*, 2000) found initial intakes of 67 and 69 μg/day for men and women respectively, aged 40-59 years in 1985. At follow-up 10 years later the intakes had fallen to 54 and 56 μg/day for men and women respectively then aged 50-69 years. For people aged 65 years and over, mean intakes for men and women were 66 and 57 μg/day respectively with considerable regional variations throughout the United Kingdom (Thane *et al*, 2002).

In The Netherlands, mean daily per capita intake was estimated to be up to 250 μg consequent on the relatively high intake of green vegetables. For menaquinone intake there are no population-based data available except for The Netherlands where menaquinones are estimated to form about 10% of total vitamin K intake (Schurgers *et al*, 1999).

Based on the average per capita food consumption in Finland (Ministry of Agriculture and Forestry, 1999; Statistics Finland, 2000), the average vitamin K intake from different foods was estimated to be 120 μg/day (Koivu-Tikkanen, 2001).

Price *et al* (1996) observed no seasonal differences when phylloquinone intake was assessed during spring, summer, autumn and winter.

Data on the vitamin K intake among children are limited.

2.2. Absorption and metabolism

Under normal physiological conditions, lipid soluble K-vitamins are absorbed in cooperation with bile acids and pancreatic enzymes. The efficacy of absorption (10-90% depending on the food matrix) (Schurgers and Vermeer, 2000) can be reduced by long-chain polyunsaturated fatty acids and badly absorbed lipid-soluble substances and hydrocarbons, like mineral oils and squalene. Vitamin K_1 and K_2 are stored in the liver. The total body pool of vitamin K (1.5 μg/kg body weight) is small compared to other fat-soluble vitamins and its turnover is rapid.

Under normal conditions, 30-40% of the absorbed vitamin K is excreted via the bile into the faeces, while approximately 15% is excreted in the urine as water soluble metabolites. Alimentary deficiency, disturbance of fat absorption, increased excretion, presence of antagonists, disturbance of bile function and liver disease, lead to decreased bioavailability of vitamin K (Suttie, 1996; Elmadfa and Leitzmann, 1998).

2.3. Physiological function

The physiological activity of phylloquinone is based on its ability to change between its oxidized (quinone and 2,3-epoxide) and reduced (hydroquinone) forms.

The major role of phylloquinone is the post-translational addition of a carboxyl-group into the γ-position of glutamate residues of specific proteins. In this respect, the prime physiological relevance of phylloquinone is the synthesis of coagulation proteins (Ferland, 1998; Olson, 1999 and 2000).

Whereas the vitamin K-dependent coagulation proteins are all synthesised in the liver, vitamin K is also essential for the synthesis of a number of proteins produced in extra-hepatic tissues. Examples of the latter group of proteins include:

- the bone Gla-protein, osteocalcin, which is exclusively synthesised by osteoblasts and odontoblasts, and which is a negative regulator of bone formation;
- matrix Gla-protein (MGP), which is synthesised in most soft tissues, but predominantly in cartilage (by chondrocytes) and in vessel wall (by vascular smooth muscle cells) and which is a potent inhibitor of soft tissue calcification;
- growth arrest-specific gene 6 protein (Gas6), which is a ligand for tyrosine kinases and has strong apoptopic activity in cultured cells.

Inadequate peak mineral bone density in young adulthood is a major contributor to later disease and may be caused by a combination of genetic and nutritional factors. In addition to total energy intake, the nutrients that promote bone synthesis include calcium, vitamin C, vitamin D, and vitamin K. Vitamin K is required for the γ-carboxylation of glutamate in 2 proteins induced by the vitamin D hormone in bone. Osteocalcin is a 49-residue protein with 3 carboxyglutamic acid residues, is water soluble, adheres to the bone mineral hydroxyapatite, and is secreted by osteoblasts. Matrix carboxyglutamic acid (Gla) protein contains 79 amino acid residues of which 5 are Gla residues. It is hydrophobic, insoluble in plasma, and is associated with the matrix of cartilage and bone as well as with the tunica media of the arterial vessel wall (Olson, 2000).

Luo *et al* (1997) demonstrated that transgenic mice, lacking the vitamin K-dependent matrix Gla protein, exhibited an excessive cartilage calcification leading to reduced growth. The most striking observation in the MGP -/- mutant, however, was excessive calcification of the large arteries leading to ruptures of the aorta before the eighth week of life in all animals.

The level of osteocalcin carboxylation has been proposed as an indicator of the nutritional state of bone with respect to vitamin K. Circulating levels of undercarboxylated osteocalcin may be a sensitive marker of vitamin K inadequacy. These levels of undercarboxylated osteocalcin have been reported to be increased both in postmenopausal women and in individuals who sustain hip fracture (Binkley and Suttie, 1995; Vermeer *et al*, 1995; Szulc *et al*, 1993 and 1994; Knapen *et al,* 1998; Luukinen *et al*, 2000).

2.4. Major criteria for assessing vitamin K status

Efforts to define the human requirement for vitamin K have been hampered by a lack of knowledge of the amount of the vitamin in various foods and by the lack of sensitive methods to assess vitamin K status (Suttie, 1992).

The major criterion for assessing the adequacy of vitamin K status in human adults is the maintenance of plasma prothrombin concentrations in the normal range (from 80 to 120 μg/mL). This classic measure of vitamin K deficiency is very insensitive but recent studies have shown that the serum concentration of under-γ-carboxylated prothrombin (PIVKA-II), the percentage of under-γ-carboxylated osteocalcin (% ucOC) in serum and the urinary γ-carboxy-glutamic acid (Gla) excretion, respond to alterations in dietary phylloquinone. Gender and age were shown to influence both osteocalcin concentrations and Gla excretion in healthy subjects (Sokoll and Sadowski, 1996). Although there is a weak correlation between serum phylloquinone and % ucOC, it was not strong enough to have predictive values as a measure of individual vitamin K status. Because of its dependence on dietary intake within the last 24 hours, serum phylloquinone is not a meaningful indicator for nutritional status (Jakob and Elmadfa 1995). Intakes of 10 μg/day for a few weeks do not prolong the prothrombin time but put subjects at risk as assessed by other measures of vitamin K deficiency.

The acquired vitamin K deficiency produced by administration of a low dose of anticoagulant warfarin was also used to assess the relative sensitivity of various measures of vitamin K status. In subjects given 1 mg warfarin/day, Bach *et al* (1996) noted elevated PIVKA-II concentrations but no significant decrease in urinary Gla. The most striking change was an increase in % ucOC. After a 14-day warfarin treatment, the subjects were given 1 mg phylloquinone for 7 days. At the end of this 7-day-period the % ucOC was lower than the baseline period. Various PIVKA-II measures respond to this low intake of phylloquinone and Gla excretion, which indicates that the total formation of vitamin K dependent proteins is decreased (Booth and Suttie, 1998).

It appears that phylloquinone intakes equal to the current Reference values of around 1 μg/kg body weight/day are sufficient to cover the hepatic K requirement and thus to ensure full gamma carboxylation of all coagulation factors. Since undercarboxylation of extrahepatic Gla- proteins seems to be common in the healthy adult population, the current recommended intake is probably insufficient fully to carboxylate these proteins.

2.5. Recommended intakes

The Committee made no recommendation for a PRI for vitamin K but considered that an intake of 1 μg/kg body weight/day appears to be adequate and would be provided by a normal diet (SCF 1993).

More recently recommended intakes in some countries have been determined based on effects on blood coagulation. A recommended daily dietary intake for vitamin K of 65-80 μg/day or 1 μg/kg body weight/day has been proposed (D-A-CH Referenzwerte, 2000). The US Food and Nutrition Board recently increased their recommendation to 120 μg/day for adult males and 90 μg/day for adult females (FNB, 2001). Because of the lack of specific information about the vitamin K requirement of children, reference values for them are set at about 1 μg/kg body weight (FNB, 2001; D-A-CH Referenzwerte, 2000).

2.6. Vitamin K deficiency

Clinical vitamin deficiency due to dietary inadequacy is rare or nonexistent in healthy adults. Several factors that protect adults from a lack of vitamin K include 1) widespread distribution of phylloquinone in plant and animal tissues, 2) the phylloquinone cycle, which regenerates the vitamin, and 3) the microbiological flora of the gut, which synthesizes menaquinones and can contribute to meeting the requirement for vitamin K.

Newborn infants have low vitamin K status, partly due to limited intestinal synthesis, and are at increased risk of developing haemorrhagic disease secondary to vitamin K deficiency. Vitamin K is routinely administered prophylactically to newborns in many countries.

The risk of vitamin K deficiency is increased by trauma, physical debilitation, renal insufficiency and chronic treatment with large doses of broad-spectrum antibiotics (Ansell *et al*, 1977).

Various drugs, including the 4-hydroxy-coumarins, salicylates, certain broad-spectrum antibiotics, and vitamin A and E in pharmacologic doses, act as antagonists of vitamin K.

The principal negative effect of vitamin E observed was on prothrombin time or other factors related to blood clotting. In several studies no effects were reported but in others there were effects on blood clotting and it was claimed that high doses of vitamin E only influenced blood clotting in cases of low vitamin K status (Steiner, 1991 and 1993; Diplock *et al*, 1998). In a review of these reports (Kappus and Diplock, 1992; Elmadfa and Leitzmann, 1998; Meydani *et al*, 1998) the conclusion was that vitamin E at high dietary intakes affects blood coagulation if vitamin K status is inadequate. High doses of α-tocopherol equivalents affected the cyclooxygenase pathway and therefore formation of thromboxane, thus impairing the thromboxane-dependent blood coagulation, and also decreased the coagulation factors II and VII (Elmadfa and Bosse, 1985).

3. HAZARD IDENTIFICATION

3.1. Acute toxicity

Acute oral toxicity studies were carried out in rats, mice and chicks. In all three species, no deaths occurred after single doses of 25,000 mg/kg body weight phylloquinone either orally or intraperitoneally (Molitor and Robinson, 1940).

3.2. Short-term studies

No adverse effects were recorded when daily oral doses of up to 2000 mg phylloquinone/kg body weight were administered to rats for 30 days (Molitor and Robinson, 1940)

3.3. Carcinogenicity

No experimental animal studies on carcinogenicity of vitamin K have been found.

One epidemiological study indicated that there was a significant association between intramuscular injection of vitamin K and childhood cancer, especially leukaemia (Golding *et al*, 1992). No significantly increased risk was associated with oral administration (Huysman and Sauer, 1994). Several other population studies have failed to confirm an association between vitamin K administration to children and cancer. A nested case-control study using data from a large, multicentre prospective study of 54,795 children showed no association between vitamin K administration and risk of any childhood cancer, or of all cancers combined (Klebanoff *et al*, 1993). A study of associations between leukaemia and prenatal or neonatal administration of vitamin K did not show any increased risk in neonates receiving vitamin K i.m. (Ansell *et al*, 1996). The latter results were confirmed in other studies (McKinney *et al*, 1998; Parker *et al*, 1998; Passmore *et al*, 1998). The evidence for an association between administration of phylloquinone to neonates and childhood cancer is therefore not convincing.

3.4. Genotoxicity

Phylloquinone was reported to reduce the mutagenicity of six heterocyclic amines in the Ames *Salmonella typhimurium* assay. There was no evidence of mutagenicity of phylloquinone in the absence of the amines (Edenharder *et al*, 1999).

Conflicting results have been obtained in studies on the ability of phylloquinone to induce sister chromatid exchanges (SCE) in human or animal leucocytes. When 5 foetal sheep were given a dose of 1 mg phylloquinone via the femoral vein, the mean number of SCEs rose from 3.94 (±0.15) prior to injection to 5.4 (±0.23) 24 hours after injection. This increase was stated to be statistically significant (Israels *et al*, 1987). In an *in vitro* study of the concentration response for SCE induction, foetal or adult sheep leucocytes were incubated with phylloquinone at concentrations of 0.1 nM to 1 μM. At 0.1 nM the number of SCEs was increased in foetal cells but the increase in adult cells was only observed at 10 nM and above. With human leucocytes taken from adult and placental blood, an increase in the mean number of SCEs per metaphase was reported in the presence of 1 μM phylloquinone. The SCEs rose from 3.32 ±0.219 to 5.76 ± 0.219 in placental leucocytes and from 5.13 ±0.273 to 7.81 ±0.326 in adult cells (Israels *et al*, 1987). Conversely, negative results were obtained when human neonates were injected with 1 mg

phylloquinone i.m. No significant difference in the mean number of SCEs and chromosomal aberrations in peripheral blood lymphocytes between treated and untreated controls was observed 24 hours after injection (Cornelissen *et al* 1991). Overall, the limited data presently available do not allow an adequate evaluation of the genotoxic potential of phylloquinone at the gene or chromosome level.

3.5. Reproductive/developmental toxicity

No data on reproductive toxicity were available.

3.6. Human data

In a study of the effect of vitamin K on bone metabolism in eight female athletes, no adverse effects were reported on administration of a supplementary 10 mg/day of phylloquinone for 1 month. In all subjects, vitamin K supplementation was associated with an increase in calcium binding capacity of osteocalcin (Craciun *et al*, 1998).

A 3 x 15-day crossover study was conducted in groups of younger (20-40 years) and older (60-80 years) healthy adults; each group contained 9 individuals of each sex. During the three 15-day periods, the participants received a diet providing 100 μg/day phylloquinone. During two periods the diet was supplemented with broccoli (377 μg/day total phylloquinone) or phylloquinone-fortified oil (417 μg/day total phylloquinone). No adverse effects were reported (Booth *et al*, 1999).

4. DOSE RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

There are no appropriate data from which to set a numerical upper limit for vitamin K.

5. RISK CHARACTERIZATION

In human studies of limited numbers, there is no evidence of adverse effects associated with supplementary intakes of vitamin K in the form of phylloquinone of up to 10 mg/day (more than two orders of magnitude higher than the recommended dietary intake of vitamin K) for limited periods of time. These limited data are supported by experimental animal studies in which no adverse effects were observed after daily administration of extremely high doses (2000 mg/kg body weight) for 30 days.

Because of the antagonistic interaction of phylloquinone and coumarin anticoagulant drugs, people taking these drugs should not significantly increase their phylloquinone intake by dietary change or by using dietary supplements without medical advice.

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OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF CHROMIUM

(EXPRESSED IN 4 APRIL 2003)

FOREWORD

This opinion is one in the series of opinions of the Scientific Committee on Food (SCF) on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Chromium is ubiquitous, occurring in water, soil and biological systems. It occurs in each of the oxidation states from Cr^{0} to Cr^{+6}. The three most stable forms in which chromium occurs in the environment are the 0, +3, and +6 valence state; metal and alloys, trivalent chromium, and hexavalent chromium, respectively. Elemental chromium (Cr^{0}) does not occur naturally. Chromium compounds with oxidation states below +3 are reducing, and above +3 are oxidising. The occurrence of hexavalent chromium compounds is rare and nearly always man-made.

The high energy needed to oxidise the trivalent to the hexavalent form of chromium results in the fact that this oxidation never occurs in biological systems. The strong oxidising property of hexavalent chromium causes its spontaneous reduction in living organisms, irrespective of its solubility.

This evaluation is limited to trivalent chromium (Cr III) because it is the form of chromium found in food and supplements. The biological effects of hexavalent chromium on both animals and man are very different from those of trivalent chromium and are not considered.

1.1. Regulations

Under European legislation (Directive 2002/46/EC), chromium (III) chloride and chromium (III) sulphate are included in the list of substances that can be used in the manufacture of foods for particular nutritional uses and in food supplements. In this list, organic complexes of chromium (e.g. chromium picolinate) are not mentioned. The Committee has concluded that an evaluation of the acceptability of chromium picolinate as a nutrient source of chromium in Foods for Particular Nutritional Uses (FPNUs) is not possible unless data on bioavailability in humans are provided (SCF, 1999).

In Germany, all special permissions for the use of chromium picolinate in food supplements were withdrawn in 2001 due to recent investigations which do not exclude adverse effects on human health (BMVEL, 2001; BgVV, 2002).

2. NUTRITIONAL BACKGROUND

2.1. Food levels and dietary intake

In the UK, a total diet study has shown that the highest concentration of chromium has been found in meat products (230 μg/kg), followed by oils and fats (170 μg/kg), bread (150 μg/kg), nuts and miscellaneous cereals (140 μg/kg), fish, sugar, and preserves (130 μg/kg). The lowest concentrations have been found in milk (10 μg/kg), fresh fruits, and green vegetables (20 μg/kg), and in eggs (40 μg/kg). The concentrations of chromium in uncontaminated drinking water mostly are below 1 μg/L (EGVM, 2002a). A number of multivitamin and mineral food supplements contain up to 100 μg chromium in a daily serving unit (EGVM 2002b). In the USA, relatively high concentrations of chromium have been found in seafood (120-470 μg/kg) followed by meat and fish (110-230 μg/kg), grains and cereals (40-220 μg/kg), fresh fruits (90-190 μg/kg), and fresh vegetables (30-140 μg/kg).

According to WHO (1996) high dietary intakes of chromium reported before 1980 are generally questionable, since the chromium analysis on which they were based, were unreliable due to contamination and by analytical problems. A number of reports indicate that many diets in the

US supply less than 50 µg of chromium per day (Anderson and Kozlovsky, 1986; Anderson, 1989; Anderson *et al*, 1988; Offenbacher *et al*, 1985).

Table 1. Dietary chromium intake in µg/day

Country	Type of survey / Method	Range	Mean
Germany[a]	Duplicate diet samples	- -	61 (M) 84 (F)
UK[b]	Food (total diet study in 1997) Supplements Drinking water	up to 170[α] up to 100[β] up to 2[γ]	100 - -
Sweden[c]	Randomly selected 24-hour diets	50-580	160
Spain	Calculated from midday meal by extrapolation to 100%[δ]	-	120
	Duplicate diets samples from Southern Spain[ε]	9,4-205	100
USA[d]	7 days self selected diets	22-48 (M) 13-36 (F)	33 (M) 25 (F)
	From supplements based on the NHANES III, 1988-1994[κ]	3,2-100[λ] (M) 4,4-127[λ] (F)	29,5 (M) 30,0 (F)

(M): males; (F): females.
[a] D-A-CH, 2000.
[b] EGVM, 2002b
[α] EGVM used the 97,5th percentile as the "maximum estimated daily intake".
[β] Related to the daily serving unit.
[γ] Estimated intake from 2 litres of water containing <1µg/L.
[c] Abdulla *et al*, 1989.
[δ] Barberá *et al*, 1989; [ε] Garcia *et al*, 2001.
[d] FNB, 2001.
[κ] Third National Health and Nutrition Examination Survey, 1988-1994.
[λ] Ranges from the 5th percentile to the 95th percentile.

2.2. Nutritional requirements and intake recommendations

The Committee stated in 1993 that since data on the essentiality and metabolism of chromium are so sparse, the Committee is unable to specify any requirements (SCF, 1993).

The UK Committee on Medical Aspects of Food Policy calculated a theoretical requirement for adults from balance studies of 23 µg/day by using regression equations and concluded that a safe and adequate level of intake lies above 25 µg for adults and between 0,1 µg/kg bw/day and 1,0 µg/kg bw/day for children and adolescents, respectively (COMA, 1991).

The Societies for Nutrition of Germany (DGE), Austria (ÖGE), and Switzerland (SGE), jointly established an adequate daily intake of 30-100 µg/day for adults (D-A-CH, 2000).

Currently, there is no formal Recommended Dietary Allowance (RDA) for chromium. The US Food and Nutrition Board derived Adequate Intakes (AI) for chromium for different age groups, e.g. 35 µg/day and 25 µg/day for 19 to 50 year old men and women, respectively (FNB, 2001).

2.3. Deficiency

Chromium deficiency has not been seen in humans except in patients during long-term parenteral nutrition without substitution of chromium. The deficiency symptoms (impaired glucose tolerance and glucose utilisation, weight loss, neuropathy, elevated plasma fatty acids, depressed respiratory quotient and abnormalities in nitrogen metabolism) disappeared rapidly after oral supplementation (200 µg/day) (Jeejeebhoy *et al*, 1977; Freund *et al*, 1979).

Chromium-deficient rats exhibit a glucose intolerance similar to clinical diabetes mellitus. Other deficiency signs in animals include impaired growth, elevated serum cholesterol and triglycerides, increased incidence of aortic plaques, corneal lesions and decreased fertility and sperm count (Anderson, 1988).

3. BIOLOGICAL CONSIDERATIONS

3.1. Function

Trivalent chromium is considered to be an essential element both in animal feeding and human nutrition. It influences carbohydrate, lipid, and protein metabolism via an effect on insulin action. However, the mechanism still is not quite clear neither is the exact structure of the biologically active form of chromium, the "Glucose Tolerance Factor" (GTF) (WHO, 1996). The GTF tentatively is identified as a chromium-nicotinic acid complex and has been suggested to operate through activation of membrane phosphotyrosine phosphatase in mammals (Mertz, 1993; Davis *et al*, 1996). Beneficial effects have been reported in presumably chromium-deficient diabetics, where supplementing the diet with chromium decreased fasting blood glucose levels, improved glucose tolerance, lower insulin levels, and decreased total cholesterol and triglyceride levels while HDL-cholesterol levels were increased (Mooradian *et al*, 1994).

3.2. Absorption, metabolism and distribution

The absorption of ingested trivalent chromium depends, among other factors, on the chemical properties of the ingested compound, on the level of dietary intake, and on the presence of other dietary components in the diet (interactions). Chromium affects the binding of iron to transferrin. Trivalent chromium ingested as chromium picolinate is better absorbed than chromium from the chloride compound. Hepatic and renal chromium concentrations in rats were roughly 2- to 6-fold greater when chromium picolinate was fed compared to chromium chloride (Anderson *et al*, 1997a). Due to the natural presence of chelating agents in the diet the bioavailability of chromium from food can vary significantly. Absorption of chromium from various sources in man is shown in Table 2.

Trivalent chromium is bound to plasma proteins such as transferrin, whereas hexavalent chromium is taken up selectively by erythrocytes, reduced to trivalent chromium by glutathione, and bound predominantly to haemoglobin. Therefore, chromium is found in both erythrocytes, and plasma, after gastrointestinal absorption of hexavalent chromium, but only in the plasma after gastrointestinal absorption of trivalent chromium.

Table 2. Absorption of chromium from various sources in man

Chromium compound	% absorption	References
Chloride	0.4 0.13	Anderson *et al*, 1983 Kerger *et al*, 1996
Picolinate	2.8 ± 1.4 (SD)	Gargas *et al*, 1994
From food	2.4 0.5 - 2.0[1] 0.4 - 2.5[2]	Bunker *et al*, 1984 ATSDR, 1993 FNB, 2001
Trivalent chromium (reduced from potassium dichromate [VI] dissolved in orange juice)	0.6	Kerger *et al*, 1996

[1] Several studies with male and female volunteers.
[2] Based on metabolic balance studies or on urinary excretion from physiological intakes.

4. HAZARD IDENTIFICATION

The toxicity of chromium compounds has been reviewed by several institutions (IPCS, 1988; IARC, 1990; WHO, 1996; EPA, 1998a, b, c and d; EGVM, 2002a and 2002b; ATSDR, 2000; FNB, 2001).

4.1. Acute toxicity

In rats the LD_{50} of orally administered trivalent chromium varies with the compound and the sex of the rat. The LD_{50} for chromium acetate is 2365 mg/kg body weight (ATSDR, 2000) and for chromium nitrate

nonahydrate 3250 mg/kg body weight (Registry of Toxic Effects, 1980). The oral LD_{50} values for water soluble trivalent chromium compounds given to rats and mice vary from 140 mg/kg to 422 mg/kg (EGVM, 2002).

4.2. Subchronic toxicity

Groups of 8 four week-old Harlan Sprague-Dawley rats were fed a stock diet to which 0, 5, 25, 50, or 100 mg of chromium per kg diet was added as chloride or picolinate for twenty weeks. For the highest dose group, the authors assumed a chromium intake of 15 mg/kg bw/day. Chromium given as picolinate showed a considerably higher bioavailability than trivalent chromium chloride which was indicated by a 2- to 6-fold greater hepatic and renal chromium concentration in animals fed chromium picolinate. Histologically, no changes in the liver and kidney have been observed but other organs were not examined histologically. However, there were no statistically significant differences in body weight, organ weights, or blood variables among all the groups tested at the age of 11, 17, and 24 weeks (Anderson *et al*, 1997a).

4.3. Chronic toxicity

Ivankovic and Preussmann (1975) performed a chronic toxicity/carcinogenicity study with BD rats (groups of 60 animals of both sexes) fed 0, 1, 2, or 5% chromium (III) oxide (Cr_2O_3) baked in bread 5 days/week for 840 days (600 feeding days in total). The highest dose corresponds to 2144 mg Cr_2O_3/kg bw/day or to about 1500 mg trivalent Cr/kg bw/day. No toxic or carcinogenic effects were noted at any feeding level. The lack of toxicity may be explained by the poor absorption of the administered pigment Cr_2O_3.

In another group of rats fed during the same study in the same way for 90 days, no changes could be detected in serum protein, bilirubin, haematology, urinalysis, and histopathology but some reductions (12-37%) in the absolute weights of the livers and spleens in the 5%-group.

4.4. Carcinogenicity

4.4.1. Oral administration

In addition to the chronic toxicity/carcinogenicity study carried out by Ivankovic and Preussman (1975) two other oral studies were conducted, one in mice and one in rats.

4.4.1.1. Mice

Swiss mice (groups of 54 males and 54 females) received 5 mg/L chromium acetate in drinking water for life. Only 60% of males survived 18 months. No increased incidence of tumours was observed (Schroeder *et al*, 1964).

4.4.1.2. Rats

Long Evans rats (groups of 46 males and 50 females) received 5 mg/L chromium acetate in drinking water for life. At least 70% of the animals survived for up to two years. No increased incidence of tumours was observed (Schroeder *et al*, 1965).

4.4.2. Other ways of administration

Several studies were conducted, mainly in rats and mice by inhalation, intratracheal instillation, intrabronchial, -pleural, -muscular, -peritoneal, -femoral and intravenous administration. No significant increased incidence of tumours was observed. All these studies are reported in the IARC Monograph no. 49 (1990); all of them present strong limitations. According to IARC (1990) “there is limited evidence in experimental animals for the carcinogenicity of chromium trioxide (chromic acid) and sodium dichromate” and “there is inadequate evidence in experimental animals for the carcinogenicity of metallic chromium, barium chromate and chromium (III) compounds”.

4.4.3. Human data

All the exposures considered by the IARC (1990) in the epidemiological studies described for hexavalent chromium include simultaneous exposure to chromium (III) and chromium (VI) compounds. The chromium (VI) species is widely considered the aetiological agent responsible for the excess cancer risk in chromium workers, but this is based on the results of animal carcinogenicity and genotoxicity as well as on biological considerations. There are no adequate data on the carcinogenicity of trivalent chromium compounds and the overall evaluation of IARC was: “metallic chromium and chromium (III) compounds are not classifiable as to their carcinogenicity to humans” (Group 3) (IARC, 1990).

4.5. Genotoxicity

4.5.1. Experimental data

A very large number of chromium compounds have been assayed with *in vitro* and *in vivo* genotoxicity tests. Comprehensive reviews are, among others, those by Levis and Bianchi (1982), IPCS (1988), IARC (1990), De Flora *et al* (1990) and EPA (1998 a and d).

When evaluating the results of the genotoxicity tests it is necessary to take into consideration several properties of the tested compound (oxidation state, solubility, ability to penetrate cell membranes, intracellular stability, and reactivity with cellular components).

A very comprehensive review on the genotoxicity of chromium compounds by De Flora *et al* (1990), showed that the large majority of the results with chromium (VI) compounds were positive for different genetic end-points *in vitro* and *in vivo*, as a function of their solubility and bioavailability to target cells.

On the other hand, chromium (III) compounds, although even more reactive than chromium VI with purified nucleic acids, generally did not produce gene mutations, sister chromatid exchanges (SCE) or cell transformation in cultured mammalian cells (IARC, 1990).

Chromium (III) and chromium (VI) compounds have been shown to decrease the fidelity of DNA synthesis (Raffetto, 1977, Snow, 1994). Trivalent chromium was not mutagenic in bacterial assays (Venitt and Levy, 1974; Petrilli and De Flora, 1978a, 1978b). In one study it was weakly mutagenic in *Bacillus subtilis* (Nakamuro *et al*, 1978). Conflicting results were obtained in *in vitro* chromosomal aberration assays in mammalian cells: positive results were shown with $CrCl_3$ (Raffetto, 1977), $CrCl_3$, $Cr(NO_3)_3$, $KCr(SO_4)_2$, or $Cr(CH_3COO)_3$ (Levis and Majone, 1979) and hydrated $CrCl_3$ in Don Chinese hamster cells (Ohno *et al*, 1982) and $Cr(CH_3COO)_3$ in human leukocytes (Nakamuro *et al*, 1978). Other compounds were not clastogenic, as $Cr_2(SO_4)_3$ in mouse FM_3A cells (Umeda and Nishimura, 1979), $CrCl_3$ or $Cr(NO_3)_3$ in human leukocytes (Nakamuro *et al*, 1978), and $Cr_2(SO_4)_3$ in Don Chinese hamster cells (Ohno *et al*, 1982). $CrCl_3 \cdot 6H_2O$ was shown to induce chromosome aberrations in human lymphocytes via indirect action (Friedman *et al*, 1987).

Blasiak and Kowalik (2000) have reported that both tri-(chromium chloride) and hexavalent (potassium dichromate) chromium were positive in the comet assay carried out in isolated human peripheral lymphocytes. The results of this study also suggest that reactive oxygen species and hydrogen peroxide may be involved in the formation of DNA strand breaks by hexavalent chromium but not by trivalent chromium; for the last compound, the authors speculate that binding to cellular ligands may be important.

Chromic chloride has been shown to covalently bind to DNA in liver and kidney of rats treated with chromium (III) chloride *in vivo* (Cupo *et al*, 1985).

No DNA damage was observed in cells of animals treated *in vivo* with chromium chloride, and no micronuclei were seen in cells of animals given chromium nitrate (IARC, 1990).

Chromium (III) picolinate was clastogenic in a range of soluble doses of 0,05-1 mM; chromosome damage was inferred to be caused by the picolinate ligand because picolinic acid in the absence of chromium was clastogenic in Chinese hamster ovary cells (CHO). Chromium (III) nicotinate and chromium (III) chloride hexahydrate did not produce chromosome damage at equivalent non-toxic concentrations (Stearns *et al*, 1995b).

Chromium (III) picolinate, and to a lesser extent chromic chloride, were mutagenic at the *hprt* locus of cultured CHO cells at the equivalent doses of 1 mM. An equivalent dose of 3 mM of picolinic acid was highly cytotoxic and at lower doses produced an increase of *hprt* mutants, not statistically significant (Stearns *et al*, 2002).

In summary, the presently available data indicate that although chromium (III) compounds may bind to DNA and produce DNA-protein cross-links under certain circumstances, differently from chromium (VI) compounds, generally they did not produce gene mutations, sister chromatid exchanges or cell transformation in cultured mammalian cells. Weak clastogenic effects have been observed in some mammalian *in vitro* systems at relatively high and cytotoxic concentrations. No induction of genetic damage or micronuclei has been observed in experimental animals.

4.5.2. Human data

A recent paper by Medeiros *et al* (2003) suggests that trivalent chromium can lead to an increase of micronucleated perypheral lymphocytes in chronically exposed tannery workers. A group of 33 tanners exposed to trivalent chromium and a small group of 5 manual metal arc stainless steel welders exposed to hexavalent chromium were examined for two end-points: a chemical one, the formation of DNA-protein crosslinks (DPC) and a biological one, the occurrence of micronuclei in perypheral lymphocytes. These determinations were paralleled by quantitative analysis of chromium in plasma and urine. A significant increase in the formation of DPC was observed in tannery workers compared with controls (0.88±0.19 versus 0.57±0.21%, P<0.001 Mann-Whitney test) and even a higher level of DPC was observed in welders (2.22±1.12%, P=0.03). Tanners showed a significant increase in micronucleated cells compared with controls (6.35±2.94 versus 3.58±1.69%°, P<0.01), whereas in welders this increase was not significant (5.40±1.67°/°°). Urinary chromium was increased in both groups, with a greater increase observed in tanners compared with controls (2.63±1.62 versus 0.70±0.38 µg/g creatinine, P<0.001) than in welders (1.90±0.37 µg/g creatinine, P<0.005). Plasma chromium was also increased in both groups.

The results of this study support the causal relationship between chromium exposure (both hexavalent and trivalent) and increased lymphocyte DPC levels.

The interpretation of the increased incidence of micronuclei in tanners is difficult; leather processing involves a considerable number of other substances including formaldehyde and benzidine, whereas in welders trivalent chromium is accompanied by variable amounts of hexavalent chromium and other metals, including nickel, a potential suppressor of chromium-dependend cytogenetic damage (Katsifis *et al*, 1998). On the other hand, these results are in contrast with the negative findings reported by IARC (1990), according to which no DNA damage was observed in animals treated *in vivo* with chromium chloride, and no micronuclei were seen in animals given chromium nitrate.

4.6. Reproductive toxicity

Chromium (III) chloride dissolved in tap water was given to sexually mature male and female Swiss mice (day 50 of age). Males received water with 1000 or 5000 mg/L chromium chloride and females with 2000 or 5000 mg/L *ad libitum* for 12 weeks. Controls were given tap water, only. Treated animals consumed less water per day than controls did. Chromium chloride reduced fertility and seminal vesicle weights significantly. Body weights were reduced in males but not in females. Testes and ovarian weights were increased whereas uterine weights were significantly reduced. The number of resorptions and dead foetuses was increased in females impregnated by males exposed to the trivalent compound and the number of resorptions in exposed females as well (Elbetieha and Al-Hamood, 1997). Unfortunately, the authors did not report the actual quantitative exposure to chromium chloride but EGVM (2002b) estimated from the given data oral doses for trivalent chromium of approximately 500 or 1250 mg/kg bw/day for females and 250 or 1250 mg/kg bw/day for males.

The fertility of male Sprague Dawley rats exposed to chromium (III) chloride in drinking water at a concentration of 1000 mg/L for 12 weeks, which is equivalent to about 50 mg $CrCl_3$/kg body weight or about 16,5 mg trivalent chromium/kg body weight, was unaffected but significant reductions in the weight of testes and seminal vesicles were observed (Bataineh *et al*, 1997).

There are no reports of developmental toxicity studies on chromium (III) compounds given orally.

4.7. Human data

In some case reports, the ingestion of chromium picolinate was associated with a number of adverse effects which might be due to the picolinate ligand (Martin and Fuller, 1998; Young *et al*, 1999; Huszonek, 1993; Wasser and Feldmann, 1997; Cerulli *et al*, 1998). In controlled clinical supplementation studies, however, no adverse effects have been observed following oral administration of daily doses up to 1 mg chromium, mostly as picolinate, for 6-64 weeks (Table 3). These studies are limited because they were primarily designed as studies on efficacy.

Only one lethal case was reported of a woman, who ingested trivalent chromium as 400 mL of a leather tanning solution containing 48 g of basic chromium sulphate ($CrOHSO_4$), equivalent to about 15 g Cr. She died of cardiogenic shock, complicated by acute renal shock, pancreatitis, haemorrhage, and gut mucosal necrosis (Van Heerden *et al*, 1994).

Table 3. Randomized Controlled Trials with chromium

Reference	Daily dose (µg Cr)	Compound	Subjects/group	Duration (weeks)
Campbell *et al*, 1999	924	CrPic	9 men (56-69 yr)	12
Walker *et al*, 1998	200	CrPic	7 wrestlers	14
Lukaski *et al*, 1996	182 172	$CrCl^3$ CrPic	12 men	8
Pasman *et al*, 1997	200	CrPic	11 obese women	64
Kato *et al*, 1998	400	CrPic	10 obese women	8
Anderson *et al*, 1997b	200[1] 1000[1]	CrPic CrPic	60 men and women free of disease other than type 2 diabetes (35-65 yr)	16
Thomas and Gropper, 1996	200	CrNic	14 healthy adults and 5 adults with non-insulin-dependent diabetes	8
Hallmark *et al*, 1996	200	CrPic	8 untrained men (23±4yr)	12
Wilson and Gondy, 1995	220	CrNic	15 (mean age 36 yr)	13
Clancy *et al*, 1994	200	CrPic	18 football players	9
Hasten *et al*, 1992	200	CrPic	18 male and 12 female college-age students	12

[1] 100 µg Cr or 500 µg Cr as CrPic two times per day.
CrPic = Chromium (III) picolinate
CrNic = Chromium (III) nicotinate

5. DOSE-RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Data on the oral toxicity of trivalent chromium are limited. Doses up to 15 mg chromium/kg bw/day did not show adverse effects in a feeding study with chromium chloride and chromium picolinate in rats for 20 weeks (Anderson *et al*, 1997). However, in this study only liver and kidney have been examined histologically. A chronic toxicity/carcinogenicity study was only performed with chromium (III) oxide. Adverse effects were not observed at concentrations up to 5% in the diet, equivalent to about 1500 mg chromium/kg bw/day, fed to rats 5 days per week for 840 days (Ivankovic and Preussmann, 1975). The study, however, can not be used to derive a NOAEL for soluble chromium salts, because the tested substance was a pigment insoluble in water, alkali, and mineral acids.

In mice, doses of 250 to 1250 mg/kg body weight chromium chloride decreased fertility significantly and reduced body weights in males. It reduced semical vesicle and uterine weights and increased testes and ovarian weights. A NOAEL was not observed (Elbetieha and Al-Hamood, 1997). In male rats, exposure to 50 mg/ $CrCl_3$ /kg bw, equivalent to 16,5 mg trivalent chromium/kg body weight decreased significantly body weights and absolute testes and seminal vesicles weights but fertility remained unaffected (Bataineh *et al*, 1997).

Adequate human data on trivalent chromium are also limited. No adverse side effects were reported in a number of supplementation trials, in which subjects received up to 1 mg chromium/day, mostly as picolinate for several months. These trials, however, were mainly studies of efficacy and not designed to find potential toxic effects.

The limited data from studies on subchronic, chronic, and reproductive toxicity on soluble trivalent chromium salts and the available human data do not give clear information on the dose response relationship. Therefore, a tolerable upper intake level can not be derived.

The UK Expert Group on Vitamins and Minerals also concluded that overall there are insufficient data from human and animals studies to derive a safe upper level for chromium. However, in the opinion of the EGVM a total daily intake of about 0.15 mg trivalent chromium per kg body weight and day (or 10 mg/person) would be expected to be without adverse health effects. This value is based (using a

100-fold margin of safety) on the study of Anderson *et al* (1997a) which indicated that 15 mg trivalent chromium/kg bw/day is not associated with adverse effects in the rat. This guidance level applies only to trivalent chromium and not to chromium picolinate which is explicitly excluded from the guidance due to the *in vitro* studies, which indicated that it may damage DNA via a mechanism which is at present still unclear (EGVM, 2002b). The US Food and Nutrition Board also concluded that the data from animal and human studies are insufficient to establish an UL for soluble chromium (III) salts (FNB, 2001).

WHO considered that supplementation of chromium should not exceed 250 µg/day (WHO, 1996).

6. RISK CHARACTERIZATION

In a number of limited human studies, there was no evidence of adverse effects associated with supplementary intake of chromium up to a dose of 1 mg chromium/day. The dietary intake of trivalent chromium in European countries, as shown in Table 1, is well below these doses.

This evaluation is not applicable to chromium picolinate.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF VANADIUM

(REQUEST N° EFSA-Q-2003-018)

(ADOPTED ON 19 FEBRUARY 2004)

SUMMARY

The European Food Safety Authority is asked to derive an upper level for the intake of vanadium from food that is unlikely to pose a risk of adverse health effects.

Vanadium has not been shown to be essential for humans.

Orally administered vanadium compounds produce adverse effects on kidneys, spleen, lungs and blood pressure in rats and show reproductive and developmental toxicity in rats and mice. In humans, gastrointestinal disturbances have been reported. The available data are inadequate, however, to derive a tolerable upper intake level.

The intake of vanadium from normal food is estimated to be of the order of 10-20 µg/day. This daily intake is at least three orders of magnitude below the lowest doses reported to cause adverse effects. In the case of supplements used by athletes and body builders, however, the intake can be similar to the doses causing adverse effects in rats and humans. Therefore, a risk can be expected to result from the prolonged ingestion of such supplements.

KEY WORDS

Vanadium, tolerable upper intake level, food safety.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested the inclusion of boron, nickel, silicon, vanadium and tin in the proposal. The Commission did not accept the Parliament's request in the absence of a positive safety evaluation by the SCF. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

1 - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

ASSESSMENT

1. INTRODUCTION

Vanadium is widely distributed in the earth's crust. It occurs naturally in the form of about 70 minerals, but does not occur as metallic vanadium. In its compounds, it forms different oxidation states, the most common being +3, +4, and +5.

2. NUTRITIONAL BACKGROUND

2.1. Food levels and dietary intake

Among foods examined for vanadium by atomic absorption spectroscopy, most concentrations were within a range from 1-30 µg/kg fresh weight. Fats and oils, fruits and vegetables contained the least vanadium, ranging from 1-5 µg/kg. Whole grains, seafood, meats and dairy products generally contained 5-30 µg/kg. Dillseeds and black pepper contained the most vanadium, 431 and 987 µg/kg, respectively (Myron *et al*, 1977).

The daily dietary intake was estimated to be of the order of a few tens of µg (WHO, 1996). Total diet studies resulted in an average intake of 13 µg/day in the UK (Evans *et al*, 1985) and an estimated dietary intake in the range of 6-18 µg/day for different age-sex-groups of adults and 6.5-11 µg/day for infants, children, and adolescents in the USA (Pennington and Jones, 1987).

In drinking water supplies in the USA, 91% of samples had below 10 µg vanadium/L, the maximum concentration was 70 µg/L and the average 4.3 µg/L (Durfor and Becker, 1963). 26% of tap water samples of 34 areas in the USA contained vanadium at concentrations of 1.3-33 µg/L with a mean of 4.85 µg/L (Greathouse and Craun, 1978).

According to data provided by the manufacturers of vitamin and mineral supplements sold in the UK and representative of at least 70% of the UK market, the highest level of vanadium in multiple-nutrient products is 25 µg/tablet or capsule (EGVM, 2003). Weight training athletes are reported, however, to use up to 60 mg/day of vanadyl sulphate, equivalent to 18.6 mg vanadium/day, to improve performance (Barceloux, 1999). Vanadium supplements aimed at body builders are reported to be available at doses between 7.5 and 10 mg/day (EGVM, 2003).

2.2. Nutritional requirements and intake recommendations

Vanadium has not been shown to be essential for humans and has no nutritional value. Therefore, nutritional requirements or intake recommendations have not been established. The SCF stated that evidence supporting the essentiality of vanadium has yet to be established (SCF, 1993). A maximum intake level of 1.8 mg vanadium/day for adults is recommended by FNB and EGVM stated that there are insufficient data to establish a safe upper level (FNB, 2001; EGVM, 2003).

2.3. Deficiency

Some signs of vanadium deficiency in goats and rats have been reported (WHO, 1996). In humans, however, vanadium deficiency has not been identified.

3. BIOLOGICAL CONSIDERATIONS

3.1. Function

A number of vanadium dependent enzymes have been found in lower organisms, such as bacteria and algae. In higher animals and humans, however, no specific biochemical function has yet been identified for vanadium. Nevertheless, the possibility has been considered that vanadium might play a role in the regulation of some enzymes, such as the Na^+/K^+-exchanging ATPase, phosphoryl-transfer enzymes, adenylate cyclase and protein kinases. Therefore, its role in hormone, glucose, lipid, bone and tooth metabolism has also been discussed (WHO, 1996).

Vanadium compounds have been shown to mimic the action of insulin in isolated cell systems, animal models and diabetic patients. Therefore, their use in the therapy of diabetes mellitus has been considered (Shechter, 1990; Shamberger, 1996).

Vanadium has also been suggested as an aid in body building, but there is no evidence that it is effective (Fawcett *et al*, 1996).

3.2. Absorption, distribution, metabolism and excretion

The low concentration of vanadium normally present in urine compared with the daily intake and the faecal levels indicate, that less than 5% of ingested vanadium is absorbed (WHO, 1996). The results of animal studies are in general in agreement with this conclusion. Uptake of radioactive vanadium pentoxide (V_2O_5) given orally to rats was 2.6% of the administered dose (Conklin *et al*, 1982). Other studies in rats have indicated that amounts greater than 10% can be absorbed from the gastrointestinal tract under some conditions (Bogden *et al*, 1982; Wiegmann *et al*, 1982).

Absorbed vanadium is transported in the serum mainly bound to transferrin. Extracellular vanadium is present in the form of vanadate (5+) and intracellular vanadium most likely in the vanadyl (4+) form. After administration by different routes to rats, the highest amounts were found in lungs (after intratracheal installation), bone, kidneys, liver and spleen. Studies on rats and mice showed a three-compartment model for elimination with plasma half-times of 15 minutes, 14 hours and 8.5 days (Lagerkvist *et al*, 1986).

3.3. Normal levels in human tissues and fluids

In contrast to earlier assumptions based on colorimetric determinations, Byrne and Kosta (1978) estimated the total body pool to be only 100 to 200 µg vanadium. Normal concentrations in human blood serum have been reported to be in the range of 0.02-0.94 µg/L (Cornelis *et al*, 1981). Thus, serum vanadium values above 1 µg/L probably indicate excessive exposure (WHO, 1996).

4. HAZARD IDENTIFICATION

This section is limited to oral toxicity data with the exception of carcinogenicity. Other toxicological aspects of vanadium, in particular hazards from inhalative exposure and aspects of occupational medicine, have been reviewed elsewhere (Browning, 1969; EPA, 1977; NIOSH, 1977; NRCC, 1980; MAK, 1984; Lagerkvist *et al*, 1986; WHO, 1988 and 2001).

4.1. Effects on cell cycle and proliferation

Vanadium compounds elicit several biological effects, affecting cell cycle, signalling pathways, and cell survival *in vitro* and *in vivo*.

Induction of apoptosis and p53 activation were observed in murine epidermal JB6 P+ cells treated with sodium metavanadate (2.24 µg/mL) or vanadyl sulphate (3.26 µg/mL) (Ye *et al*, 1999; Huang *et al*, 2000).

In newborn rat brain cells, sodium metavanadate induced apoptosis and the activation of extracellular-signal regulated kinases (Luo *et al*, 2003).

In vivo, a marked increase of apoptotic cells was observed in lung cells of BALB/cJ mice treated orally with sodium metavanadate (50 µg/mouse). In general the addition of NADPH, sodium formate or superoxide dismutase enhanced vanadate-induced apoptosis, which was inhibited by catalase or deferoxamine, suggesting a role for oxidative species in this process (Wang *et al*, 2003).

In the human breast cancer cell line Mcf-7, vanadate stimulated cell proliferation and activated oestrogen receptor-α (ER-α), inducing the expression of oestrogen-regulated genes (Martin *et al*, 2003).

Exposure to noncytotoxic levels of vanadate stimulated interleukin-8 production in human promyelocyte cells (Sonoda *et al*, 1997), and inhibited tyrosine phosphatases in the human bronchial epithelial cell line BEAS 2B, resulting in increased levels of tyrosine phosphorylation (Samet *et al*, 1999) and activation of mitogen activated protein kinases (MAP kinases) (Samet *et al*, 1998).

In the human alveolar epithelial cancer cell line A549, exposure to vanadate led to cell growth arrest at G2/M and caused up-regulation of cell growth regulatory proteins (p21, phospho cdc2 and cdc25) and activation of MAP kinases. Different reactive oxygen species were shown to affect specific MAP kinases and cell cycle regulatory proteins (Zhang *et al*, 2003).

4.2. Acute toxicity

The oral acute toxicity of vanadium varies with the species and the nature of the compound. In general, vanadium is said to be better tolerated by the rat and the mouse than by larger animals including the rabbit and horse (WHO, 1988). Acute vanadium poisoning in animals is characterized by marked nervous disturbance, haemorrhagic enteritis and a fall of temperature. Death is preceded by paralysis of hind legs, laboured respiration and convulsions (Browning, 1969).

In rats of unknown strain, vanadium pentoxide and ammonium metavanadate have been reported to have an oral LD_{50} of 10.4 and 18.3 mg/kg body weight, equivalent to 5.8 and 8.0 mg vanadium/kg body weight, respectively (Massmann, 1956). In male Sprague-Dawley rats, sodium metavanadate and vanadyl sulphate pentahydrate were less toxic with oral LD_{50} values equivalent to 41 mg vanadium/kg body weight and 90.3 mg vanadium/kg body weight, respectively. In male Swiss mice, the LD_{50} values of these compounds were equivalent to 31 mg vanadium/kg body weight and 94 mg vanadium/kg body weight, respectively (Llobet and Domingo, 1984).

4.3. Subacute/Subchronic toxicity

Groups of 10 male Sprague-Dawley rats received 0, 5, 10, and 50 mg/L sodium metavanadate in drinking water for a period of three months. The concentrations were equivalent to approximately 0.8, 1.5 and 7.7 mg vanadium/kg body weight/day as calculated by FNB (2001). Appearance, behaviour, food and water consumption, growth, mortality and the weights of liver, kidneys, heart, spleen and lungs were not affected. The histopathological examination of organs in 3 rats per group, however, showed mild lesions in kidneys (corticomedullar microhaemorrhagic foci), spleen (hypertrophy and hyperplasy in the white pulp) and lungs (mononuclear cell infiltration, mostly perivascular), more evident in animals receiving the highest dose. These lesions are described as dose-dependent, their significance, however, is not clearly specified. The vanadium concentrations in a number of organs were elevated at the highest dose and in kidneys and spleen at the dose of 10 mg/L. In addition, significantly increased plasma concentrations of urea and uric acid were found at the highest dose level (Domingo *et al*, 1985).

Groups of 12 male streptozotocin-induced diabetic rats were exposed to drinking water containing NaCl (80 mM) and sodium metavanadate (150 mg/L), sodium orthovanadate (230 mg/L) or vanadyl sulphate pentahydrate (310 mg/L) for 28 days. The groups were compared to controls, either diabetic or non-diabetic, receiving drinking water containing NaCl (80 mM) only. At the ingested doses, equivalent to 6.1, 15.6 and 22.7 mg vanadium/kg body weight/day, the daily food and fluid intake as well as the blood glucose levels were significantly reduced relative to the diabetic controls, with sodium metavanadate being the most effective compound tested. Signs of toxicity including decreased weight gain, increased serum concentrations of urea and creatinine and some deaths were observed in all vanadium-treated animals (Domingo *et al*, 1991).

Groups of 10 male Sprague-Dawley rats received drinking water with 0 and 100 mg vanadium/L as sodium metavanadate for 7 months. Heart rate and systolic and diastolic blood pressure were increased significantly in the vanadate exposed animals. Vanadate strongly increased also the levels of urinary Na+ and K+ and affected the kidneys, where the lumen of the proximal tubule cells was narrowed and contained amorphous protein material (Carmignani *et al*, 1991).

Groups of 6 male weaning Sprague-Dawley rats were given sodium metavanadate in drinking water at concentrations of 0, 10 and 40 mg vanadium/L for 210 days and 0 and 1 mg vanadium/L for 180 days. All vanadium exposed rats showed a significant increase in systolic and diastolic blood pressure, but not of heart rate and cardiac inotropism. These changes were not dose-dependent. Rats exposed to 40 mg/L had effects on kidney morphology (narrowed proximal tubules containing amorphous material and hydropic degeneration in proximal, distal and straight tubules). Such abnormalities were less evident in rats treated with 10 mg/L and absent in those treated with 1 mg/L of vanadium. At 10 and 40 mg/L, plasma renin, urinary kallikrein and urinary kininase I and II activities as well as urinary potassium were increased (Boscolo *et al*, 1994).

Groups of 5 male unilateral-nephrectomized Sprague-Dawley rats received normal rat chow containing 0.3 mg vanadium/kg and normal rat chow supplemented with sodium orthovanadate, equivalent to 100 mg vanadium/kg diet, and drank either tap water or a 1% solution of sodium chloride for a period of 9 weeks. In a second experiment, groups of 9 male unilateral-nephrectomized Sprague-Dawley rats drank tap water and ate normal rat chow or normal rat chow supplemented with sodium orthovanadate,

equivalent to 100 or 200 mg vanadium/kg diet for a period of 56 weeks. In the vanadate exposed rats drinking tap water, systolic blood pressure gradually increased over several weeks and then was sustained in a dose-related manner. This effect was correlated positively with plasma vanadium levels from 0.04 to 0.27 µg/mL and associated with an increased heart-to-body-weight ratio (Steffen *et al*, 1981).

Groups of about 12 two-month old Wistar rats of both sexes received 0 and 150 mg vanadium/L as ammonium metavanadate in their drinking water for a period of 4 weeks, equivalent to 0 and 13 mg vanadium/kg body weight/day, respectively. A small decrease in food and water consumption and body weight gain was observed in treated animals with transient diarrhoea in two cases. In the peripheral blood, a significant decrease in erythrocyte count and haemoglobin level and significant increases in the percentage of reticulocytes and polychromatophilic erythrocytes and in the neutrophilic granulocyte and lymphocyte count were noted. Some inhibitory influence on the phagocytic activity of granulocytes in females was also observed (Zaporowska and Wasilewski, 1992).

Groups of about 15 two-month old Wistar rats of both sexes received 0, 10 and 50 mg vanadium/L as ammonium metavanadate in their drinking water for a period of 4 weeks, equivalent to 0, 1.5 and 5 to 6 mg vanadium/kg body weight/day, respectively. The erythrocyte count was significantly decreased at both dose levels associated with a significant decrease of the haemoglobin level and increased percentage of reticulocytes in the peripheral blood at the highest dose level. In addition, the L-ascorbic acid level in the plasma of males was significantly decreased at both dose levels (Zaporowska *et al*, 1993).

In a study with non-diabetic and streptozotocin-diabetic male Wistar rats, groups of 8-32 animals were untreated or exposed to vanadyl sulphate hydrate for 52 weeks at doses of 500 mg/L stepwise increased to 1250 or 1500 mg/L (Dai *et al*, 1994a), equivalent to 8-36 mg vanadium/kg body weight/day (Dai *et al*, 1995). The treatment caused significant decreases in body weight gain and plasma insulin level in non-diabetic rats (Dai *et al*, 1994b), but did not produce persistent impairment of hepatic or renal function and significant changes in organ weights, gross and microscopic findings in either non-diabetic or diabetic animals. However, increased amounts of vanadium were retained in various organs for months after cessation of treatment in the following rank order: bone > kidneys > testis > liver > pancreas > brain (Dai *et al*, 1994a). In contrast to other studies, systolic blood pressure, pulse rate and selected haematological indices were not significantly changed (Dai and McNeill, 1994).

In another study, groups of 8 male Wistar rats were exposed to ammonium metavanadate (10 mg vanadium/kg body weight/day), vanadyl sulphate hydrate (8 mg vanadium/kg body weight/day) and bis (maltolato)oxovanadium (IV) (9 mg vanadium/kg body weight/day) for 12 weeks. Again, there were no significant differences of haematological parameters between controls and exposed animals (Dai *et al*, 1995).

4.4. Chronic toxicity/Carcinogenicity

Only limited carcinogenicity studies by oral route on vanadyl sulfate are presently available.

Groups of 23 male and 29 female Charles River CD mice were given 5 mg/L vanadium as vanadyl sulfate in the drinking water from weaning until natural death. The diet fed to the mice for the first 6 months contained 1.4 mg/kg vanadium and thereafter 3.2 mg/kg vanadium, on a wet basis. Dead animals were autopsied, gross lesions noted and abnormal tissues sectioned for microscopic analysis. No effects in terms of growth, survival, longevity, increased incidence of tumours or other pathological signs were observed (Schroeder and Balassa, 1967).

Groups of 50 Long-Evans rats of each sex received drinking water containing 5 mg/L vanadium as vanadyl sulfate from the time of weaning until natural death. The diet contained 3.2 mg/kg vanadium. Seventeen animals of each sex were killed by an epidemic of virulent pneumonia and removed from the series. No toxicity was observed. Growth, survival and incidence of visible tumours at necropsy were not significantly different from the controls (Schroeder *et al*, 1970).

Groups of 54 males and 54 females of Swiss mice of the Charles River CD strain were given 5 mg/L vanadium as vanadyl sulfate in the drinking water for life span. Body weights were examined at intervals and animals that died naturally were dissected, gross tumours detected and sections made of heart, lung, liver, kidney and spleen for microscopic examination. Males given vanadium had significantly higher body weights than controls and longevity was increased by vanadium in both sexes. A slight increase in the number of tumour-bearing female mice was not considered relevant (Schroeder and Mitchener, 1975).

The results of these studies are hampered by strong limitations such as: a non-standard protocol; only one single dose level; no signs of toxicity; high content of vanadium in the diet (more than half of that compared with the vanadium concentration in the drinking water); high spontaneous total incidence of tumours. Therefore, the results of these studies should be considered inconclusive.

Recently, the results of 2-year standard bioassays with vanadium pentoxide particles in F344/N rats and B6C3F1 mice exposed by inhalation have been made available (NTP, 2002).

Groups of 50 male and 50 female rats were exposed to particulate aerosols of vanadium pentoxide at concentrations of 0, 0.5, 1 or 2 mg/m^3 by inhalation, 6 hours per days, 5 days per week for 104 weeks. Survival and body weights were generally similar to those of the controls; only mean body weights in females exposed to 2 mg/m^3 were lower than those of the controls. In male rats, the incidences of lung alveolar/bronchiolar neoplasms (adenomas or carcinomas) often exceeded the historical control ranges: adenomas (4/50, 8/49, 5/48, 6/50); carcinomas (0/50, 3/49, 1/48, 3/50); adenomas/carcinomas (4/50, 10/49, 6/48, 9/50). Some tumours also occurred, at lower extent, in the females: adenomas (0/49, 3/49, 1/50, 0/50); adenomas plus 1 carcinoma at the top dose (0/49, 3/49, 1/50, 1/50). Non-neoplastic lesions in the respiratory tract (e.g. hyperplasia, inflammation, fibrosis) were also observed, generally increased at higher concentrations. The NTP conclusions were: “some evidence of carcinogenic activity” in male rats, and “equivocal evidence of carcinogenic activity” in female rats.

Groups of 50 male and 50 female mice were exposed to particulate aerosols of vanadium pentoxide at concentrations of 0, 1, 2 or 4 mg/m^3 by inhalation, 6 hours per day, 5 days per week for 104 weeks. Survival of males at the top concentration was significantly less than that of controls. Mean body weights of 4 mg/m^3 males and all exposed groups of females were generally less than in controls. The incidences of lung alveolar/bronchiolar neoplasms were significantly increased in all exposed groups both in males and in females. Males: adenomas (13/50, 16/50, 26/50, 15/50); carcinomas (12/50, 29/50, 30/50, 35/50); adenomas/carcinomas (22/50, 42/50, 43/50, 43/50). Females: adenomas (1/50, 17/50, 23/50, 19/50); carcinomas (0/50, 23/50, 18/50, 22/50); adenomas/carcinomas (1/50, 32/50, 35/50, 32/50). Non-neoplastic lesions were also observed with increased severity at higher concentrations. The NTP conclusion was: “clear evidence of carcinogenic activity” in male and female mice.

Based on these inhalation studies, vanadium pentoxide has been evaluated by IARC as “possibly carcinogenic to humans” (Group 2B) on the basis of “sufficient evidence” in experimental animals, in the absence of human data (IARC Monographs, Vol. 86, 2003). The relevance of the results of the NTP inhalation studies in rats and mice for oral ingestion of vanadium pentoxide or other vanadium compounds is unclear.

4.5. Genotoxicity

4.5.1. In vitro studies in bacteria

In a screening of metal compounds with a semiquantitative spot test procedure without metabolic activation, vanadium pentoxide (+5) and ammonium metavanadate (+5) were reported not to be mutagenic in the *Escherichia coli* strains B/r and WP2, and in *Salmonella enterica var.* Typhimurium TA1535, TA1537, TA1538, TA98 and TA100 (Kanematsu *et al*, 1980). In the same study, vanadium oxydichloride, vanadium pentoxide and ammonium metavanadate were evaluated as positive in the rec assay in *Bacillus subtilis.*

Ammonium metavanadate was found to be weakly mutagenic in *Salmonella enterica var.* Typhimurium TA1535 in a plate incorporation assay with a modified Vogel-Bonner medium, inducing a doubling of revertants at the highest dose tested (43.3 µg/plate, without S9) (Arlauskas *et al*, 1985).

Negative results were obtained in the standard *Salmonella enterica var.* Typhimurium reversion test (Ames test) with vanadium pentoxide at concentrations from 0.03 to 333 µg/plate in strains TA97, TA98, TA100, TA102, and TA1535, with or without Aroclor induced rat or hamster liver S9 (NTP, 2002).

4.5.2. In vitro studies in eukaryotic cells

Mitotic gene conversion and point mutations were induced in *Saccharomyces cerevisiae* strain D7 by ammonium metavanadate at concentrations from 9.4 to 24.6 µg/mL (Bronzetti *et al*, 1990). Positive results were also obtained in the same test system with vanadyl sulfate (+4) at concentrations from 26.1 to 68.5 µg/mL, only in experiments with cells from logarithmic growth phase (Galli *et al*, 1991). In the same study, both ammonium metavanadate (0.47-0.58 µg/mL) and vanadyl sulfate (1.22-1.63 µg/mL) induced mitotic aneuploidy in the *S. cerevisiae* strain D6.1M.

In Chinese hamster V79 cells, no induction of forward mutations at the hprt locus was observed after treatment with vanadyl sulfate (+4) (0.081-0.81 µg/mL, both in the presence or in the absence of metabolic activation (Galli *et al*, 1991), or after treatment with vanadium pentoxide (1-3 µg/mL) in the absence of metabolic activation (Zhong *et al*, 1994). In experiments without exogenous metabolic activation, ammonium metavanadate, at low non-cytotoxic concentrations (up to 0.58 ng/mL), increased the frequency of mutations at the *hprt* locus in V79 cells, and, at higher concentrations (0.585 µg/mL), at the bacterial *gpt* gene in transgenic G12 cells (Cohen *et al*, 1992).

Vanadium trioxide (+3), vanadyl sulfate and ammonium metavanadate, at concentrations respectively of 0.1-1 µg/mL, 0.5-6 µg/mL and 0.5-4 µg/mL, induced significant increases in SCE frequencies in Chinese hamster ovary cells (CHO-K1). The genotoxic activities of ammonium metavanadate and vanadyl sulphate were decreased in the presence of hepatic S9 (Owusu-Yaw *et al*, 1990). In the same study, all three vanadium compounds induced significant increases of structural chromosomal aberrations, with a prevalence of breaks and exchanges, after a 2-hour treatment with 4, 8, 16 µg/mL (ammonium metavanadate), 6, 12, 24 µg/mL (vanadyl sulphate), and 12, 18 µg/mL (vanadium trioxide), both in the absence and in the presence of metabolic activation.

In another study, no significant increase in sister chromatid exchange (SCE) frequency was observed in Chinese hamster V79 cells after 24h treatment with vanadium pentoxide at concentrations up to 4 µg/mL. However, a dose-related increase of endoreduplication and micronucleated cells containing CREST-positive micronuclei was observed, indicating an aneugenic effect (Zhong *et al*, 1994).

No induction of structural chromosome aberrations and SCE was observed in human lymphocytes treated for 48h with 2, 4 and 6 µg/mL of vanadium pentoxide in the absence of metabolic activation (Roldán and Altamirano, 1990). However, a statistically significant increase in the frequency of polyploid cells was reported at all dose levels.

A study by Migliore *et al* (1993), investigated the ability of sodium ortho-(0.41-13.12 µg/mL) and metavanadate (0.28-8.96 µg/mL), ammonium metavanadate (0.29-9.36 µg/mL) and vanadyl sulphate (0.41-13.04 µg/mL) to produce structural and numerical chromosome aberrations, micronuclei and SCE in human lymphocytes *in vitro*. Treatments with vanadium compounds did not induce any significant increase in the frequency of cells with structural chromosomal aberrations. On the other hand, statistically significant increases in SCE rates were observed with all compounds at the two highest concentrations tested, and large increases in the incidence of micronuclei from 1.64 µg/mL onwards. The characterization of micronuclei by fluorescence in situ hybridization showed that centromeres were present in a large fraction of micronuclei, indicating an aneugenic potential for these vanadium compounds. The same authors also demonstrated the preferential loss of acrocentric and sex chromosomes after treatment of hyman lymphocytes *in vitro* with vanadyl sulphate and sodium orthovanadate (Migliore *et al*, 1995 and 1999).

Immunostaining of the spindle apparatus using an anti-β-tubulin antibody, and an *in vitro* assay measuring the polymerization/depolymerization of purified tubulin, confirmed that vanadium pentoxide at doses as low as 1.82 ng/mL affect spindle organization and function in human lymphocytes through the disruption of microtubules (Ramírez *et al*, 1997).

In a recent study, Rodríguez-Mercado *et al* (2003) evaluated the genotoxicity of vanadium tetraoxide (+4) in human peripheral blood cells *in vitro*. A dose-related increase of structural chromosome aberrations and SCE, as well as an inhibition of mitotic and replicative index, were observed in cultures treated with vanadium tetraoxide (+4) (2-16 µg/mL, without metabolic activation). On the other hand, no increase in comet test parameters was observed after short (2 h) treatments with V_2O_4, possibly because of the limited ability of V+4 to cross cell membrane.

In other tests for DNA damage using single cell gel electrophoresis (comet assay), vanadium pentoxide induced DNA single strand breaks and/or alkali labile sites in non-stimulated human leukocytes at doses from 0.54 µg/mL or more. On the other hand, in stimulated lymphocytes only the highest concentration tested (540 µg/mL) induced a significant increase in DNA damage (Rojas *et al*, 1996).

In another study, orthovanadate significantly increased comet tail length in non-stimulated human lymphocytes (0.25-0.5 µg/mL) and in cultured human fibroblasts (from 0.025 µg/mL); moreover, co-exposure of human fibroblasts to vanadate (0.025 µg/mL) and UV or bleomycin resulted in non-repairable DNA double-strand breaks (Ivancsits *et al*, 2002).

The induction of morphological transformation by vanadium +5 and +4 has been studied in mouse BALB 3T3 cells. Ammonium and sodium vanadate (+5) showed transforming activity at concentrations of 0.351 µg/mL or higher, while vanadyl sulphate (+4) was ineffective (Sabbioni *et al*, 1991 and 1993).

Sodium orthovanadate (0.033-1.14 µg/mL) was assayed for cell transformation in Syrian hamster embryo cells. A marked increase in cell transformation was noted only at the highest dose, without any effect on cloning efficiency (Rivedal *et al*, 1990; Kerckaert *et al*, 1996).

Finally, ammonium metavanadate, added at the concentration of 0.585 µg/mL, amplified the yields of type II and type III foci in methylcholanthrene-initiated C3H/10T1/2 cells (Parfett and Pilon, 1995).

4.5.3. In vivo studies

Treatment of Drosophila larvae with vanadyl sulphate (163-407 µg/mL) induced somatic mutations, detected as aberrant red sectors in the w+/w adult eye (Barrera Ferrer and Villalobos Cabrera, 1998).

Single cell gel electrophoresis (comet assay) was used to assess the ability of V_2O_5 to induce DNA damage *in vivo* in six different organs (liver, kidney, lung, spleen, heart and bone marrow) of CD-1 mice. V_2O_5 was given by intraperitoneal injection at 5.75, 11.5 and 23 mg/kg body weight (LD_{50}) 24 h before sacrifice. A significant, although not dose-dependent, increase of DNA migration was observed in kidney, liver, heart and lung cells, whereas only slight damage was induced in spleen cells, and no effect in bone marrow (Altamirano-Lozano *et al*, 1999). With the same protocol significant, dose-related increase of DNA migration was observed in testicular cells with the lowest dose assayed (5.75 mg/kg) onwards (Altamirano-Lozano *et al*, 1996).

Vanadyl sulphate, sodium orthovanadate and ammonium metavanadate were tested for the induction of micronuclei, and structural and numerical chromosome aberrations in bone marrow of male CD-1 mice. Micronuclei and hyperploidy were induced by all compounds when given by single intragastric administration at 100, 75 and 50 mg/kg body weight, respectively, but only vanadyl sulphate induced a significant increase of structural chromosomal aberrations at the dose of 100 mg/kg body weight, corresponding approximately to 1/5 of the LD50 (Ciranni *et al*, 1995).

No increase in the frequency of micronucleated normochromatic erythrocytes, and no alteration of the PCE/NCE ratio, were seen in peripheral blood samples of male and female B6C3F1 mice exposed for 3 months by inhalation to 1-16 mg/m3 vanadium pentoxide (NTP, 2002).

Sodium orthovanadate, administered intraperitoneally to ICR female mice during oocyte maturation at doses of 0, 5, 15, and 25 mg/kg body weight, induced different cytogenetic abnormalities in oocytes and in bone marrow cells. In oocytes, vanadate induced premature anaphase, whereas in bone marrow sodium orthovanadate increased tetraploidy, hyperploidy and premature centromere separation (Mailhes *et al*, 2003).

4.5.4. Summary of genotoxicity test results

Overall, genotoxicity test results suggest that vanadium compounds do not induce gene mutations in bacterial cells as well as, with the possible exception of ammonium metavanadate, in mammalian cells. There is clear evidence that pentavalent and tetravalent forms of vanadium produce aneuploidy *in vitro* and *in vivo*, very likely through interference with microtubule assembly and spindle formation (Winkelhaus and Hauser, 1997; Ochi, 2002), as well as polyploidy, endoreduplication and other aneugenic-related effects. The production of micronuclei, *in vitro* and/or *in vivo*, appears to be caused by chromosome loss (aneuploidy), and not by chromosome-breaking mechanism (clastogenicity).

Less clear is the evidence that vanadium compounds can produce structural chromosomal aberrations, although vanadium tetraoxide was positive *in vitro*, and vanadyl sulphate was weakly positive *in vivo*.

The DNA damaging activity, observed *in vitro* and/or *in vivo* by the Comet assay, is probably due to reactive oxygen species generated through a Fenton-like reaction (Sakurai, 1994; Shi *et al*, 1996; Altamirano-Lozano, 1998).

In conclusion, the nature of the genotoxicity database indicates that indirect, threshold-based modes of action are probably responsible of the aneugenic and DNA damaging activity of vanadium compounds.

4.6. Reproductive and developmental toxicity

Groups of 20 female Sprague-Dawley rats were administered intragastrically 0, 5, 10 and 20 mg sodium metavanadate/kg body weight/day, equivalent to about 0, 2, 4, and 8 mg vanadium/kg body weight/day for 14 days before mating with groups of 20 Sprague-Dawley males which had received the same doses for 60 days. No significant adverse effects were observed on numbers of corpora lutea, implantations, live and dead foetuses, or resorptions. However, the average body weight/litter of the rat pups nursed by vanadium-treated mothers was significantly reduced on post-natal day 21 at 10 and 20 mg/kg body weight/day. In addition, average body weight, body length and tail length as well as relative organ weights of liver, kidneys and spleen showed significant decreases even at the lowest dose (Domingo *et al*, 1986).

Groups of 20 pregnant rats were given intragastrically doses of 0, 5, 10 and 20 mg sodium metavanadate/kg body weight/day on days 6-14 of gestation. The highest dose equivalent to about 8 mg vanadium/kg body weight/day was found to be embryotoxic but was not embryolethal or teratogenic (Paternain *et al*, 1987).

Groups of 16 to 20 pregnant Swiss mice were administered doses of 0, 37.5, 75 and 150 mg/kg body weight/day of vanadyl sulphate pentahydrate, equivalent to about 0, 7.5, 15 and 30 mg vanadium/kg body weight/day once daily by gavage on gestational days 6-15. Maternal toxicity was observed as evidenced by reduced weight gain, reduced body weight and decreased absolute liver and kidney weights at 75 and 150 mg/kg body weight/day. The number of total implants, live and dead foetuses, late resorptions, the sex ratio and the post-implantation losses were not significantly different from the controls. However, there was a significant increase in the number of early resorptions per litter, a reduction of body weights and body length, an increase of the number of stunted foetuses and an increased incidence of poorly ossified skeletal elements at all dose levels. In addition, an increase of external malformations was seen with cleft palates and micrognathia as the major gross malformations at 30 mg vanadium/kg body weight/day (Paternain *et al*, 1990).

Groups of 14-20 Swiss mice received sodium orthovanadate by gavage at doses of 0, 7.5, 15, 30, and 60 mg/kg body weight/day on days 6-15 of pregnancy. Maternal toxicity was observed at 30 and 60 mg/kg body weight/day and reduced food consumption and weight gain at 15 and 30 mg/kg body weight/day. Embryolethality and teratogenicity were not observed, but foetal toxicity in the form of a significant delay in the ossification process of skeletal districts was seen at 30 mg/kg body weight/day. The no observed adverse effect level (NOAEL) for maternal toxicity was 7.5 mg/kg body weight/day, equivalent to about 2 mg vanadium/kg body weight/day, and the NOAEL for developmental toxicity was 15 mg/kg body weight/day, equivalent to about 4 mg vanadium/kg body weight/day, under the conditions of this study (Sanchez *et al*, 1991).

Groups of 24 male Swiss mice received for 64 days sodium metavanadate in the drinking water at dosages of 0, 20, 40, 60 and 80 mg/kg body weight/day, equivalent to about 0, 8, 17, 25 and 33 mg vanadium/kg body weight/day. At the end of the exposure period, each group was divided into two subgroups: 8 animals were used for a mating trial and 16 animals for testes pathology and sperm examination. A significant decrease in the pregnancy rate was observed at 60 and 80 mg/kg body weight/day. Decreased body and epididymis weight was observed at 80 mg/kg body weight/day. Spermatozoa counts were significantly reduced at 60 and 80 mg/kg body weight/day, but the sperm motility was unaffected. The NOAEL in this study was 40 mg/kg body weight/day, equivalent to about 17 mg vanadium/kg body weight/day (Llobet *et al*, 1993).

4.7. Human data

Twelve patients, 10 of whom had coronary heart disease and 9 were hypercholesterolaemic, were treated orally with diammonium vanadotartrate (25 mg, 3 times daily for 2 weeks, increased to 125 mg daily during the following fortnight and maintained for a further 5 months in 10 patients). Persistent abdominal pain, anorexia, nausea and loss of weight were reported in 5 patients. A green tongue appeared in 5 men and one other developed pharyngitis with marginal ulceration of the tongue (Somerville and Davies, 1962).

Six patients received ammonium vanadyl tartrate in increasing doses between 25 and 125 mg/day for 45 to 68 days. All patients experienced gastrointestinal difficulties with cramps and diarrhoea including one patient who did not receive more than 50 mg/day, equivalent to about 0.17 mg vanadium/kg body weight/day. Doses of 50 mg/day or more resulted also in a green tint on the tongue. No effects were seen at 25 mg/day in 4 subjects, equivalent to approximately 0.08 mg vanadium/kg body weight/day (Dimond *et al*, 1963).

125 mg sodium metavanadate/day, equivalent to 0.83 mg vanadium/kg body weight/day, administered orally to 10 diabetic patients for two weeks resulted in an improvement of insulin sensitivity in non-insulin- and insulin-dependent diabetes mellitus patients and a decrease in serum cholesterol levels. The major adverse side effect was gastrointestinal intolerance including mild diarrhoea in 4 subjects. No evidence of toxicity was detected by screening electrolytes, blood urea nitrogen and creatinine, by liver and thyroid function studies, urine analysis and a complete blood count (Goldfine *et al*, 1995).

Gastrointestinal effects including nausea, diarrhoea, abdominal cramps and flatulence were reported in patients with non-insulin dependent diabetes mellitus given 100 mg vanadyl sulphate/day for a few weeks, equivalent to approximately 0.52 mg vanadium/kg body weight/day (Cohen *et al*, 1995; Boden *et al*, 1996; Halberstam *et al*, 1996).

In a double blind, placebo-controlled trial, vanadyl sulphate capsules were given to 11 male and 4 female weight training athletes at mean daily doses of 9 mg vanadium, i.e. 0.12 mg vanadium/kg body weight/day, for a period of 12 weeks. 5 subjects in the treatment group and 4 in the placebo group withdrew for various reasons. Body weight and blood pressure remained stable during the trial. No treatment effects were seen on haematocrit, blood viscosity, haematological and biochemical indices, compared to the placebo group (Fawcett *et al*, 1997).

DNA strand breaks, 8-OHdG, and SCE were analysed in blood leukocytes and lymphocytes of 46 male workers employed in a vanadium factory (vanadium concentration in serum 2.18-46.35 µg/L), and compared with 12 non-exposed controls. No exposure-related increase in genotoxic or oxidative DNA damage was observed (Ivancsits *et al*, 2002).

CONCLUSIONS

1. DOSE RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

According to the available subacute and subchronic studies with rats, orally administered vanadium compounds produced adverse effects on kidneys, spleen, lungs and blood pressure. In one study, effects occurred even at 0.8 mg vanadium/kg body weight/day after administration for a period of 3 months (Domingo *et al*, 1985). A NOAEL cannot be derived from these studies.

Similarly, there is no NOAEL for developmental toxicity in rats. Toxic effects of sodium metavanadate were seen in the offspring of rats nursed by vanadium-treated mothers at and above doses of 2 mg vanadium/kg body weight/day (Domingo *et al*, 1986). In mice, however, NOAELs for maternal toxicity of about 2 mg vanadium/kg body weight/day and for developmental toxicity of about 4 mg vanadium/kg body weight/day were reported (Sanchez *et al*, 1991).

An adequate evaluation of the carcinogenic potential of vanadium by the oral route is not possible, due to strong limitations of the presently available feed studies. The relevance of the NTP inhalation studies in rats and mice for oral ingestion of vanadium is unclear.

There is clear evidence that both pentavalent and tetravalent vanadium compounds induce aneuploidy *in vitro* due to interference with the spindle apparatus. These forms, as well as trivalent vanadium, can also produce DNA damage *in vitro* and/or (V_2O_5) *in vivo*, probably due to reactive oxygen species. Overall, the available data suggest that vanadium compounds do not produce gene mutations. The evidence for them also being able to produce structural chromosomal aberrations is unclear.

Clinical experience in humans is limited to studies with small numbers of volunteers and patients. In these studies, gastrointestinal disturbances including diarrhoea and abdominal cramps were observed as major side effects. The lowest dose of a vanadium compound reported to cause such an effect, was equivalent to about 0.2 mg vanadium/kg body weight/day (Dimond *et al*, 1963).

On the basis of these limited dose-response data, a tolerable upper intake level cannot be derived.

2. RISK CHARACTERIZATION

Vanadium has not been shown to be essential for humans.

Studies in humans have shown that the oral intake of vanadium compounds can cause gastrointestinal disturbances. In addition, vanadium compounds affect kidneys and other organs of rats at relatively low doses and have adverse effects at higher doses on the reproduction and the development of the offspring of rats and mice. These effects have not been demonstrated in humans, but there is no evidence that they cannot occur in humans.

The mean dietary intake of vanadium is about of 10-20 µg/person/day or 0.2-0.3 µg/kg body weight/day. This daily intake is at least three orders of magnitude below the lowest doses reported to cause adverse effects in rats (800 µg vanadium/kg body weight/day) and in humans (about 200 µg vanadium/kg body weight/day).
The available data, however, are inadequate to define the highest level of oral intake that can be regarded as tolerable. In the case of supplements taken by athletes and body builders, with daily doses up to 18 mg vanadium/person/day, equivalent to about 300 µg vanadium/kg body weight/day, the intake is similar to the doses reported to cause gastrointestinal effects in humans and kidney lesions in rats. Therefore, a risk of adverse effects could be expected to result from the prolonged ingestion of such supplements.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF SILICON

(REQUEST N° EFSA-Q-2003-018)

(ADOPTED ON 28 APRIL 2004)

SUMMARY

The European Food Safety Authority is asked to derive an upper level for the intake of silicon from food that is unlikely to pose a risk of adverse health effects.

Silicon has not been shown to be essential for humans. Silicon occurs naturally in foods as silicon dioxide (silica) and silicates, and may also be added as an anti-caking and anti-foaming agent in the form of silica, silicates and dimethylpolysiloxane. Silicate-containing antacids have been widely used for a number of decades.

Short-term oral ingestion of sodium or magnesium silicate produces adverse renal effects in dogs and guinea pigs, but not in rats; similar doses of silicon dioxide and aluminum silicate did not produce adverse renal effects in dogs or rats. Long-term oral administration of silica at high dose levels inhibits growth in rats and mice. This effect was not regarded as a toxic effect, but was rather due to nutritional imbalance because of the high dose of silica added to the diet. In humans, apart from occasional reports of renal stones, mainly associated with long-term use of silicate-containing antacids, there is little evidence of adverse effects of orally ingested silicon. The available data are inadequate to derive a tolerable upper intake level.

The estimated typical dietary intake (20-50 mg silicon/day) corresponds to 0.3-0.8 mg/kg body weight/day in a 60 kg person. These intakes are unlikely to cause adverse effects.

KEY WORDS

Silicon, tolerable upper intake level, metabolism, function, food safety.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.
With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

1. *Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.*

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Silicon is a non-metallic element with atomic weight of 28. It occurs in the earth's crust at an average concentration of about 28%, but does not exist in nature in forms other than as silicon dioxide (silica) or as silicates (Friedberg and Schiller, 1988). Silica consists of free silicon dioxide, which is amorphous (e.g. diatomaceus earth) or crystalline (e.g. quartz, tridymite and cristobalite), or in combination with various cations as silicates (e.g. Fuller's earth, asbestos, talc and mica). Silicon in water is present as ortho-silicic acid $Si(OH)_4$. Silicic acid exists as monosilicic acid. A saturated solution contains 0.1% silicic acid. Silicic acid can also exist as oligomers and as polysilicic acid, which is colloidal. The chemistry of silicon has many similarities to that of carbon. It forms bonds with silicon, hydrogen, oxygen, nitrogen and carbon. The substitution of carbon for silicon in organic compounds results in molecules with different properties due to a larger size and electronegativity of silicon.

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

The essentiality of silicon for man has not been established and a functional role for silicon in humans has not yet been identified. In animals silicon is found in bound form that has never been fully characterised. Probably, it is present as silicic acid or silanolate, which may play a role in the structural organisation of some mucopolysaccharides (Nielsen, 1994). In chicken and rats, silicon appears to be involved in bone formation and metabolism (Nielsen, 1994).

Silicon is found in connective tissues, including aorta, trachea, tendon, bone and skin. In blood silicon is not bound to protein and exists as silicic acid (Carlisle, 1984). Both the distribution of silicon in animals and the effect of silicon deficiency on the form and composition of connective tissue support the view that silicon functions as a biological cross-linking agent contributing to the architecture of connective tissue (Carlisle, 1984 and 1988). In 1972, Carlisle and Schwartz and Milne reported that silicon deprivation in chicks and rats led to abnormally shaped bones and cartilagenous tissue, which both were restored upon supplementation with soluble silicon. Subsequent studies confirmed these findings and also extended them to calves (Carlisle 1980a and b; Calomme and Vanden Berghe, 1997). Silicon was detected in small areas of ossifying bone during mineralisation, but less silicon was found in bone at a later stage in bone development. Rats deprived in silicon show decreased bone hydroxyproline and alkaline- and acid phosphatase activity (Seaborn and Nielsen, 1993 and 1994). Furthermore, silicon deprivation in rats decreases collagen formation in wounds and bone and liver ornithine transaminase activity a key enzyme in proline synthesis (Seaborn and Nielsen, 2002). Silicon appears to increase prolyl-4-hydroxylase, galactosyl-hydroxyl-lysyl glucosyl-transferase and lysyloxidase activities, enzymes that modify collagen (Nielsen, 1994). Silicon is apparently also involved in bone calcification, but the mechanisms are unclear (Nielsen, 1994). While a role for silicon has not been defined in humans, recent *in vitro* studies show that orthosilicic acid at physiological concentrations stimulated collagen type 1 synthesis, probably by modulating prolyl hydroxylase activity, in human osteoblast-like cells and to a lower degree in skin fibroblasts, and promoted osteoblastic differentiation (Reffitt *et al*, 2003).

A significant portion of dietary silicon (20-50 mg/day) is excreted in the urine (8.7 to 33.1 mg/24 hours) suggesting that silicon in the diet is fairly well absorbed (literature reviewed in Reffitt *et al* 1999). Silicon from beverages is apparently well absorbed as Bellia *et al* (1994) found that 42-75% of silicon in beer was excreted in the urine. However, there are only a few reports where the uptake and excretion of silicon ingested as orthosilicic acid in water are described. Popplewell *et al* (1998) used ^{32}Si and determined silicon in urine by accelerator mass spectrometry in one healthy male. Within 48 hours 36% of the dose was excreted into the urine and the elimination appeared to be near to complete. Two first-order phases of elimination with half-lives of 2.7 and 11.3 hours were found. Reffitt *et al* (1999) studied silicon kinetics following intake of orthosilicic acid in water (27-55 mg/L) in healthy individuals, six men and two women. Based on urinary excretion, the uptake was about 50% (range: 21-74%). Silicon peaked in blood after about 1 hour. Renal clearance was 82-90 mL/min suggesting high renal filtration and a significant correlation was found between creatinine clearance and silicon levels in urine

or serum. In a second study they compared the bioavailability of monomeric and oligomeric silicic acid (Jugdaohsingh *et al*, 1999). Following administration of monomeric silicic acid 53% was excreted in urine, whereas ingestion of oligomeric silicic acid only caused a marginal increase of silicon in urine. Recently the bioavailability of silicon from solid foods, excluding silicon from fluids, was studied, and Jugdaohsingh *et al* (2002) found that a mean of 41% was excreted in urine. This is contrary to the common belief that bioavailability of silicon from phytolithic silica in plant-based food is low.

Fasting concentrations of silicon in plasma are 2-10 μM, increasing to 20-30 μM after meals. Urinary excretion is approximately 700 μmol/day, equivalent to 19.6 mg silicon/day (Jugdaohsingh *et al*, 2000; Reffitt *et al*, 1999). The significance of renal elimination of silicon is demonstrated by higher serum concentrations of silicon in patients with chronic renal failure compared to healthy controls (Dobbie and Smith, 1986).

Mean silicon intakes in US population groups were estimated on the basis of the original Framingham and Framingham Offspring cohorts by Jugdaohsingh *et al* (2002), who found intakes to be 30 and 33 mg/day in men and 24 and 25 mg/day in women in the two cohorts, respectively. Silicon intake decreased with age.

The daily intake from the British diet has been estimated to 20-50 mg (Bellia *et al*, 1994; Pennington, 1991). The relative contributions were 55% from water, coffee and beer, 14% from grain products and 8% from vegetables.

Silicon in the form of silica is found in supplements and, according to the recommended doses by the producers, provides 1-75 mg silicon/day, corresponding to 0.017-1.5 mg silicon/kg body weight/day (e.g., products on the Norwegian market, according to the Norwegian Institute of Public Health).

Silicon in the form of amorphous silica, silicates and dimethylpolysiloxane is added to food as anti-caking and anti-foaming agents. Dimethylpolysiloxane is used for the treatment of infant colic. JECFA established ADIs as non-specified for silica and a number of silicates and the SCF established a group ADI as non-specified for silica and a number of silicates (SCF, 1991; WHO, 1969, 1974a and b).

A Recommended Intake for silicon has not been established due to insufficient data and lack of functional criteria (FNB, 2001; SCF, 1993).

3. HAZARD IDENTIFICATION

3.1. Genotoxicity

Silica is considered not to be genotoxic *in vitro* or *in vivo* (IARC, 1987).

3.2. Animal toxicity data

Virtually no studies on the toxic effects of soluble silica have been identified. A few quite old studies in rabbits showed that intravenous injection of polysilicic acid ("colloidal silica") 100 mg/kg body weight caused immediate death due to blood clotting. Daily intravenous injection of ≥5 mg "silica sol" in rabbits caused liver fibrosis, enlargement of the spleen and interstitial nephritis. Focal areas of necrosis were seen in midzones of the liver lobules and an increase in transaminases in serum (Friedberg and Schiller, 1988).

Ruminants consuming plants with a high content of silicon may develop silicate renal calculi (Bailey, 1981).

Amorphous silicon dioxide, aluminium, calcium, magnesium and sodium aluminium silicates were evaluated by JECFA in 1969 and 1974 (WHO, 1969, 1974a and b). In these reports several studies on oral amorphous silica fed to rats were reported. Generally no adverse effects were seen except for a reduction in body weight gain in rats fed 2.5% micronised silica gel in the diet or in rats fed a hydrophobic preparation of amorphous polymeric silica, in which silanol groups on the surface had been reacted with dimethyl-dichlorosilane, when the dose had been raised to 8000 mg/kg body weight. No adverse effects were observed in rats fed 100 mg amorphous silica/kg body weight/day for 2 years.

Kawate (1969) studied groups of rats fed 0.375 to 3 g of silica per day and rat for seven days. No fatalities, clinical signs or changes in gross pathology were reported.

Dobbie and Smith (1982) investigated nephrotoxicity of magnesium trisilicate, crushed and ball-milled quartz and granite in guinea pigs. The three substances were suspended in their drinking water (250 mg/L) and given five days per week for four months. The concentrations of silicon in the supernatants of the suspensions in drinking water were 10, 267 and 29 μmol/L for tap water, magnesium trisilicate and granite, respectively. Renal lesions involving distal tubule and collecting ducts including interstitial inflammation between affected tubuli were found in all animals given magnesium trisilicate (estimated dose: 50-100 mg magnesium trisilicate/kg body weight/day) and two of six animals given crushed quartz.

Newberne and Wilson (1970) showed that oral administration of sodium silicate and magnesium trisilicate (1.8 g/kg body weight/day) for four weeks produced renal tubular damage and chronic interstitial inflammation in dogs, but no nephrotoxicity in rats. No renal lesions were found in dogs following equivalent doses of silicon dioxide and aluminium silicate.

Takizawa *et al* (1988) fed groups of 40 B6C3F1 mice 0, 12500, 25000 or 50000 ppm (mg/kg) of food grade micronised silica (SYLOID) (an anti-caking agent) in the diet for up to 21 months. Mean cumulated intakes by week 93 were 38.5, 79.8 and 160.2 g/male mouse and 37, 72.5 and 157.6 g/female mouse. The growth in the groups on the top dose (corresponding to 7500 mg silica/kg body weight/day or 3500 mg silicon/kg body weight/day) was reduced. No other observations or adverse effects were reported following gross and microscopic pathology. Lymphomas and leukaemia occurred in controls and treated groups, but tests for positive dose-related trends were not significant, and these malignancies were therefore not considered to be treatment-related.

Takizawa *et al* (1988) fed groups of 40 Fischer rats 0, 12500, 25000 or 50000 ppm (mg/kg) of food grade micronised silica (SYLOID) (an anti-caking agent) in the diet for up to 21 months. Mean cumulated intakes by week 103 were 143.5, 179.6 and 581.2 g/male rat and 107.3, 205 and 435.3 g/female rat. Liver weights were reduced in the mid- and high dose only in females at 12 and 24 months, but not dose-related. However, the body weights were also reduced and no specific changes were reported on liver histopathology. The high dose corresponds to 2500 mg silica/kg body weight/day or 1170 mg silicon/kg body weight/day).

3.3. Reproductive and developmental toxicity

A two-generation reproduction study was performed in rats with oral administration of 100 mg amorphous silica/kg body weight/day. No adverse effects were observed (WHO, 1974).

3.4. Human data

There are no reports on human toxicity following intake of silicon occurring naturally in food.
Humans have for decades consumed low levels of amorphous silicates as food additives used for anti-foaming and anti-caking purposes without any reported deleterious effects (Nielsen, 1994).

Humans have used silicon in the form of magnesium trisilicate extensively as an antacid for several decades. The only adverse effect that has been reported is the formation of renal silicate stones (Farrer and Rajfer, 1984; Lee *et al*, 1993). Silica urolithiasis is generally rare and up to mid 1980 was reported only in patients exposed to magnesium trisilicate (Haddad and Kouyoumdjian, 1986). In only one case urinary silicon was determined and it was 1 mmol/L (Haddad and Kouyoumdjian, 1986). More recently a few cases with silica stones were reported in subjects not ingesting trisilicate antacid (Ichiyanagi *et al*, 1998). In these cases the source of silicon was unknown. Very recently, renal silica calculi were reported in an infant in whom the aetiology was ascribed to consumption of milk diluted with silicon-rich spring water (172 mg silicon/L) (Nishizono *et al*, 2004). The silicon concentration in the water was approximately 7- to 34-fold higher than the average concentrations in tap water in Japanese prefectures and far above concentrations of silicon in drinking water in the UK, which were all below 6 mg silicon/L (Dobbie and Smith, 1986). It was estimated that the intake of silicon in this infant was approximately 172-206 mg/day. Urinary concentration of silicon was not measured.

Inhaled silica, particularly crystalline forms, is well known to cause the lung disease silicosis. Chronic inhalation may apparently also result in renal toxicity characterised by glomerular and tubular changes and a number of cases and some epidemiological studies have been reported (Stratta *et al*, 2001). Nephropathy occurs in silica-exposed individuals both with and without silicosis. Affected patients may also show elevated levels of silicon in the kidneys (Saldanha *et al*, 1975). Ceramic workers exposed to silica dust were found to have an increased risk of end stage renal disease (Rapiti *et al*, 1999). Increased prevalence of renal disease was also seen in fluorspar miners exposed to silica (Fenwick and Main, 2000). The mechanism of silicon induced nephropathy is not known.

Patients with renal failure generally have a higher concentration of silicon in plasma than healthy subjects. In two dialysis patients with high silicon levels, 137 and 84 μmol/L, painful skin eruptions, folliculitis, and disturbed hair growth were observed. The authors suggested that these symptoms could be caused by silicon (Saldanha *et al*, 1997). Abnormal hair growth following exposure to organic silicon compounds has been reported previously. Penetrating folliculitis has been reported in dialysis patients previously, but was not specifically related to high silicon concentrations in plasma (Saldanha *et al*, 1997). Such lesions were, however, not reported in another report on haemodialysis patients who had silicon plasma concentrations in the same range (Parry *et al*, 1998). Thus, a causal relationship between these symptoms and silicon exposure cannot be established.

3.5. Interactions with aluminium

Silicon is thought to act as an antidote to aluminium toxicity by reducing the bioavailability of aluminium. Even modest levels of silicon in water can protect against aluminium toxicity in fish (Parry *et al*, 1998). Silicon reduces aluminium accumulation in the brain of aluminium-exposed rats (Belles *et al*, 1998), but does not apparently protect against aluminium-induced developmental effects in rats (Belles *et al* 1999). Silicon addition to drinking water containing aluminium reduces the plasma peak of aluminium in humans (Edwardson *et al*, 1993). Oligomeric, but not monomeric silicic acid prevents aluminium absorption in humans (Jugdaohsingh *et al*, 2000). Silicon in beer has been shown to promote the excretion of aluminium from body stores (Bellia *et al* 1996), but this was not confirmed in a recent study on monosilicic acid (Jugdaohsingh *et al* 2000). It has been proposed that silicon in drinking water might protect against the neurotoxicity of aluminium; however, there are no clear conclusions (Rondeau, 2002).

In patients undergoing renal transplantation, serum silicon, unlike aluminium, progressively decreased with improving renal function (Bellia *et al*, 1994). In a study on haemodialysis patients, Parry *et al* (1998) found that patients with high serum silicon had a lower serum concentration of aluminium. The authors suggested that patients with high serum aluminium levels might be protected from aluminium-related toxicity provided they had a serum concentration of silicon in the range of 100-150 μmol/L.

Red cell superoxide dismutase (SOD) is reduced in haemodialysis patients (Shankin-Kestenbau *et al*, 1990a). It is well known that aluminium can inhibit SOD; however, recently it was also found that silicon concentrations similar to those found in serum of uraemic patients inhibit SOD *in vitro* (Shankin-Kestenbau *et al*, 1990b).

4. DOSE-RESPONSE ASSESSMENT

There are no data demonstrating a dose-response relationship for adverse effects, such as silicate renal stones or other renal effects, in humans.

Short-term oral exposure with daily intakes of 1.8 g/kg body weight of sodium or magnesium silicate produces adverse renal effects in dogs, but not in rats (Newberne and Wilson, 1970). Similar doses of silicon dioxide and aluminium silicate did not produce adverse renal effects in either species. Renal effects have been reported in guinea pigs exposed orally to high doses of magnesium trisilicate (50-100 mg/kg body weight/day) (Dobbie and Smith, 1982).

Long-term toxicity studies in rats and mice (Takizawa *et al*, 1988), show apparent effects on growth at 2500 and 7500 mg silica/kg body weight/day, corresponding to 1170 and 3500 mg silicon/kg body weight/day, respectively. This effect was not regarded as a toxic effect, but was rather due to nutritional imbalance because of the high dose of silica added to the diet. These studies do not provide any information on the bioavailability of water-soluble forms of silicon from silica, which presumably is low, and hence the systemic load of silicon is not known. The extrapolation of these data to other forms of silicon (such as silicates) is inappropriate.

CONCLUSIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

There are no suitable data for dose-response for establishment of an upper level.

2. RISK CHARACTERISATION

Silicon has not been shown to be essential for humans.

In addition to naturally occurring silicon in the diet, food also contains silicon in form of additives. The systemic availability of silicon from these additives varies, but is generally low. The estimated typical dietary intake (20-50 mg silicon/day) corresponds to 0.3-0.8 mg/kg body weight/day in a 60 kg person. These intakes are unlikely to cause adverse effects.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN C (L-ASCORBIC ACID, ITS CALCIUM, POTASSIUM AND SODIUM SALTS AND L-ASCORBYL-6-PALMITATE)

(REQUEST N° EFSA-Q-2003-018)

(ADOPTED ON 28 APRIL 2004)

SUMMARY

The European Food Safety Authority is asked to derive an upper level for the intake of vitamin C from food that is unlikely to pose a risk of adverse health effects.

Vitamin C is a water soluble vitamin that is an important anti-oxidant in the body. Insufficient intake results in the deficiency condition scurvy.

The vitamin is of low acute toxicity as indicated by the limited data available from studies in animals and humans. Despite the extensive use of high doses of vitamin C in some vitamin supplements, there have been few controlled studies that specifically investigated adverse effects. Overall, acute gastrointestinal intolerance (e.g., abdominal distension, flatulence, diarrhoea, transient colic) is the most clearly defined adverse effect at high intakes, but there are limited data on the dose-response relationship for adults or for groups such as children or the elderly. While there is uncertainty whether high intakes of vitamin C increase renal excretion of oxalate which could increase the risk of renal stones, an increased risk of kidney stones was not found in individuals with habitual intakes of 1.5 g/day. There are insufficient data to establish a tolerable upper intake level for vitamin C.

The available human data suggest that supplemental daily doses of vitamin C up to about 1 g, in addition to normal dietary intakes, are not associated with adverse gastrointestinal effects, but that acute gastrointestinal effects may occur at higher intakes (3-4 g/day). The absorption of vitamin C is saturated at high doses, and therefore intakes above 1 g/day would be associated with negligible increased uptake and tissue levels, but an increased risk of adverse gastrointestinal effects.

The average daily intakes reported in surveys in European countries are above the recommended daily intakes, with the 95th percentile intakes from food and supplements ranging up to about 1 g/day. These dietary intakes do not represent a cause for concern.

There has not been a systematic assessment of the safety of the long-term use of high dose vitamin C supplements.

KEY WORDS

Vitamin C, ascorbic acid, tolerable upper intake level, gastrointestinal effects, food safety.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

1 - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Vitamin C (3-oxo-L-gulofuranolactone or L-threo-hex-2-enonic acid) is a 6-carbon hydroxy-lactone that is structurally related to glucose. It is a micronutrient essential to humans, primates and guinea pigs, but which is synthesised by other mammalian species from glucose and galactose. It is readily oxidised to L-dehydroascorbic acid, in which the unsaturated 2,3-dihydroxy group is replaced by a saturated 2,3-diketone function; L-dehydroascorbic acid can be reduced back to ascorbic acid.

Vitamin C is highly water soluble, and in solution can be oxidised by atmospheric oxygen to give an equilibrium mixture of ascorbic and dehydroascorbic acids. Vitamin C has important anti-oxidant properties, and protects cells against oxidative stress. Because of this general cytoprotective role, its importance has been investigated in a variety of clinical conditions, including cancer, vascular disease, and cataracts.

Vitamin C deficiency in humans leads to clinical syndromes known as scurvy in adults and Moeller-Barlow disease in children (SCF, 1993), conditions which are associated with intakes of less than 10 mg/day. Early or prescorbutic symptoms in adults include fatigue, weakness, anaemia and aching joints and muscles, while there are important effects on bone tissue in children. Later stages of deficiency are characterised by capillary fragility causing bleeding from the gums and haemorrhages, and delayed wound healing due to impaired collagen synthesis.

Advice by Linus Pauling that daily intakes of 1 g or more of vitamin C can protect against the common cold (see Miller and Hayes, 1982) was followed by other claims of beneficial effects on a variety of conditions. Because of the media attention given to these claims and the apparently low toxicity of vitamin C there has been extensive human exposure to intakes up to 10 g/day (Miller and Hayes, 1982). However despite this extensive human exposure, there are only limited data that are appropriate for use in risk assessment.

Recent reviews of vitamin C by the Food and Nutrition Board in the USA (FNB, 2000) and the Expert Group on Vitamins and Minerals in the UK (EGVM, 2003) have recommended an upper level of 2 g/day and a guidance level of 1 g/day, as supplemental intake, respectively.

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

Major food sources of vitamin C are plants such as citrus fruits, soft fruits and green vegetables. Animal tissues also contain vitamin C, with kidney and liver representing good sources. The amounts of vitamin C present in the food when consumed may be reduced, because it is readily lost due to dissolution in water and oxidation during cooking processes such as boiling.

Ascorbic acid is a permitted anti-oxidant additive in food, with no specified limits on the level of use. Vitamin C is present in numerous dietary supplements with manufacturer recommended daily intakes of 60-3000 mg in single vitamin preparations and 10-1000 mg/day in multi-vitamin preparations (EGVM, 2003).

In 1992, the Scientific Committee for Food (SCF, 1993) recommended a Population Reference Intake of 45 mg/day for adults, with an increase to 55 mg/day in pregnancy, and to 70 mg/day during lactation.

Vitamin C has a number of biochemical roles in the body (Basu and Dickerson, 1996). It is a strong reducing agent and antioxidant, which is important in preventing the damaging effects of free radicals. Vitamin C is an enzyme co-factor for many biochemical reactions, especially those involving oxidations, such as the synthesis of hydroxyproline from proline for collagen biosynthesis, mono-oxygenases, dioxygenases and mixed function oxygenases. It is important in the synthesis and stabilisation of neurotransmitters and carnitine, and increases the gastrointestinal absorption of non-haem iron by reducing ferric to ferrous iron (SCF, 1993).

Gastrointestinal absorption of low doses of vitamin C is efficient, and occurs in the small intestine via a sodium-dependent active transport mechanism. The extent of absorption of vitamin C is 80-90% at the usual intakes from food of 30-180 mg/day (SCF, 1993), but because the transporter is saturable, absorption efficiency gradually decreases at higher intakes (Kallner *et al*, 1979 and 1985; Hornig and Moser, 1981; Blanchard *et al*, 1997). There is a non-linear relationship between daily intake of vitamin C and plasma concentrations, with a 5-fold increase in intake from 0.5 g/day to 2.5 g/day producing only a 20% increase in plasma levels (Levine *et al*, 1996 and 1999). Ascorbyl palmitate is probably hydrolysed in the lumen of the gastrointestinal tract prior to absorption, but data defining the *in vivo* fate of this synthetic form of vitamin C have not been identified.

Ascorbic acid is widely distributed in all tissues of the body, with higher levels found in the adrenal and pituitary glands and the retina, and lower levels in kidney and muscle tissue. Vitamin C can be detected in most tissues and exists as an equilibrium mixture of ascorbic acid and dehydroascorbic acid, dependent on the redox status of the cells. Plasma and urinary vitamin C are not reliable indicators of body stores of vitamin C because they are influenced by recent dietary intake. Leukocytes contain higher concentrations of vitamin C than plasma, blood or serum (Levine *et al*, 1996), and may provide a more reliable indicator of status. A vitamin C concentration in leukocytes below 0.01 mg per 10^8 cells is generally regarded as indicative of deficiency.

Vitamin C is readily oxidised to dehydroascorbic acid, which can be reduced back to ascorbic acid or hydrolysed to diketogulonic acid and then oxidised to oxalic and threonic acid, xylose, xylonic acid and lyxonic acid (Basu and Dickerson, 1996). Some oxidation to carbon dioxide occurs at high doses, possibly due to metabolism of unabsorbed ascorbate by the intestinal microflora (Kallner *et al*, 1985). Ascorbic acid may also undergo limited conjugation with sulphate to form ascorbate-2-sulphate, which is excreted in urine. Unchanged ascorbic acid and its metabolites are excreted in the urine. Approximately 3% of a 60 mg oral dose is eliminated in the faeces. At intakes above 80-100 mg/day, most of the additional absorbed vitamin is excreted unchanged in the urine, indicating that tissue reserves are saturated at this intake level (SCF, 1993; FNB, 2000). This increasing renal elimination of ascorbic acid with increase in dose results in an inverse relationship between the elimination half-life and the dosage (Kallner *et al*, 1979) and probably arises from saturation of reabsorption from renal tubule (Blanchard *et al*, 1997).

The average daily intakes in European countries are above the reference intake established by the SCF in 1993, with relatively consistent data in different countries (Table 1). The 97.5th percentiles of intake are about 5-6 times the reference intake.

3. HAZARD IDENTIFICATION

3.1. Genotoxicity

The genetic toxicology of ascorbic acid was reviewed by Shamberger (1984) at which time there was evidence for indirect mutagenic effects via the generation of oxidative damage in the presence of transition metals, and also for anti-mutagenic effects in a variety of systems.

Vitamin C would be expected to be anti-mutagenic, because of its antioxidant properties, and there are data consistent with this. For example, ascorbic acid reduces the spontaneous mutation rate in mismatch repair-defective cells (Glaab *et al*, 2001), protects against gamma-ray induced damage (Konopacka and Rzeszowska-Wolny, 2001) and reduces the activity of some genotoxic compounds (Blasiak *et al*, 2001; Nefic, 2001; Rao *et al*, 2001; Kaya *et al*, 2002; Chang *et al*, 2002), including important food-borne mutagens such as patulin (Alves *et al*, 2000) and toxins such as zearalenone and ochratoxin A (Grosse *et al*, 1997 - based on a reduction in DNA adducts measured by ^{32}P-post labelling).

Table 1. The daily intakes of vitamin C in EU countries (mg/day)

	Population	N	Method	Supplements	Mean	97.5%
Austria[a]	men + women	2488	24 h recall	Not defined	88	276
Germany[b]	men women	854 1134	7-day record	Not defined Not defined	70 83	270 282
Germany[c]	men women men women	1268 1540 240 347	Computer-assisted dietary interview	- - + +	150 151 168 156	180 176 309 285
Italy[d]	household	2734	7-day record	+	113	268
Ireland[e]	men women men women	662 717 662 717	7-day record	- - + +	81 72 116 108	212 187 588 588
Netherlands[f]	household	5958	2-day record	-	78	204
Sweden[g]	men women men women	1897 2223 770 1655	8-day record	- - + +	87 103 151 169	201 224 1056 1117
UK[h]	men women men women	833 891 833 891	7-day record	- - + +	83 (71) 81 (69) 101 (74) 112 (76)	217 205 329 473

[a] Elmadfa *et al* (1998)
[b] Heseker *et al* (1992) - median not mean value
[c] Mensink and Ströbel (1999); Mensink *et al* (2002) - values are the mean and 75th percentile
[d] Turrini (INRAN)
[e] IUNA (2001)
[f] Hulshof and Kruizinga (1999)
[g] Elmstahl *et al* (1994) - values are the median and 95th percentile
[h] Henderson *et al* (2003) - values are the mean (with the median in parentheses)

Although ascorbic acid is an antioxidant via its conversion to dehydroascorbic acid, the reversibility of the reaction can lead to the generation of reactive oxygen species via redox cycling (Ballin *et al*, 1988; Stadtman, 1991). This may be involved in some positive genotoxic effects reported with vitamin C *in vitro*, such as single strand breaks produced by sodium ascorbate (Singh, 1997), weak sister chromatid exchange (SCE) activity (Galloway and Painter, 1979; Speit *et al*, 1980; Best and McKenzie, 1988) and activity in the comet assay (Blasiak *et al*, 2000). Ascorbic acid has been reported to increase the genotoxicity of mitomycin C (Krishnaja and Sharma, 2003), cadmium chloride (Blasiak *et al*, 2000) and cobalt chloride (Kaya *et al*, 2002). The results obtained may depend on the concentrations of vitamin C studied, with protective activity at low concentrations but cytotoxicity and an increase in genotoxic activity at concentrations more than 200 μg/mL (Antunes and Takahashi, 1999). Oral and intraperitoneal doses of up to 10 g/kg in hamsters did not induce SCEs (Speit *et al*, 1980).

Positive genotoxicity results tended to occur *in vitro* when vitamin C was tested in the presence of metal ions such as iron and copper, which may be related to its reduction of the metal followed by the formation of highly reactive hydroxyl radicals via a Fenton reaction (Carr and Frei, 1999). However, DNA-reactive species can be generated by interaction between vitamin C and lipid hydroperoxide decomposition in the absence of transition metal ions (Lee *et al*, 2001); the *in vitro* concentrations used in this study were similar to those present in plasma after doses of 200 mg/day, but the toxicological significance of this observation is unclear.

Podmore *et al* (1998a) reported that the administration of 500 mg/day of vitamin C supplements to 28 human volunteers for 6 weeks caused an increase in 8-oxoadenine but a decrease in 8-oxoguanine in the DNA of isolated lymphocytes measured by gas chromatography-mass spectrometry (GC-MS), possibly due to its pro-oxidant effect. Two letters were published as a consequence of this report, which pointed out that the dosage given would not have increased the intracellular concentrations of ascorbate in lymphocytes, and that the increased oxidation could be an artefact formed by monocytes

in the lymphocyte preparation (Levine *et al*, 1998), that artefacts were not adequately excluded, the study was not a randomised double-blind placebo controlled investigation, and the authors did not give information about smoking habits or cite previous publications (Poulsen *et al*, 1998). In their reply to these letters, Podmore *et al* (1998b) provided further support for the validity of their findings and pointed out that because 8-oxoadenine is 10-times less mutagenic than 8-oxoguanine, their results are consistent with an "overall profound protective effect".

An increase in total damage to lymphocyte DNA bases (but not in 8-hydroxyadenine or 8-hydroxyguanine) was reported in a study in which one group of 20 volunteers who were given 14 mg iron plus either 60 or 260 mg of vitamin C, but not in a second group of 18 subjects who were treated in the same way but had slightly lower pre-treatment plasma levels of ascorbic acid (Rehman *et al*, 1998). In a subsequent paper by the same research group, treatment of 20 healthy adults with ascorbate (280 mg/day), with or without supplemental iron (14 mg/day), for periods of 6 weeks in a cross-over design showed no significant rise in oxidative DNA damage as measured by GC-MS (Proteggente *et al*, 2000): significant decreases in 8-oxo-7,8-dihydroguanaine and 5-hydroxymethyl uracil were found during treatment with ascorbate and the authors concluded that there was no compelling evidence of a pro-oxidant effect resulting in DNA damage. *In vivo* administration of 1g vitamin C daily for 42 days to human volunteers did not influence the extent of endogenous DNA damage in peripheral lymphocytes measured using an Elisa technique, but reduced the extent of *ex vivo* peroxide-induced damage (Brennan *et al*, 2000). The significance of these observations is unclear and currently under further study.

Overall, the data currently available do not allow an adequate evaluation of the genotoxic potential of high intakes of vitamin C, and in particular its capacity to induce gene and chromosomal mutations. The significance of oxidative DNA damage observed *in vitro* or *in vivo* at high concentrations is unclear.

3.2. Animal toxicity data

Vitamin C has low acute toxicity. High doses of vitamin C (100 mg per 160 g animal per day) are associated with decreased growth rates in guinea pigs fed a nutritionally inadequate diet of unfortified wheat flour, but weight gain was not altered if the treated animals were fed a wheat flour diet fortified with casein (Nandi *et al*, 1973). No effects on reproductive or developmental parameters were found in guinea pigs, rats and hamsters given oral doses of up to 400 mg/kg body weight/day during pregnancy (Alleva *et al*, 1976) or in rats and mice given up to 1000 mg/kg body weight/day (Frohberg *et al*, 1973). A conditioned increase in vitamin C requirements has been reported in guinea pigs (Sorensen *et al*, 1974).

There have been a number of studies in which rats have been given high dietary concentrations of the sodium salt of ascorbic acid and the free acid in relation to the role of sodium ions in the generation of bladder hyperplasia and cancer in male rats. Using a 2-stage model of bladder carcinogenesis, in which male rats were treated with possible promoters of bladder carcinogenesis for 6 weeks, Cohen *et al* (1991) showed that sodium ascorbate at 5% in the diet (equivalent to about 2500 mg/kg body weight/day) increased the incidence of bladder cancers, but that an equimolar dietary concentration of ascorbic acid (4.44%) was inactive. In a subsequent study in which sodium ascorbate was given in the diet to rats without pre-treatment with a carcinogen, significant increases in simple, papillary and nodular hyperplasia in the urinary bladder were detected in rats fed diets containing 5% or 7% sodium ascorbate, but these effects were abolished by co-treatment with ammonium chloride which acidified the urine (Cohen *et al*, 1998); there was a small and non-significant increase in the numbers of papillomas and carcinomas in the urinary bladder at dietary levels of 5% (n=1) and 7% (n=2) compared to control (n=0) or 1% dietary level (n=0). In 1993, the JECFA concluded that similar effects produced by the sodium salt of saccharin were related to sodium-induced changes in urine volume, osmolality and pH, and were not relevant to human health (JECFA, 1993).

Dietary administration of ascorbyl palmitate at levels of 2000 ppm and 4000 ppm (equivalent to daily intakes of about 100 and 200 mg/kg body weight per day) to male rats treated with the colon carcinogen azoxymethane caused a significant reduction in the incidence (% of animals with tumours) and the multiplicities of invasive and total adenocarcinomas of the colon (Rao *et al,* 1995). In contrast, administration of 2% ascorbyl palmitate in the diet of mice (equivalent to daily intakes of about 2000 mg/kg body weight per day) did not attenuate the hyperplastic and dysplastic effects of azoxymethanol in the colon (Huang *et al*, 1992).

3.3. Human data

Despite a number of clinical studies in which high doses of vitamin C (up to 1 g or more per day) have been given, there is a limited database on tolerability or adverse effects. A number of studies with different doses and durations have not reported adverse effects, but it is difficult to determine the significance of these because it is frequently unclear how any adverse effects were investigated (reviewed in Carr and Frei, 1999) (EGVM, 2003). In addition many studies have used a combination of vitamins and minerals, and identifying any effect of vitamin C *per se* is not possible because other parts of the treatment could mask adverse effects.

Adverse effects were not reported in recent studies in which 12 healthy adult volunteers received 500 mg/day for 8 weeks (McArdle *et al*, 2002), 19 patients with hypertension received 500 mg/day for 30 days (Duffy *et al*, 1999), 28 male smokers received 500 mg/day for 4 weeks (Aghdassi *et al*, 1999), 18 healthy male adults given 2 g/day for 6 weeks (Tofler *et al*, 2000), 130 healthy adults given 250 mg of slow release vitamin C for 3 years (Salonen *et al*, 2000), 8 adults received increasing daily doses up to a maximum of 2 g/day for 2 weeks (Johnston and Cox., 2001), 5 adults received 1 g/day for 6 months (Pullin *et al*, 2002) and 30 adults given daily doses of 500 mg for 6 weeks (Hamilton *et al*, 2000). Most of these studies had primary endpoints related to a health benefit, and assessment of adverse effects or tolerability was not a part of the study design.

A double-blind, cross-over study on the effects of daily doses of 3 g of vitamin C, combined with very high doses of nicotinamide (3 g), calcium pantothenate (1.2 g) and pyridoxine (0.6 g), in 41 children with attention deficit disorders (Haslam *et al*, 1984) reported an increase in serum transaminases. However, this cannot be assigned to the vitamin C component, because of the complex megavitamin regimen. There was little information reported on general tolerability, but 3 children did not complete the study because of excessive vomiting, abdominal discomfort or an inability to swallow the vitamin capsules and “some patients experienced nausea and vomiting during the course of treatment”.

Vitamin C was administered to 10, 269 adults aged 40-80 with coronary disease at a daily dose of 250 mg/day (in combination with 600 mg vitamin E and 20 mg β-carotene) for up to 5 years. The subjects showed good compliance and there were no significant differences in mortality or morbidity compared with a placebo group of equal size (Heart Protection Study, 2002). No effects on inflammatory markers were reported in a recent long-term multi-vitamin study (Bruunsgaard *et al*, 2003) in which 52 men aged 47-70 were treated for 3 years with a combination of 500 mg vitamin C and 182 mg of α-tocopherol daily, but it is unclear to what extent other effects, such as gastrointestinal problems, would have been recorded.

A retrospective cohort study of 994 women, of whom 277 were regular users of vitamin C supplements for up to 12 years, reported a significant increase in bone mineral density of the neck of the femur; no side effects were reported but no parameters other than bone mineral density were assessed (Morton *et al*, 2001).

Adverse effect data were not reported in a 4-year double-blind, placebo-controlled study in which patients with a history of adenoma of the large bowel were given vitamin C (1 g/day) with either vitamin E (205 patients) or vitamin E plus β-carotene (175 patients) (Greenberg *et al*, 1994). Adherence to the prescribed regimen, and information about symptoms, illnesses and hospitalisations were assessed every 6 months. The numbers of patients who withdrew from the study were similar in all groups, but 4 subjects in the two vitamin C treatment groups “stopped taking the medications because of their presumed toxicity”; no other information was provided and tolerability during the study was not reported.

No subjective side effects were reported in the study of Cook *et al* (1984) in which 17 adults were given 2 g/day with meals for 16 weeks.

No adverse effects were reported in a randomised, double-blind, placebo-controlled study in which 21 patients with coronary artery disease were given a single dose of 2 g vitamin C followed by 500 mg/day for 30 days (Gokce *et al*, 1999).

3.3.1. Gastrointestinal effects

Gastrointestinal effects are the most common adverse clinical events associated with acute, high doses of vitamin C (above 1 g daily), but these can be reduced by taking the vitamin after meals (reviewed in Miller and Hayes, 1982). The incidences of stomach pains, nausea and diarrhoea in children given 1 g/day for 3 months were similar to those in the control groups (Ludvigsson *et al*, 1977). Abdominal

distension, flatulence, diarrhoea and transient colic were reported as “fairly frequent” in a study in healthy human volunteers given daily doses which increased by 1000 mg per day each week, with adverse effects reported at doses of 3-4 g/day, although no details were given of the exact dosing regimen or the numbers of subjects studied or their age, sex or body weight (Cameron and Campbell, 1974). Two out of 15 volunteers experienced diarrhoea when consuming 10 g of vitamin C daily for 5 days in a clinical study on oxalate excretion, despite the fact that the subjects were advised to take the vitamin C tablets at mealtimes to minimise the potential for adverse gastrointestinal effects (Wandzilak *et al*, 1994).

3.3.2. Renal effects

Adverse effects related to the renal system have been reported, including renal stones, renal tubular disease and oxaluria. Vitamin C consumption has been suggested to increase oxalate excretion and the risk of urinary stone formation, but the available data are both confusing and contradictory. An early report stated that there could be wide inter-subject differences in the excretion of oxalate following high doses of vitamin C (Briggs, 1976). An additional problem is that urinary oxalate can be produced from urinary ascorbic acid as an artefact of the analytical procedure, so that the validity of the analytical data depends on the extent to which this was controlled by the use of preservatives. Increased oxalate excretion would represent a risk factor for the formation of bladder stones, and there have been anecdotal case reports of kidney stones or other nephropathy (Nakamoto *et al*, 1998) in patients who have taken high daily doses of vitamin C.

Groups of 3 patients who had unilateral nephrostomy tubes after lithotripsy for renal stones were given supplemental doses of 100, 500, 1000, or 2000 mg ascorbic acid on days 2 and 3 postoperatively (Urivetzky *et al*, 1992). Urine specimens were collected from the nephrostomy catheter and also from the contralateral kidney directly into EDTA and sodium thimerosol preservative to stabilise ascorbic acid and oxalate; oxalate was measured following the removal of ascorbic acid with sodium nitrite. There was a statistically significant increase in urinary oxalate at doses of 1000 and 2000 mg. The authors estimated that there was a 6-13 mg/day increase in urinary oxalate excretion per 1000 mg/day ascorbic acid intake, and concluded that there was an increased risk of calcium oxalate renal stones.

Urinary excretion of oxalate was measured in 15 volunteers given ascorbic acid supplementation (1, 5 and 10 g/day for 5 days in a cross-over design) (Wandzilak *et al*, 1994). The 24-hour urine samples were preserved by reducing the pH to 2 by adding 20 mL of concentrated hydrochloric acid. Ascorbate was reported to be converted non-enzymatically into oxalate during analytical measurement. The study did not find an increase in urinary oxalate excretion after *ex vivo* non-enzymatic conversion of ascorbate to oxalate had been taken into account.

Supplemental vitamin C intakes of 1 g/day in 7 volunteers caused statistically significant increases in urinary excretion of oxalate (determined by an enzymatic method reported to be free from interference by ascorbic acid) (Levine *et al*, 1996). An increase in urinary oxalate excretion, measured by an enzymatic assay on urine collections stabilised with acid, was also reported in 6 subjects given 1 g of supplemental ascorbate with 2.85 litres of orange juice containing 0.62 g of ascorbate daily for 4 days, but not in the same subject given 2 g of ascorbate per day for 4 days (Liebman *et al*, 1997).

Auer *et al* (1998a) investigated the urinary excretion of oxalate in the presence and absence of EDTA preservation of the urine samples in 10 healthy male volunteers (with no history of stone formation) given 4 g of vitamin C daily for 5 days. Erroneously high oxalate concentrations were found in the absence, but not in the presence of EDTA. There was no significant increase in oxalate excretion at any stage of the protocol in EDTA preserved samples and it was concluded that large doses of vitamin C did not affect the principal risk factors associated with calcium oxalate kidney stone formation. In contrast, the same authors (Auer *et al*, 1998b) reported increased excretion of oxalate in EDTA treated urine samples from a single volunteer who took 8 g daily for a period of 8 days, at which time the study was terminated because of the detection of haematuria, which was associated with crystalluria.

A prospective study on the relationship between vitamin C intake and the risk of symptomatic kidney stones in a group of 45,251 men (Curhan *et al*, 1996) found no association with vitamin C intake in 751 cases of kidney stones. The age-adjusted relative risk for subjects with intakes of 1.5 g/day or more compared with less than 0.25g/day was 0.78 (95% confidence intervals 0.54-1.11), indicating that even if such doses do increase oxalate excretion, it is not a clinically significant effect. A similar study in a cohort of 85,557 women in whom there were 1078 incidences of kidney stones showed a relative risk of 1.06 (95% confidence intervals 0.69-1.64) for subjects with intakes of 1.5 g/day or more compared with less than 0.25 g/day (Curhan *et al*, 1999).

No significant relationships were found in an analysis of data from 5214 men and 5785 women between serum vitamin C concentrations and the prevalence of kidney stones, serum vitamin B_{12} levels, or serum ferritin levels in men, but a negative correlation with serum ferritin was found for women (Simon and Hudes, 1999).

Increased excretion of uric acid has also been reported after the ingestion of 4 g or 8 g of ascorbic acid (Stein *et al*, 1976); although the available data at lower doses are limited and conflicting, in all studies hyperuricosuria was absent at doses of less than 1 g (Levine *et al*, 1999).

3.3.3. Other effects

Other anecdotally reported adverse effects include metabolic acidosis and changes in prothrombin activity, but a double-blind trial in patients given 200 mg/day showed no significant effect on the incidence of thrombotic episodes (Hornig and Moser, 1981).

A low incidence of adverse effects was reported during a study in patients with multiple sclerosis who were randomised to receive either supplements providing 2 g/day vitamin C, together with 6 mg/day sodium selenite and 480 mg/day vitamin E, or placebo for 5 weeks. The patients were interviewed about side effects after 2 weeks and 4 weeks of treatment. One out of the 10 patients receiving the active supplement reported slight facial erythema at week 2, which subsequently subsided during continued treatment, one reported a peculiar urine smell and another reported an increased number of headaches; three of the 10 patients receiving the placebo reported an increased number of headaches (Mai *et al*, 1990). It cannot be determined from the data whether these were caused by the high doses of vitamin C or by the other constituents.

There is a suggestion in the literature of conditioned need-scurvy, in which scurvy-like symptoms occur soon after cessation of ingestion of high amounts of vitamin C (1 g or more per day) (Siegel *et al*, 1982). High intakes during pregnancy may result in neonatal scurvy by conditioning the offspring to require greater than the expected or recommended daily intakes, but the evidence for this is very limited (Cochrane, 1965). The reports of conditioned scurvy in humans are anecdotal and it does not represent a significant risk (Hornig and Moser, 1981).

Vitamin C increases iron uptake considerably from the gut when given to humans in single-meal studies in amounts from 25 to 1000 mg (Hallberg, 1985; Cook and Monsen, 1977). Studies of longer duration show a less marked effect (Hunt and Roughead, 2000), but even a small increase could be important in subjects with conditions such as haemochromatosis (Gerster, 1999) or in subjects heterozygous for this condition. A dose of 2 g/day vitamin C taken with meals for 16 weeks in 17 healthy volunteers, and up to 24 months in 9 subjects, had no significant effect on body iron stores (Cook *et al*, 1984); this study was limited by the small numbers of participants and their variable iron status.

Large amounts of vitamin C were reported to destroy the vitamin B_{12} content of food (Herbert and Jacob, 1974), and reduced vitamin B_{12} levels in serum were reported in 3 out of 90 individuals consuming more than 1000 mg/day of vitamin C over a minimum of 3 years (Hind, 1975). However, subsequent reports showed that these observations arose from inadequate assay methods (Newmark *et al*, 1976 and 1979), and that ascorbic acid in blood can interfere with the measurement of vitamin B_{12} (Herbert *et al*, 1978).

An increase in serum cholesterol was reported in 25 patients with atherosclerosis following treatment with 1 g vitamin C daily for 6 weeks, but not in healthy volunteers (Spittle, 1971); the authors suggested that this may have arisen due to mobilisation of arterial cholesterol deposits (which would be a benefit), but there was no direct evidence to support this. In contrast, a 10% decrease in total plasma cholesterol levels, but with no change in the cholesterol/HDL ratio, was reported in 18 healthy adult males given 2 g vitamin C daily for 6 weeks (Tofler *et al*, 2000).

There is conflicting evidence about the relationship between vitamin C intake and breast cancer. A prospective study in a large cohort (n=62,573) of postmenopausal women had found a lower risk of breast cancer in women with the highest intakes of vitamin C from food, but not from supplements (Verhoeven *et al*, 1997). However a recent nested case-control study found an increased risk of breast cancer among a cohort of postmenopausal Danish women (Nissen *et al*, 2003). A significantly increased risk was observed at intakes above 300 mg/day in comparison with intakes 60-150 mg/day. The numbers of cases and controls in the high-intake comparison were 62 and 41, respectively. When women who were taking supplemental vitamin C were excluded, the association between increasing vitamin C intake and breast cancer was weaker and no longer statistically significant.

In conclusion, the effects of vitamin C on cholesterol levels, conditioned need due to high intake, iron absorption, prothrombin time and vitamin B_{12} degradation, breast cancer and the possible pro-oxidant activity of vitamin C are not sufficiently well documented or substantiated to be used as the basis for risk assessment. There have been conflicting reports on the influence of vitamin C supplements on the presence of oxidised bases in DNA (Podmore *et al*, 1998; Rehman *et al*, 1998; Proteggente *et al*, 2000). These have been performed at relatively low doses (280 mg/day, 60 or 260 mg/day and 500 mg/day for 6 weeks respectively), and there are no data currently available at higher intakes.

4. DOSE-RESPONSE ASSESSMENT

Adequate data defining the dose-response relationships for each adverse effect described above are not available, because many studies used a single dose level only. Despite the extensive use of vitamin C supplements (up to 10 g/day) for the prevention of colds and other conditions, the tolerability of such intakes has not been subject to systematic assessment. Therefore there are few data to support the widely held view that high intakes of vitamin C are safe.

There have been a small number of studies that have investigated dose-response relationships in a controlled and scientific manner.

4.1. Gastrointestinal effects

Two out of 15 volunteers experienced diarrhoea when consuming 10 g of vitamin C daily for 5 days (Wandzilak *et al*, 1994). In a study in healthy human volunteers given increasing doses of vitamin C, abdominal distension, flatulence, diarrhoea and transient colic were reported as “fairly frequent” at doses of 3-4 g daily (Cameron and Campbell, 1974). Lower intakes appear to be tolerated without gastrointestinal effects since no subjective side effects were reported in 17 adults given 2 g/day for 16 weeks (Cook *et al*, 1984). The Miller and Hayes (1982) review concluded that doses greater than 1 g/day could result in adverse gastrointestinal effects. The data of Ludvigsson *et al* (1977) indicate that 1 g/day would not produce adverse gastrointestinal effects in children.

4.2. Renal effects

A review of the early investigational studies on the relationship between ascorbic acid intake and oxalate excretion (Hornig and Moser, 1981) concluded that there were methodological problems with many of the studies.

A statistically significant increase in urinary oxalate excretion was reported in groups of 3 patients with calcium oxalate renal stones given 1 g or 2 g of supplemental ascorbic acid daily. Precautions were taken to prevent artefactual formation of oxalic acid by collection of intrarenal urine specimens from a catheter into EDTA and sodium thimerosol preservative (Urivetzky *et al*, 1992).

The more extensive cross-over study by Wandzilak *et al* (1994) in which 15 subjects were given 1000, 5000 and 10,000 mg vitamin C each for 5 days reported no increase in oxalate excretion after correction for non-enzymatic *ex vivo* formation. However the data are difficult to interpret because of the highly acidic preservative used.

The two studies by Auer *et al* (1998a and b) indicate no increase in the urinary excretion of oxalate in 10 healthy male volunteers (with no history of stone formation) given 4 g of vitamin C daily for 5 days, but a marked increase associated with haematuria and crystalluria in a single individual who took 8 g daily for a period of 8 days.

In summary, one study reported that high intakes of vitamin C (1 or 2 g per day) increased the urinary excretion of oxalic acid in patients with renal stones, but this was not found in studies in healthy volunteers. Data from the cohort studies (Curhan *et al*, 1996 and 1999) show that intakes of 1.5 g/day do not increase the risk of kidney stone formation.

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The vitamin is of low acute toxicity as indicated by the limited data available from studies in animals and humans. Despite the extensive use of high doses of vitamin C in some vitamin supplements, there

have been few controlled studies that specifically investigated adverse effects. Based on the limited data, acute gastrointestinal intolerance is the most clearly defined adverse effect at high intakes, but there are limited data on the dose-response relationship for adults or for groups such as children or the elderly. There are insufficient data to establish a tolerable upper intake level for vitamin C.

2. RISK CHARACTERISATION

The available human data suggest that supplemental daily doses of vitamin C up to about 1 g in addition to normal dietary intakes are not associated with adverse gastrointestinal effects, but that acute gastrointestinal effects may occur at higher intakes (3-4 g/day). While there is uncertainty whether high intakes of vitamin C increase renal excretion of oxalate, which could increase the risk of renal stones, an increased risk of kidney stones was not found in individuals with habitual intakes of 1.5 g/day. The absorption of vitamin C is saturated at high doses, and therefore intakes above 1 g/day would be associated with negligible increased uptake and tissue levels, but an increased risk of adverse gastrointestinal effects. There are no data on the gastrointestinal absorption or tolerability of esterified forms of vitamin C, such as ascorbyl palmitate, but such esters might be expected to show similar properties, and therefore this conclusion applies to these forms as well as ascorbic acid and its salts.

The average daily intakes reported in surveys in European countries (Table 1) are above the Population Reference Intake, with the 95th percentile intake from food and supplements ranging up to about 1 g/day. These dietary intakes do not represent a cause for concern.

There has not been a systematic assessment of the safety of the long-term use of high dose vitamin C supplements.

3. RECOMMENDATIONS FOR FURTHER WORK

Any future studies on possible benefits of high intakes of vitamin C should investigate the nature and incidence of adverse effects. Very few data are available on esterified forms of vitamin C, such as ascorbyl palmitate, and these forms should be included in future studies.

The potential for vitamin C to induce gene or chromosomal mutations *in vivo* in humans at high doses (1 g or more) should be investigated especially pro-oxidant effects on DNA bases, using sensitive methods, because there are inadequate data to ensure the safety of long-term high-dose intakes.

Subgroups of the population at increased risk have not been investigated; individuals who are predisposed to gastrointestinal problems, kidney stones or who are unable to regulate iron absorption, due to haemochromatosis or thalassaemia, should be included in future studies on the possible beneficial and adverse effects of vitamin C.

The conflicting evidence about vitamin C intake and breast cancer is noted and no conclusion is possible at this time. The possible association warrants further research to clarify any relationship for both dietary sources and vitamin C supplements.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF BORON (SODIUM BORATE AND BORIC ACID)

(REQUEST N° EFSA-Q-2003-018)
(ADOPTED ON 8 JULY 2004)

SUMMARY

Boron occurs in foods as borate and boric acid. Boron has not been established to be an essential nutrient for humans and no specific biochemical function for boron has been identified in higher animals or man. There is some evidence that, in humans, boron intake within the usual dietary range may influence the metabolism and utilisation of other nutrients, particularly calcium, and may have a beneficial effect on bone calcification and maintenance. Recommended intakes for boron have not been established.

Studies of dietary deprivation of boron in animals have reported adverse effects (e.g. on growth, serum steroid hormone concentrations and bone calcification) that can be corrected by increasing boron intake. The effects of low boron intakes are more marked when accompanied by low status for other nutrients (e.g. vitamin D, magnesium).

Data on boron intake in EU countries are limited. In the UK mean intake in adults from food is estimated at 1.5 mg/day, with the 97.5 percentile of 2.6 mg/day, while mean intake from water is estimated to be in the range of 0.2-0.6 mg/day. The main dietary sources of boron are plant foods, and foods rich in boron include fruits, leafy vegetables, mushrooms, nuts and legumes, as well as wine, cider and beer. Supplements may contain 1.5-10 mg boron/dose.

Boron as borate is readily absorbed (>90%) from the human gut and is evenly distributed throughout the tissues and organs, and can cross the placenta, of animals and humans. Absorbed boron is readily excreted in urine.

Ingestion of boron at dose levels of greater than 13 mg/kg body weight/day in short and long term studies in a number of animal species (e.g. mouse, rat, dog, pig) has been shown to result in a range of adverse effects, with developmental and reproductive effects being the most critical. Studies of boron in mice and rats and *in vitro* showed no evidence of carcinogenicity or genotoxicity.

There are many case reports of boron intoxication in humans. Ingestion of boric acid at daily dose levels of 0.14-0.43 g boric acid/kg body weight (equivalent to about 25-76 mg boron/kg body weight) over periods ranging from days to weeks, resulted in a variety of symptoms, the most common being gastrointestinal effects such as vomiting, diarrhoea and abdominal pain.

It was considered that the data on adverse effects of boron in humans were not adequate for establishing a tolerable upper intake level (UL). The UL was based on the adverse effect occurring at the lowest intake levels in animals, i.e. decreased foetal body weight in rats resulting from maternal boron intake during pregnancy. The no observed adverse effect level (NOAEL) for this effect (9.6 mg/kg body weight/day) was extrapolated to humans by dividing by an uncertainty factor of 60 (to allow for variability between rats and humans and between-person variability in humans) to give an UL of 0.16 mg/kg body weight/day, which is equivalent to an UL of 10 mg/person/day in adults. This UL also applies to pregnant and lactating women. UL values for children were derived by extrapolating from the UL for adults on a body surface area basis, giving values (mg/day) of 3, 4, 5, 7 and 9 for children aged 1-3, 4-6, 7-10, 11-14 and 15-17 years of age, respectively. These UL values apply only to the intake of boron as boric acid and borates.

Based on the limited data available, boron intakes from food and water in EU are below the UL. The consumption of some supplements containing boron may lead to intakes which exceed the UL.

KEY WORDS

Boron, sodium borate, boric acid, tolerable upper level.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Boron is a naturally occurring element that is found in the form of borates in the oceans, sedimentary rocks, coal, shale, and some soils. Boron has an atomic number of 5 and a relative atomic mass of 10.811; it exists in two allotropic forms, a brown amorphous powder and very hard brilliant crystals. In nature it is found only in compounds, for example with sodium and oxygen in borax ($Na_2B_4O_7 \cdot 10H_2O$). In aqueous solution at near-neutral pH, monomeric boric acid [$B(OH)_3$] is the most common species present, regardless of whether the boron source is boric acid (H_3BO_3) or borate. Boron occurs in food as borate or boric acid. For comparative purposes doses of boron compounds are expressed as boron equivalents.

Boron is widely distributed in nature, with concentrations of about 10 mg/kg in the earth's crust (range 5 mg/kg in basalts to 100 mg/kg in shales) and about 4.5 mg/litre in the ocean. The most important commercial borate products and minerals are borax pentahydrate, borax, sodium perborate, boric acid, colemanite, and ulexite.

Boron compounds can be found in the form of boric acid, borax and other borates in a wide range of consumer products, including boron-silicate glass, soaps, detergents, preservatives, adhesives, porcelain, cosmetics, enamel, leathers, carpets, artificial gemstones, high-contrast photographic material, wicks, electric condensers, fertilisers, insecticides, and herbicides (Moore *et al*, 1997).

Boron has long been known to be essential for the growth of vascular plants, with a number of functions which include sugar transport, cell wall synthesis and RNA metabolism. It is

1 - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

proposed that boron has an unique membrane function in plants (Nielsen,1986; FNB, 2001; EGVM, 2002 and 2003). There is also some evidence to support the essentiality of boron in animals.

The WHO established an acceptable safe range of population mean intakes for boron of 1-13 mg/day (WHO, 1996; IPCS, 1998).

In 1996 the SCF adopted an opinion on trace elements including boron in natural mineral waters, in which a NOAEL of 9.6 mg/kg body weight was established. The usual safety factor of 100 was applied leading to the establishment of a tolerable daily intake (TDI) of 0.1 mg boron/kg body weight for humans. The SCF concluded that assuming a consumption of 2 litres of natural mineral water/person/day and the allocation of 10% of the TDI to this source of exposure would lead to a guideline value of 0.3 mg/L. The Scientific Committee on Toxicity and Ecotoxicity set a TDI of 0.3 mg boron /kg body weight (CSTE, 1996).

Council Directive 80/778/EEC on the quality of water intended for human consumption specifies a maximum level of 1 mg/L.

The FNB established a tolerable upper intake level of 20 mg boron/day for adults (FNB, 2001). The EGVM upper safe level is 0.16 mg boron/kg body weight/day (EGVM, 2003).

2. NUTRITIONAL BACKGROUND

2.1. Physiological effects

Boron has not been established to be an essential nutrient for humans and no specific biochemical function for boron has been identified in higher animals or man (FNB, 2001). Recommended intakes for boron have not been established (SCF, 1993; FNB, 2001).

Studies of low boron diets (0.3-0.4 mg/day) in man have reported adverse effects on biological functions that can be corrected by increasing boron intake to about 3 mg/day (Nielsen *et al*, 1987a and b, and 1988a; Nielsen, 1990a.). Several studies have reported that intake of boron may influence the metabolism and utilisation of calcium, copper, magnesium, nitrogen, glucose, triglycerides, reactive oxygen and oestrogen and may have effects on the function of several body systems, including blood, brain and bone (Nielsen, 1997; Hunt, 1997; Penland, 1998; FNB, 2001). Perhaps the best-documented effect of boron is on calcium metabolism or utilization, and thus, bone calcification and maintenance (Nielsen, 1998; Nielsen *et al*, 1987b; Hunt *et al*, 1997; Pizzorno and Murray, 1999; Meacham *et al*, 1994).

Studies of dietary deprivation of boron in animals have reported adverse effects on biological functions, including depressed growth, reduced serum steroid hormone concentrations, changes in plasma and organ calcium and magnesium concentrations, plasma alkaline phosphatase and bone calcification, that can be corrected by increasing boron intake (Nielsen, 1989 and 1990b; Nielsen *et al* 1988a, b and c, and 1990; Hunt and Nielsen, 1981; Hunt, 1988). The effects of low boron intakes are more marked when accompanied by low status for other nutrients (e.g. cholecalciferol, magnesium) (Benderdour *et al*, 1998; McCoy *et al*, 1994; Hunt, 1989; Hegsted *et al*, 1991; Nielsen, 1987a and b).

Although boron deprivation has been reported to cause developmental defects in fish (Rowe and Eckhert, 1999; Eckhert, 1998) and frogs (Fort *et al*, 1999 and 2002), such effects have not been found consistently in rodent models (Lanoue *et al*, 1998 and 1999).

2.2. Absorption, distribution, metabolism and elimination

There is no evidence from metabolic studies for homeostatic control of boron in humans (Samman *et al*, 1998; FNB, 2001), although some reports have suggested that regulation of body content occurs (Hunt and Stoecker, 1996; Sutherland *et al*, 1998).

Boron as borate is readily, and almost completely absorbed (>90%) from the human gut. The mechanism has not been defined. Essentially 100% of boron ingested in the range 0.4-3 mg/day is excreted in faeces and urine and there is no evidence of boron accumulation (Hunt *et al*, 1997). Supplementation with 10 mg of boron/day resulted in the recovery of 84% of the dose in the urine (Samman *et al*, 1998).

At low concentrations, inorganic borates can be converted to boric acid at physiological pH in the aqueous layer overlying mucosal surfaces prior to absorption. Boron as boric acid is evenly distributed throughout the body fluids, via passive diffusion. Boron administered at various dose levels is distributed throughout the tissues and organs of animals and humans at concentrations between 0.05 and 0.6 mg/kg fresh weight and several times these concentrations in bones (Nielsen, 1989 and 1986). In rats dosed with 2, 12.5 and 25 mg boron/rat/day most tissues appeared to reach steady-state boron levels (12-30 mg boron/kg tissue) by 3-4 days; these levels were 3- to 20-fold above control values (IPCS, 1998; Naghii and Samman, 1993, 1996 and 1997b). Adipose tissue took up only 20% as much boron as other tissues (3.78 mg/kg tissue). Bone boron levels (47.4 mg/kg tissue) indicated greater uptake in bone than in other tissues (30-40 mg/kg tissue) after a 7 day feeding experiment in rats. In addition boron levels continued to increase throughout the 7 days.

It has been shown that boron compounds can cross the human placenta (IPCS, 1998).

Borate compounds are not metabolised by biological systems, because of the considerable energy required to break the boron-oxygen bond (Emsley, 1989).

The primary route of elimination is by glomerular filtration (Murray, 1998; Dourson *et al* 1998) and >90% of the administered dose is excreted via urine, regardless of the route of exposure or administration. The 3- to 4- fold higher clearance in rats compared to humans arises from the higher glomerular filtration rate in rats. In humans, excretion is relatively rapid, with a half-life of elimination of 24 hours or less (Nielsen, 1986 and 1988; Litovitz *et al*, 1988). Boron does not accumulate in the blood of regularly exposed workers (Culver *et al*, 1994).

Elimination kinetics of boron from bone is different from soft tissue and body fluids (EGVM, 2002; IPCS, 1998; Chapin *et al*, 1997), suggesting a second kinetic compartment in bone in which a small percentage of absorbed boron is stored.

2.3. Food sources and other sources

Boron is present in aquatic and terrestrial plants but does not bioaccumulate through the food chain. The greatest exposure to boron for most populations comes from food. The daily intake of boron by humans can vary widely depending on the proportions of various food groups in the diet (Nielsen, 1988; Naghii and Samman, 1996; EGVM, 2002 and 2003). Foods rich in boron include fruits, leafy vegetables, mushrooms, nuts and legumes as well as wine, cider and beer. Meat, fish and dairy products are poor sources (Meacham and Hunt, 1998; Anderson *et al*, 1994; Hunt *et al*, 1991; MAFF, 1994).

Water, in particular mineral waters, can be an important source of boron. The average boron content of drinking water in Germany was estimated as 23.1 μg/L (Becker *et al*, 1997). In one study it was reported that bottled water can contain up to 4.35 mg boron/litre, with an average boron content of 0.75 mg/litre (Moore *et al*, 1997).

2.4. Typical intakes

Data on dietary intakes of boron are limited. Boron is not included in the nutrient databases for dietary surveys.

Mean (1.5 mg/day) and 97.5 percentile (2.6 mg/day) boron intakes in adults in the UK have been estimated from analysis of samples from the 1994 Total Diet Study using consumption data from the 1986/87 Dietary and Nutritional Survey of British Adults (MAFF, 1997). In the report of the UK Expert Group on Vitamins and Minerals (EGVM, 2003), the exposure assessment revealed a mean intake for water (0.2-0.6 mg/day) for supplements (up to 2.0 mg/day), and for cosmetics and consumer products (up to 0.47 mg/day). Thus the estimated maximum daily intake of boron was 5.67 mg/day. Vegetarians were identified as a potential high intake group.

Rainey *et al* (1999) have calculated the mean dietary intakes for a typical US population as 1.17 mg/day for men, 0.96 mg/day for women and 1.01 mg/day for pregnant women. Vegetarian adults had a mean intake of 1.47 mg/day for men and 1.29 mg/day for women. The IPCS (1998) reported an average intake of boron for humans as 0.44 μg/day from ambient air, 0.2-0.6 mg/day from drinking water, and 1.2 mg/day from the diet. Coffee and milk are low in boron, but make up 12% of the total boron intake by virtue of the volume consumed (Rainey *et al*, 1999).

Body building supplements have been reported to contain 1.5-10 mg boron/dose, resulting in possible daily intakes of 1.5-30 mg boron (EGVM, 2003).

3. HAZARD IDENTIFICATION

3.1. Animal toxicity data

3.1.1. Acute and short-term toxicity

The oral LD_{50} values for boric acid and borax for mice and rats are in the range of 400-900 mg boron/kg body weight. Acute oral LD_{50} values in the range of 200-350 mg boron/kg body weight for boric acid or borax exposure for guinea pigs, dogs, rabbits and cats (IPCS, 1998; Wang *et al*, 1984; Weir and Fisher, 1972; Smyth *et al*, 1969; Pfeiffer *et al*, 1945).

A short-term experiment (4 weeks) was performed to determine the specificity of the effect of boron on steroid hormones and to determine subsequent changes in plasma lipids in rats. Addition of boron (as boric acid) to the drinking water to provide 2 mg boron/rat/day, did not affect body or testicular weight. The addition of boric acid (2 mg boron/rat/day) to the drinking water resulted in significant elevations in the plasma 1,25-dihydroxyvitamin D concentration at week 2 and the plasma testosterone at week 4 relative to the control group. After 2 weeks, there was a significant decrease in plasma triacylglycerol and HDL-cholesterol concentrations in rats fed boric acid relative to their counterparts in the control group. However, at week 4 only HDL_3-cholesterol was significantly lower (Naghii and Samman, 1997a).

In a 13-week study conducted by the US National Toxicology Program (NTP, 1987) mice (10/sex/dose) were exposed to boric acid in the diet at concentrations sufficient to produce estimated intakes of approximately 0, 34, 70, 141, 281 or 563 mg boron/kg body weight/day for males and 0, 47, 97, 194, 388 or 563 mg boron/kg body weight/day for females. Deaths occurred at high doses (8/10 high dose males and 6/10 high dose females and 1/10 males of the 281 mg boron/kg body weight/day died). Clinical signs of toxicity were a thin, hunched appearance, dehydration, foot lesions, and scaly tails. A dose-related decrease in body weight gain was observed. Histological effects included a dose-related incidence of minimal to mild extramedullary haematopoiesis of the spleen in males and females, hyperkeratosis and acanthosis of the stomach at the highest dose level, and testicular lesions.

Non-accidental mortality at the end of the toxicity study was increased significantly in the males. The only significant lesions were seen in the testes of male mice (NTP, 1987; Dieter, 1994; IPCS, 1998).

Lee *et al* (1978) fed borax in the diet to male Sprague-Dawley rats (18/dose) at dose levels equivalent to 0, 30, 60 or 125-131 mg boron/kg body weight/day for 30 or 60 days. Body weights were not consistently affected by treatment. Organ weights were not affected in the 30 mg/kg body weight/day, but at 60 and 125-131 mg/kg body weight/day, absolute liver and epididymis weights were significantly lower than the controls after 60 days but not after 30 days.

In a 90-day study, Sprague-Dawley rats (10/sex/dose) received 0, 2.6, 8.8, 26.3, 87.5 or 262.5 mg boron/kg body weight/day in the diet as boric acid or borax. All high dose animals died within 3-6 weeks. Body weights in males and females were reduced in animals receiving 87.5 mg boron/kg body weight/day. Absolute organ weights -including the liver, spleen, kidneys, brain, adrenals and ovaries- in this dose groups were also decreased. Relative weights of the adrenals and kidneys were significantly increased, but the relative weights of liver and ovaries were significantly decreased at the 87.5 mg boron/kg body weight/day. A pronounced reduction in testicular weights was also seen (Weir and Fisher, 1972).

In a 90-day drinking water study with male rats, the highest dose of 6 mg boron/L (as borax) (0.426 mg boron/kg body weight/day) caused no effects on fertility and reproduction or the weights of the testes or prostate (Dixon *et al*, 1979).

In a toxicity study beagle dogs were fed a diet containing boric acid or borax for 90 days or 2 years. In the 90-day study the dose levels were 0, 0.44, 4.58 or 43.75 mg boron/kg body weight/day. Testis weights were significantly lower than controls in the middle and upper dose groups. Testicular microscopic structure was not different from control and middle dose, however in the high dose group (43.75 mg boron/kg body weight/ day) 4 out of 5 dogs had complete atrophy, and the remaining dog had one-third of tubules showing some abnormality. In the 2-year study the dogs (4/sex/group) received boric acid or

borax in the diet at dose levels equivalent 0, 1.5, 2.9, or 8.8 and an additional group of dogs received 29 mg boron/kg body weight/day for 38 weeks. No effects were observed on general appearance, body weight, food consumption, organ weights, haematology, or serum chemistry. Changes in testicular morphology occurred in males in the highest dose groups (Weir and Fisher, 1972).

The effects of boron on bone growth were studied (Seffner *et al*, 1990) in growing pigs exposed to boron (4 or 8 mg/kg body weight per day) in two studies. They reported dose-related thinning of the cortex of the humerus and a reduction (significant at 8 mg/kg body weight/day) in bone-derived serum alkaline phosphatase, suggesting reduced osteoblast activity.

3.1.2. Long-term toxicity and carcinogenicity

A 2-year study was conducted in mice (50/sex/day) which received approximately 0, 275, or 550 mg boric acid/kg body weight/day (0, 48.1, or 96.3 boron/kg/day) in the diet. No clinical signs of toxicity were observed. Body weights were 10-17% lower in males of the high dose group after 32 weeks and in females after 52 weeks. Non-accidental mortality at the end of the study was 9/50, 20/50, and 23/50 in control, low-, and high-dose and this increase was statistically significant in males. The lesions in male mice appeared in the testes which showed testicular atrophy and interstitial cell hyperplasia at both dose levels. Also a dose-related increase in incidence of splenic lymphoid depletion in males was observed. Survival of the male mice was significantly reduced. An increased incidence of hepatocellular tumours in low dose male mice was considered by NTP as non-associated to boric acid. Overall, NTP concluded that this study produced no evidence of carcinogenicity of boric acid, although the low number of surviving males may have reduced the sensitivity of the study (NTP, 1987; Dieter, 1994; IPCS, 1998).

In a 2-year study, rats (35/sex/dose) were administered doses equivalent to 0, 5.9, 17.5, or 58.5 mg boron/kg body weight/day in the diet as borax or boric acid. High-dose animals had coarse hair coats, scaly tails, hunched posture, swollen and desquamated pads of the paws, abnormally long toenails, shrunken scrotum, inflamed eyelids, and bloody eye discharge. These signs became frequent and more pronounced during the first year but did not change thereafter. Serum chemistry and urine values were normal; the packed cell volume (PCV) and haemoglobin levels were significantly lower than in controls. The absolute and relative weights of the testes were significantly lower, and relative weight of the brain and thyroid were higher, than in controls. The seminiferous epithelium was atrophied, and the tubular size in the testes was decreased at 58.5 mg boron/kg body weight/day. No treatment-related effects were observed at lower dose levels, the NOAEL for this study was 17.5 mg boron/kg body weight/day. An increased incidence of tumours was not observed (Weir and Fischer, 1972; IPCS, 1998).

Based on the lack of human data and on the results of the aforementioned animal studies, boron was classified by the US EPA as a Group D chemical (not classifiable as to human carcinogenicity) (US EPA, 1994).

3.1.3. Reproductive and developmental studies

In a multigeneration continuous-breeding experiment (Fail *et al*, 1990 and 1991), Swiss CD-1 mice (F_0 generation) were fed boric acid in the diet at 0, 1000, 4500, or 9000 mg/kg feed for 27 weeks, which gave calculated doses of 0, 19.2, 104.7, and 222.1 mg boron/kg body weight/day for males and 0, 31.9, 148.1 and 290.5 mg boron/kg body weight/day for females. Treatment with boric acid significantly impaired fertility; all males and females in the high-dose groups were infertile. At the middle dose, the number of litters per pair, number of live pups per litter, proportion of pups born alive, and pup weight adjusted for litter size were all decreased. The trend towards a lower fertility index at this dose level was more apparent with subsequent matings. Animals from different treatment groups were cross-mated. When mid-dose males were mated with controls females, mating and fertility indices were significantly depressed, with only one pair in that group producing a live litter; these indices were not affected when control males were mated with mid-dose females, confirming that the male was the affected sex. At the F_0 necropsy, sperm motility was significantly reduced in all exposed groups (by 12%, 32%, and 47%, from low- to high-dose groups, respectively). Low dose and mid-dose animals from the F_1 generation were exposed during gestation and lactation. The fertility of the low-dose F_1 mice was not affected, but the litter-adjusted body weights of the F_2 pups were significantly decreased (by 3.3%) relative to controls. The low dose was considered a LOAEL for decreased sperm motility in the F_0 males, 26% increased uterine weights and 8% kidney weight/adrenal weight in the F_1 females and a 3.3% reduction in litter-adjusted birth weight in the F_2 pups. This study provides no NOAEL, but the changes in the low dose were minor and this indicates that this dose level is close to the NOAEL (IPCS, 1998).

Fail *et al* (1989 and 1990) utilised CD-1 mice both to characterise the effects of boric acid on fertility and to test the reversibility of these effects. Adult CD-1 mice were exposed to boric acid in the feed for 27 weeks at 0, 1000, 4500, or 9000 mg/kg diet (doses were not given). The males at the high and mid-doses had testicular atrophy and decreased spermatogenesis. Fertility was diminished in animals receiving the mid-dose and completely absent in the high-dose group (IPCS, 1998).

Secondary to the loss of germ cells, the activities of enzymes found primarily in spermatogenic cells were significantly decreased and enzyme activities associated with premeiotic spermatogenic cells were significantly increased in Sprague-Dawley rats at dose levels of 60 and 125-131 mg boron/kg body weight/day for 30 or 60 days (Lee *et al*, 1978). Mean plasma follicle stimulating hormone (FSH) levels were significantly elevated in a dose-dependent manner in all treatment groups in this study (60 to 125-131 mg boron/kg body weight/day) after 60-days of intake. FSH levels in animals receiving the highest dose tested (125-131 mg boron/kg body weight/day) were still elevated 12 months after treatment termination, owing to atrophied testes and no recovery of spermatogenesis. Plasma luteinizing hormone (LH) levels were not significantly elevated, and mean plasma testosterone levels were within the normal range throughout the study (Lee *et al*, 1978; IPCS, 1998).

The reversibility of testicular lesions was evaluated by Ku *et al* (1993a) in an experiment in which F-344 rats were dosed at 3000, 4500, 6000, or 9000 mg boric acid/kg diet (26, 38, 52 and 69 mg boron/kg body weight/day) in the feed for 9 weeks and assessed for recovery up to 32 weeks post treatment. Inhibited spermiation was exhibited at 38-52 mg boron/kg body weight/day (5.6 μg boron/mg tissue), whereas inhibited spermiation progressed to atrophy at 52-68 mg boron/kg body weight/day (11.9 μg boron/mg tissue). Boron did not accumulate in the testes to levels greater than found in blood during the 9-week period. After treatment, serum and testis boron levels in all dose groups fell to background levels. Inhibited spermiation at 38 mg/boron/kg body weight/day was reversed at 16 weeks post-treatment, but focal atrophy was detected that did not recover up to 32 weeks post-treatment (IPCS, 1998).

The development of the boron-induced testicular lesion was investigated by Treinen and Chapin (1991), who fed boric acid at a level of 0 or 60.9 mg boron/kg body weight/day (estimated by the authors) to male F-344 rats and sacrificed six treated and four control male rats at intervals from 4 to 28 days after the start of the boric acid intake. In half of the treated rats, there was inhibition of spermiation in 10-30% of stage IX tubules at 7 days and inhibition in all stages IX and stage X tubules after 10 days of exposure. At 28 days there was significant loss of spermatocytes and spermatids from all tubules in exposed rats, and basal serum testosterone levels were significantly decreased from 4 days on (IPCS, 1998).

In a three-generation reproduction study performed in conjunction with the long-term toxicity study, it was found that 58.5 mg boron/kg body weight/day produced testicular atrophy and complete suppression of fertility in rats. Lower doses (17.5 or 5.9 mg boron/kg body weight/day) did not reduce fertility (Weir and Fisher, 1972). Due to the small group size the data of the study are of limited value for a risk assessment (IPCS, 1998).

A study by Ku *et al* (1993a) found no detectable treatment-related changes in testicular structure in rats following consumption of 17.5 mg boron/kg body weight/day for up to 9 weeks. In a follow-up study to explore and identify the mechanism for the testicular toxicity of boric acid, Ku *et al* (1993b) evaluated several end-points in cell culture systems following *in vitro* boric acid exposure. The data suggest an effect of boric acid on the DNA synthesis activity of mitotic and meiotic germ cells and, to a lesser extent, on energy metabolism in Sertoli cells. The effect on DNA synthesis occurred at boron concentrations that were similar to the serum levels recorded when testis atrophy was observed. These observations show that boric acid interferes with the production and or maturation of early germ cells, and offers an explanation for atrophy, but not for inhibited spermiation (IPCS, 1998).

Additional mechanistic studies by Ku and Chapin (1994) showed that testicular toxicity and CNS hormonal effects were not due to selective boron accumulation in testis or brain/hypothalamus. Changes in testis phosphorus, calcium, and zinc levels did not precede atrophy. *In vitro* studies showed no effect on steroidogenic functions of isolated Leydig cells. The authors showed that inhibited spermiation was not due to increased testicular cyclic adenosine monophosphate or reduced serum protease plasminogen activators. Effects of boric acid were also seen in Sertoli-germ cells co-cultures on Sertoli cell energy metabolism (lactate secreted by Sertoli cells is a preferred energy source for germ cells) and DNA/RNA synthesis (germ cells synthesise DNA/RNA, and boric acid impairs the synthesis of these nucleic acids in the liver). The most sensitive *in vitro* end-point was DNA synthesis in mitotic/meiotic germ cells; energy metabolism in germ cells was affected to a lesser extent, which

was manifested *in vivo* as a decrease in early germ cell/Sertoli cell ratio prior to atrophy of the testes. The mechanisms of inhibited spermiation are still not defined (IPCS, 1998).

Heindel *et al* (1992) also investigated the development toxicity and teratogenicity of boric acid in mice at 0, 43, 79, or 175 mg boron/kg body weight/day in the diet. There was a significant dose-related decrease in average foetal body weight per litter at 79 and 175 mg boron/kg body weight/day. Offspring of mice receiving 79 or 175 mg boron/kg body weight/day during gestation days 0-17, showed an increased incidence of skeletal (rib) malformations. These changes occurred at doses for which there were also signs of maternal toxicity (increased kidney weights and pathology); the LOAEL for developmental effects (decreased foetal body weight per litter) was 79 mg boron/kg body weight/day, and the NOAEL for developmental effects was 43 mg boron/kg body weight/day (IPCS, 1998).

Sprague-Dawley rats were fed diets giving intakes of 0, 13.6, 28.5 or 57.7 mg boron/kg body weight/day as boric acid from gestation days 0 to 20 (Heindel *et al*, 1992). An additional group of rats received boric acid at 94.2 mg boron/kg body weight/day on gestation days 6-15 only. Maternal effects included a significant and dose-related increase in relative liver and kidney weights at 28.5 mg/kg body weight/day and higher. Treatment with 94.2 mg boron/kg body weight/day significantly increased prenatal mortality. Average foetal body weight per litter was reduced significantly in a dose-related manner in all treated groups compared with controls. The percentage of malformed foetuses per litter and the percentage of litters with at least one malformed foetus were significantly increased at 28.5 mg boron/kg body weight/day. Malformations consisted primarily of abnormalities of the eyes, the CNS, the cardiovascular system, and the axial skeleton. The most common malformations were enlargements of lateral ventricles in the brain and agenesis or shortening of rib XIII. The percentage of foetuses with variations per litter was reduced relative to controls at 13.6 and 28.5 mg boron/kg body weight/day, but was significantly increased in rats receiving the 94.2 mg boron/kg body weight/day. The LOAEL of 13.6 mg boron/kg body weight/day for rats occurred in the absence of maternal toxicity; a NOAEL was not established (IPCS, 1998).

Price *et al* (1996a) did a follow-up to the Heindel *et al* (1992) study in Sprague-Dawley (CVD) rats in order to determine a NOAEL for foetal body weight reduction and to determine whether the offspring would recover from prenatally reduced body weight during postnatal development. Skeletal malformations and variations were also studied to further characterise the low end of the dose-response curve (phase 1) and to determine whether the incidence of skeletal defects in offspring changed during postnatal life (phase 2). Boric acid was administered in the diet to CD rats from gestation day 0 to 20. In phase 1, uterine contents were examined on gestation day 20. During phase 1, the intake of boric acid was 0, 3.3, 6.3, 9.6, 13.3 or 25 mg boron/kg body weight/day. For these treatment dose groups, foetal body weights were 99, 98, 97, and 88% of controls; the reduction was significant only at 13.3 and 25 mg boron/kg body weight/day on gestation day 20. During phase 1, the incidences of short rib XIII (a malformation) and wavy ribs (variation) were increased at 13.3 mg boron/kg body weight/day or more relative to the control litters. During phase 2, the intake of boric acid during gestation was 0, 3.3, 6.5, 9.8, 12.9 or 25.3 mg boron/kg body weight/day. At birth, boric acid intake stopped and dams were allowed to deliver and rear their litters until postnatal day 21. On post-natal day 0 of phase 2, there were no effects of boric acid on offspring body weight, nor were any differences seen through postnatal day 21. On postnatal day 21 of phase 2, the percentage of pups per litter with short rib XIII was elevated only in the 25.3 mg boron/kg body weight/day group, and there was no-treatment-related increase in wavy ribs or extra ribs on lumbar 1 observed in these pups on day 21. The NOAEL for phase 1 is 9.6 mg boron/kg body weight/day based on a decrease in foetal body weight and the LOAEL was 13.3 mg boron/kg body weight/day. The NOAEL for phase 2 was 12.9 mg boron/kg body weight/day, and the LOAEL was 25.3 mg boron/kg body weight/day (IPCS, 1998).

Price *et al* (1996b) investigated the developmental toxicity and teratogenicity of boric acid in rabbits at doses of 0, 10.9, 21.9, or 43.7 mg boron/kg body weight/day given by gavage. Developmental effects in rabbits exposed to 43.7 mg boron/kg body weight/day included a high rate of prenatal mortality, increased number of pregnant females with no live foetuses, and fewer live foetuses per live litter on postnatal day 30. Malformed live foetuses per litter were increased significantly at 43.7 mg boron/kg body weight/day, primarily because of the incidence of foetuses with cardiovascular defects, the most prevalent of which was interventricular septal defect. Skeletal variations observed were extra rib on lumbar 1-position and misaligned sternebrae. The NOAEL for maternal and developmental effects was 21.9 mg boron/kg body weight/day and the LOAEL was 43.7 mg boron/kg body weight/day (IPCS, 1998).

In a 2-year study (Weir and Fisher, 1972) groups of 4 male and 4 female beagle dogs were fed diets containing boric acid or borax to provide doses of 0, 1.45, 2.93, or 8.75 mg boron/kg body weight/day.

No evidence of toxicity was observed. An additional group of dogs (4 male and 4 female) was fed diets containing boric acid or borax doses of 0 or 29.3 mg boron/kg body weight/day for 38 weeks. The authors stated that boric acid caused testicular degeneration in dogs, including spermatogenic arrest and atrophy of the seminiferious epithelium. The background lesions in testis and its accessory organs were also high, so the results were equivocal (IPCS, 1998).

3.2. Genotoxicity

Boric acid was not mutagenic in *Salmonella enterica var.* Typhimurium with or without exogenous metabolic activation (S-9 fraction) (Haworth *et al*, 1983; Benson *et al*, 1984; NTP, 1987; IPCS, 1998). Boric acid was not mutagenic in the L5178Y mouse lymphoma *tk* assay with or without S-9 (NTP, 1987; Rudd, 1991). Boric acid did not induce unscheduled DNA synthesis (UDS) in primary cultures of male F344 rat hepatocytes (Bakke, 1991).

Crude or refined borax were negative in assays for mutagenicity in V79 Chinese hamster cells, C3H10T ½ mouse embryo fibroblasts and diploid human foreskin fibroblasts (Landolph, 1985).

Similarly, boric acid was negative in *in vitro* assays for chromosomal aberrations or sister chromatid exchanges (SCEs) in Chinese hamster ovary cells with or without metabolic activation systems (NTP, 1987).

In vivo, boric acid was negative in a micronucleus assay on Swiss-Webster mice (O'Loughlin, 1991). In this study, boric acid was administered in deionized water orally for 2 consecutive days at 900, 1800 or 3500 mg/kg. No induction of chromosomal aberrations or mitotic spindle abnormalities in bone marrow erythrocytes was observed.

Overall, the available data indicate that boric acid and borax are not genotoxic.

3.3. Human toxicity data

3.3.1. Acute and short-term toxicity

The lowest lethal dose after single accidental oral ingestion of boron acid by humans ranged from approximately 98-650 mg boron/kg body weight (Stokinger, 1981; Teshima *et al*, 1992), dermal exposure revealed a lower lethal dose of 1457 mg boron/kg body weight, whereas death has been reported when boron was given intravenously at 0.5 mg boron/kg body weight. Litovitz *et al* (1988) stated that the potential lethal dose is 3-6 g for infants and 15-20 g for adults expressed as boric acid.

There are data from several case reports of intoxication with boron as boric acid from inappropriate use of medical preparations (Goldbloom and Goldbloom, 1953; Linden *et al*, 1986; Litovitz *et al*, 1988; Stokinger, 1981; Kliegel, 1980; Stokinger and Spiegl, 1953; Naghii and Samman, 1997b; IPCS, 1998; Teshima *et al*, 1992; Stein *et al*, 1973; Gordon *et al*, 1973). Symptoms included gastrointestinal disturbances, generalised or alternating focal seizure disorders, irritability, granular degeneration of tubular cells, exfoliate dermatitis, epilepsy, cardio-circulatory collapse, CNS effects such as oedema and congestion of the brain, hair loss, lethargy, anorexia and mental confusion.

The toxic dose varied with the duration of intake, 4-10 weeks of intake revealed toxic effects at an average daily ingestion of 0.143-0.429 g boric acid/kg body weight, equivalent to 25-76 mg boron/kg body weight (O'Sullivan and Taylor, 1983).

Linden *et al* (1986) published a retrospective review of 364 cases of boric acid ingestion reported to the Rocky Mountain Poison and Drug Center in Denver, CO, USA, between 1983 and 1984. Vomiting, diarrhoea, and abdominal pain were the most common symptoms given by 276 cases. Of the 72 cases reported in 1984, 79% were asymptomatic, whereas 20% had mild gastrointestinal symptoms.

The average oral dose of boric acid required to produce clinical symptoms of intoxication with boron is still unclear (Goldbloom and Goldbloom, 1953; IPCS, 1998). In a 3-week study in post-menopausal women receiving either a low boron diet (0.33 mg/day) or this diet supplemented to 3.33 mg/day, the supplement had no effect on blood levels of minerals, or steroids (Beattie and Peace, 1993).

3.3.2. Reproductive effects

An ecological study assessed boron exposure from drinking water and fertility among residents in two geographical regions in Turkey. Drinking water from Region I contained 2.05-29 mg boron/L and

Region II had a range of 0.03-0.40 mg boron/L (IPCS, 1998; Sayli *et al*, 1998). No statistical analyses were performed. The results of this descriptive study suggest that fertility, as measured by the ability to produce a live birth, was not adversely affected for residents of high boron drinking water and soil area. Fertility following inhalation exposure to boron was assessed in a descriptive study. Whorton *et al* (1994) estimated the standardised birth rate (SBR) to assess fertility in 542/720 occupational workers in a borax mine in California, USA. No significant trend was observed for SBR in relation to exposure for quintiles of mean exposure levels ranging from <0.82 mg boron/m^3 to >5.05 mg boron/m^3.

4. DOSE-RESPONSE ASSESSMENT

The data on the toxicity of boron in humans are sparse and not suitable for dose-response assessment.

Limited long-term carcinogenicity studies in mice and rats showed no evidence of carcinogenicity. In addition, *in vitro* and *in vivo* studies at gene or chromosome level showed no evidence of genotoxicity.

There are several short-term and long-term toxicity studies in a number of animal species (e.g. mouse, rat, dog and pig). The animal experiments reveal developmental and reproductive effects as the most critical adverse effects. The reproductive effects were observed both in repeated dose toxicity studies and reproduction studies. The reproductive effects found at dose levels of 58.5 mg boron/kg body weight/day in a 2-year toxicity study in rats consisted of atrophy of seminiferous epithelium and decreased size of testicular tubules, which was not observed at 17.5 mg boron/kg body weight/day (Weir and Fisher, 1972). Developmental effects produced by boron included short ribs, variation in the number of ribs and decrease in foetal body weight. The LOAEL for decreased foetal body weight was 13.3 mg boron/kg body weight/day with a NOAEL of 9.6 mg/kg body weight/day (Price *et al*, 1996a).

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

1.1. Adults

The human database on boron is not adequate for establishing an UL and there are no human data on developmental and reproductive effects comparable to those observed in animals. In consequence, the UL is based on the most sensitive end-point detected in the animal studies, i.e. the NOAEL for decreased foetal body weight in rats following maternal exposure during pregnancy. Normally, a default uncertainty factor of 100 would be applied to a NOAEL derived from an animal study, but this factor should be modified if relevant data are available. In the case of boron, critical analysis of the existing data on boron toxicokinetics allows use of the approach proposed by the International Programme on Chemical Safety (IPCS, 1994) in which the 10-fold interspecies and human variability factors are subdivided into toxicokinetic and toxicodynamic aspects. The default toxicokinetic uncertainty factor for extrapolation from animals to humans was appropriate for boron and was retained. The glomerular filtration rate (GFR) in pregnant women is 144 ± 32 mL/min (Dourson *et al*, 1998). Human variability in GFR during pregnancy was calculated as the ratio of the mean GFR divided by (the mean GFR minus two times the standard deviation) (144/80), i.e. 1.8. The default toxicokinetic uncertainty factor for human variability was then adjusted from the default value of 3.2 to 1.8 based on variability in GFR, which is the critical physiological process involved in boron clearance. There are no data on species differences or human variation in boron toxicodynamics, and the default factors for these aspects were retained. The resulting combined overall uncertainty factor was 60. Dividing the NOAEL of 9.6 mg boron/kg body weight/day by the uncertainty factor of 60 gives a daily intake of 0.16 mg/kg body weight/day, which gives a UL of 10 mg boron/person/day for adults.

The UL only applies to the intake of boron in the form of boric acid and borates.

1.2. Pregnancy and lactation

As the UL is based on a NOAEL for adverse effects from reproductive studies and multigeneration studies, the UL of boron applies also to pregnant and lactating women.

1.3. Children and adolescents

There are no data on adverse effects of boron intakes on children and adolescents. However, the multigeneration studies in animals do not indicate that young animals are more susceptible than adults. Therefore, in the absence of adequate data the Panel chose to extrapolate the UL from adults to children on a surface area (body weight$^{0.75}$) basis. The reference weights derived by the SCF (SCF, 1993; SCF, 2000) are used as a basis for the calculations of surface area and UL.

Age (years)	Tolerable Upper Intake Level (UL) for boron (mg/day)
1-3	3
4-6	4
7-10	5
11-14	7
15-17	9

2. RISK CHARACTERISATION

The limited data on boron intake in EU countries indicate that the intakes of boron from food and drinking water are below the UL.

The intake of some supplements containing boron may lead to intakes that exceed the UL.

3. RECOMMENDATIONS

Data on intake of boron from different sources in Europe is very limited and should be collected for different European countries.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF IRON

(REQUEST N° EFSA-Q-2003-018)
(ADOPTED ON 19 OCTOBER 2004)

SUMMARY

Iron is an essential trace element that has important metabolic functions, including oxygen transport and storage and many redox reactions. Insufficient intake results in the deficiency condition anaemia, adverse outcomes of pregnancy, impaired psychomotor development and cognitive performance and reduced immune function.

Case reports of accidental poisoning with medicinal iron, especially in young children, indicate acute damage of gastrointestinal, hepatic, pancreatic and cardiovascular structures after ingestion of very high doses. An acute oral dose of 60 mg iron/kg body weight can be lethal but oral doses below about 10-20 mg iron/kg body weight do not cause acute systemic toxicity.

Adverse gastrointestinal effects (e.g. nausea, epigastric discomfort, constipation) have been reported after short-term oral dosage at 50-60 mg daily of supplemental non-haem iron preparations, particularly if taken without food.

Iron overload with clinical symptoms, including liver cirrhosis, has been reported in individuals receiving long-term, high-dose medical treatment with iron (160-1200 mg iron/day). Iron overload with clinical symptoms has also been found in subjects homozygous for hereditary haemochromatosis (a genetic disorder of iron storage), even at normal dietary iron intakes. Bantu siderosis, with liver cirrhosis and diabetes, has been attributed to chronic excess intake of highly available iron (50-100 mg iron/day) in beer; however, these adverse effects may be confounded by chronic alcohol intake and possibly by a genetic disorder.

Although a proportion of the population has serum ferritin levels indicative of elevated iron stores (above 200 μg/L for women and 300 μg/L for men), the point at which an elevated serum ferritin level becomes associated with an increased risk of adverse effects (such as liver fibrosis) is not known. The risk of adverse effects from iron overload in the general population, including those heterozygous for hereditary haemochromatosis, is considered to be low.

Epidemiological studies have reported associations between high iron intake and/or stores with increased risk of chronic diseases such as cardiovascular disease, type II diabetes and cancer of the gastrointestinal tract. However, these data are conflicting and do not provide convincing evidence of a causal relationship between iron intake or stores and such chronic diseases.

The Panel considered that the available data are insufficient to establish a tolerable upper intake level for iron.

Based on estimates of current iron intakes in European countries, the risk of adverse effects from high iron intake from food sources, including fortified foods in some countries, but excluding supplements, is considered to be low for the population as a whole, except for those homozygous for hereditary haemochromatosis (up to 0.5% of the population). However, intake of iron from food supplements in men and postmenopausal women may increase the proportion of the population likely to develop biochemical indicators of high iron stores. Some groups at special risk for poor iron status, such as menstruating women or children, could benefit from additional iron intake and/or improved availability of dietary iron.

KEY WORDS

Iron, tolerable upper intake level, gastrointestinal effects.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Iron is a metal with an atomic mass of 55.8. It is present in biological systems in one of two oxidation states, and redox interconversions of the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms are central to the biological properties of this mineral. Iron is an essential constituent of oxygen carriers, such as haemoglobin and myoglobin, and the iron contained within haem is essential for the redox reactions of numerous cytochromes.

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

2.1. Functions of iron, deficiency symptoms, and prevalence of iron-deficiency

Iron deficiency is associated with reduced function of an array of iron-dependent enzymes and proteins (Beard and Dawson, 1997). The most important effect of deficiency is impaired physical performance due to reduced levels of haemoglobin and myoglobin and lower activity of iron-dependent cytochromes, leading to reduced cellular concentrations of ATP. Lack of iron-dependent ribonucleotide-reductase and aminoacid-monoxygenases may impair RNA synthesis and neurotransmitter metabolism. Iron-deficiency anaemia increases the risk for low birth weight (Baynes and Bothwell, 1990), and appears to impair psychomotor development and cognitive performance (Grantham-McGregor and Ani, 2001). Cellular immune function and thermoregulation may also be impaired in severe iron-deficiency (Beard and Dawson, 1997).

1 - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

Iron deficiency anaemia is one of the most common nutritional disorders world-wide. It has the highest prevalence in women of childbearing age and in infants, affecting an estimated 25-46% of babies in the third world (DeMaeyer and Adiels-Tegman, 1985; Dewey *et al*, 1997). Anaemia is found in 2% of males and 5% of females in Germany (VERA-Schriftenreihe, 1995). In the USA the prevalence of iron-deficiency anaemia is 0.2%, 2.6% and 1.9% in men and in pre- and post-menopausal women, respectively (Cook *et al*, 1986); corresponding data from Northern Ireland are 0.5%, 6.6% and 4.6% respectively (Strain *et al*, 1990).

Groups vulnerable to iron deficiency are infants over 6 months, toddlers, adolescents and pregnant women (due to high requirements), older people and people consuming foods high in iron absorption inhibitors (see below) (due to poor absorption) and menstruating women or individuals with pathological blood loss (due to high blood losses) (EGVM, 2003).

2.2. Iron homeostasis

Ferrous iron in low molecular weight forms may increase oxidative stress, because reactive oxygen species (ROS) may convert superoxide anions into highly reactive hydroxyl radicals by an iron-catalysed Fenton reaction (McCord and Turrens, 1994). *In vitro* studies in cultured mammalian cells have demonstrated a direct interrelationship between the intracellular free, iron pool and oxidative stress. To minimise oxidative stress the body contains a number of high affinity iron-binding proteins so that there are very low levels of free non-bound iron in the circulation or within cells. The different binding proteins dominate the fate of iron in the body.

The adult human body contains 2.2-3.8 g iron under iron-adequate conditions (Lynch, 1984). Homeostatic mechanisms have evolved that can alter intestinal iron absorption and supply iron preferentially to functional compartments in response to deficiency or excess.

2.2.1. Iron absorption, distribution and regulation of tissue stores

Tissue concentrations and body stores of iron are controlled at three different levels:

i. Luminal iron: the extent of uptake of iron by the cells of the gastrointestinal tract affects how much remains unabsorbed and passes to the lower bowel, prior to elimination in faeces;

ii. Mucosal iron: the mucosa is the main site of regulation of iron uptake in relation to liver stores and ferritin levels;

iii. Post-mucosal iron: relates to the impact of iron intake on iron status and body stores.

2.2.1.1. Luminal iron

Non-haem iron is present in foods largely as salts, which are made soluble in the stomach, and absorption from foods depends on its dissolution as ferric salts and subsequent reduction to the ferrous form. Any elemental iron in the diet is probably absorbed as non-haem iron following its dissolution in the acid stomach contents. The absorption of non-haem iron can be increased substantially by the presence of ligands, such as ascorbate, citrate and fumarate, as well as the presence of amino acids (e.g. cysteine) and oligopeptides resulting from meat digestion (Mulvihill *et al*, 1998). In contrast, very stable complexes, for example with phytates, phosphates and oxalates, impair non-haem iron absorption. Depending on the concentration of supportive or inhibitory ligands in the intestinal lumen the absorption of non-haem iron can vary by a factor of 10 in single-meal studies, but the effects are less pronounced in more long-term studies (Hallberg and Rossander, 1984; Rossander, 1987; Hunt and Roughead 2000).

2.2.1.2. Mucosal iron

In addition, homeostatic regulation will influence the extent of non-haem iron absorption. Body iron content is linked to demand by regulated intestinal non-haem iron absorption which, in turn, is regulated to a major extent by the uptake of iron into the cells of the intestinal mucosa (Schümann *et al*, 1999a and b). This step is mediated by the divalent metal transporter (DMT-1) (Gunshin *et al*, 1997), the expression of which is higher in iron deficient animals (Fleming *et al*, 1999) and in hereditary haemochromatosis (Byrnes *et al*, 2002). The activity of DMT-1 decreases after a period of high oral iron intake (Oates *et al*, 2000), and such down-regulation of iron uptake may be regarded as protection against iron overload. There is evidence of iron-zinc interaction at the mucosal level. Large doses of supplemental non-haem iron inhibit zinc absorption if they are both taken without food, but do not inhibit zinc absorption if they are consumed with food (FNB, 2001).

Transfer of information on hepatic iron stores to the gut may be mediated via an acute phase hepatic protein, hepcidin (Nicolas *et al*, 2001), or possibly the pro-hormone form pro-hepcidin (Kulaksiz *et al,* 2004), which influences the extent of iron absorption (Leong and Lonnerdal, 2004) and can result in a mucosal block. Hepcidin levels are inappropriately low in patients with hereditary haemochromatosis in relation to their iron stores and ferritin levels (Robson, 2004), and genetic variants of which may contribute to iron accumulation in some types of haemochromatosis (Bridle *et al*, 2003; Ganz, 2003).

Haem iron enters the mucosa via a pathway that is not regulated by DMT-1, but involves interaction of iron in a porphyrin complex with a haem-receptor (Tenhunen *et al*, 1980; Roberts *et al*, 1993). Haem iron absorption is less regulated (Finch, 1994), and varies between 15% in iron-replete individuals and 35% in iron-deficient subjects. The increased haem-iron absorption in iron-deficiency is partly due to higher haem-binding to the brush-border (Roberts *et al*, 1993) and partly to induction of mucosal haem-oxygenase, an enzyme that cleaves the porphyrin ring and liberates iron for transfer into the body. Haem-iron absorption is largely independent of dietary ligands, although a high luminal calcium concentration reduces absorption (Hallberg *et al*, 1992).

A recent study showed that iron absorption does not appear to be altered in heterozygotes for hereditary haemochromatosis; HFE C282Y-heterozygous subjects did not absorb dietary iron more efficiently, even when foods were highly fortified with iron from ferrous sulfate and ascorbic acid, than did control subjects (Hunt and Zeng, 2004).

Due to the various exogenous and endogenous factors affecting iron absorption, a clear relationship is generally not found between total iron intake and iron status. Total daily iron absorption is about 0.9 mg in males and 0.5 and 0.6 mg higher in menstruating women and blood donors. The mucosal barrier can be overwhelmed by high iron doses, such as occurs in acute iron intoxication (Ellenham and Barceloux, 1988).

2.2.1.3. Post-mucosal iron

The concentration of free iron in blood is extremely low due to the high affinity binding of iron to transferrin. Transferrin is an 80 kDa plasma protein that binds two Fe3+ ions per molecule with high affinity (binding constant: 10^{-30}). The total iron binding capacity (TIBC) of transferrin in the plasma of healthy adults is approximately 56µmol/L. About 30% and 10% of TIBC is occupied in normal iron status and in iron deficiency respectively. The plasma iron pool is approximately 3 mg, but its daily turnover is more than 30 mg/day (Bothwell *et al*, 1979). Free iron, or non-transferrin-bound iron (NTBI), can occur in the presence of normal transferrin saturation values, and this has been demonstrated using the method of Hider (2002) in both homozygotes (Loreal *et al*, 2000; Gosriwanata *et al*, 1999) and in heterozygotes for hereditary haemochromatosis (de Valk *et al*, 2000).

A low iron concentration in plasma is essential for the bacteriostatic and bactericidal systems in blood and lymph (Ward *et al*, 1996). Iron overload, with its associated increased plasma iron levels, suppresses immune cell function, allows increased growth rates of infectious organisms and increases the risks of morbidity and mortality due to infectious disease (Collins, 2003; Failla, 2003; Ward *et al*, 1996).

Iron is transferred from the plasma to the tissues via binding of iron-loaded transferrin to transferrin receptors (TfR) at the cell surface, which are subsequently internalised within endocytotic vesicles. High serum TfR values indicate high activity of cellular iron uptake, which in most cases is due to high erythropoietic iron demand. Transferrin releases its iron under acidic conditions in the endocytotic vesicles, and the transferrin and TfR return subsequently to the cell surface.

Within cells, iron is preferentially distributed to iron-dependent enzymes and iron-binding proteins. Excessive intracellular iron is sequestered in the storage protein ferritin (500 kDa) or in its degradation product called haemosiderin. One ferritin molecule can store up to 4500 iron atoms as ferric hydroxyphosphate micelles. Such iron can be remobilised and utilised on demand. The serum ferritin concentration is an indication of body iron stores, except when high levels arise due to inflammation (Ponka *et al*, 1998).

Homeostatic mechanisms involving cytosolic iron-regulatory proteins (IRPs) have developed to maintain low concentrations of free iron, in order to provide iron for essential functions and to protect the cells from oxidative damage. IRP-1 is activated by cellular iron-deficiency, but also by reactive oxygen species (ROS). At normal cellular levels of free iron, IRP-1 contains a 4Fe-4S cluster but in iron-deficient cells the 4Fe-4S cluster is lost and the molecule binds to specific base loops of mRNAs for DMT-1,

TfR and ferritin, called “iron-response elements”. In deficiency IRP binding to TfR-mRNA increases TfR expression, while the ferritin mRNA is broken down more rapidly, which restores the intracellular free iron concentration by a combination of increased uptake and decreased sequestration. IRP activity is also induced by ROS and during hypoxia-reoxygenation stress (Rouault and Klausner 1997; Hanson and Leibold, 1999) when any increase in free iron could lead to increased oxidative damage (Martins *et al*, 1995; Hentze and Kühn, 1996; Pantopoulos and Hentze, 1995; Mueller *et al*, 2001; Rouault and Klausner, 1997, Hanson and Leibold, 1998; 1999). This is prevented, because the tendency towards an increased free intracellular pool causes an increase in the expression of ferritin and a decrease in TfR expression (Kakhlon *et al*, 2001), thereby reversing the effects of IRP activation and decreasing both the free intracellular pool and oxidative stress (Picard *et al*, 1998).

The low pH values in inflamed tissues may mobilise free iron from ferritin and from its haem-bound form (Britton *et al*, 1994; Biemond *et al*, 1984; Koishinako *et al*, 1996). Iron-related increases in ROS production may contribute to pathogenic processes, such as myocardial infarction and intermittent claudication, which can follow hypoxia and re-oxygenation, (see Schümann, 2001), and also in the induction of cirrhosis, as exemplified in iron-overloaded rat livers (Bacon *et al*, 1983). Hepcidin (see above) is an acute phase protein and its increased release from the liver may result in a mucosal block, thereby contributing to the effects of inflammation on iron homeostasis.

Under physiological conditions iron status is almost exclusively regulated by adaptation of intestinal iron absorption according to the demand (see 2.2.1.2). In healthy iron-replete humans with iron stores of 800-1200 mg, non-haem iron absorption increases when iron stores are depleted and vice versa (Finch, 1994). This mode of regulation is highly predictable in normal subjects, and iron stores as determined by plasma ferritin values have been used to predict iron absorption (Magnusson *et al*, 1981). Duodenal non-haem iron absorption is linked to body iron status via the supply of serum iron to intestinal crypt cells. At adequate supply levels, the activity of IRPs in these cells is low, whereas in iron-deficiency IRP activity in the enterocytes is increased and intestinal iron absorption is high. Iron absorption adapts to changes in plasma iron concentration with a lag time of 48h, which corresponds to the time required for young enterocytes to express enzymes and transport proteins, such as DMT-1 and iron regulated gene (IREG), and to migrate up the villi to the site of absorption (Schümann *et al*, 1999b).

In anaemia, an undefined and unidentified “erythrocyte regulator” can increase iron absorption to 20-40 mg Fe/day from oral iron preparations (Finch, 1994). In addition iron absorption is increased by an unknown mechanism to up to 66% in pregnant women and returns to normal at 16-24 weeks after delivery (Barrett *et al*, 1994). Iron absorption is also increased during lactation and growth.

Regulation of non-haem iron absorption protects from iron overload at normal and moderately increased iron-intake levels. A group of 12 Swedish male blood donors and 19 non-donors received standard meals with radioactively labelled non-haem iron (12 mg Fe/day) and haem-iron (2 mg/day). Total iron absorption increased when serum ferritin concentrations were less than 60 µg/L, but decreased when serum ferritin exceeded this level, and the homeostatic equilibrium point of the system was estimated to be 60 µg ferritin/L (Hallberg *et al*, 1997). This study did not investigate the maximum regulatory capacity by the administration of high levels of additional iron, but such data are available from fortification studies. Fortification of curry powder with sodium-iron-EDTA giving additional intakes of 7.5 mg Fe/day for 2 years in iron-replete male subjects (Ballot *et al*, 1989) did not increase serum ferritin levels. There were no changes in iron stores as estimated by serum ferritin following the addition of 10 mg Fe/day as ferrous sulphate to the food of a healthy male subject for 500 days, or as determined via serial phlebotomies after the end of the iron substitution period (Sayers *et al*, 1994). In these two studies serum ferritin levels did not change despite long-term challenge with 7.5-10 mg Fe/day in addition to normal dietary iron intake of approx. 10 mg Fe/day. Iron status in the elderly, as indicated by elevated serum ferritin levels, was increased after intake of an additional 30 mg Fe/day as supplements (Fleming *et al*, 2002), in a study in which subjects with abnormal results for blood leukocytes, C-reactive protein (CRP), and 3 liver enzymes, as indicators of inflammation and liver diseases, were excluded. Theoretical calculations of the accumulation of iron in a fertile woman given different daily intakes (Borch-Iohnsen and Petersson Grawe, 1995) indicated that a daily intake of 60 mg for 5 years would lead to a serum ferritin value close to that seen in iron overload.

2.2.2. Iron excretion

Iron excretion via the kidneys is very low, and body iron is highly conserved. Renal elimination is not controlled as part of iron homeostasis or the control of excess body stores. Normally, only about 0.1 mg is lost daily in urine. The sloughing of mucosal enterocytes results in elimination of absorbed iron before it reaches the systemic circulation and accounts for the loss of 0.6mg per day into the intestinal

lumen. About 0.2-0.3 mg is lost daily from the skin. The total daily loss is equivalent to about 0.05 % of body iron content (Green *et al*, 1968). Menstrual losses are variable and may be almost as high as the total loss in non-menstruating women (FNB, 2001).

2.2.3. Biomarkers for the characterisation of iron status

Approximately 70% of body iron content is present in haemoglobin (Hb) and the Hb concentration closely reflects the amount of iron utilised in the organism. Serum iron concentration, total iron binding capacity (TIBC), serum transferrin and transferrin saturation may be used to indicate the level of iron supply to the tissues. Because serum transferrin binds iron very tightly, TIBC is essentially a measure of the amount of serum transferrin. Transferrin saturation gives the percentage of TIBC that is occupied by serum iron. Serum transferrin and TIBC are increased in iron-deficiency, during infancy and in pregnancy.

Ferritin binds and sequesters intracellular iron which is not utilised in the functional iron pool. Small amounts of ferritin are also found in the serum and can be used as an indicator of body iron stores (Halliday *et al*, 1994). Changes in serum ferritin correlate with changes in iron-stores (Skikne *et al*, 1990). Because of this reproducible relationship, ferritin is regarded as the most reliable indirect measure of iron stores over a wide range (Finch, 1994), and 1 µg ferritin/L in the serum represents 8-10 mg of storage iron (Walters *et al*, 1973; Finch *et al*, 1986).

Although it is an indicator for body iron stores serum ferritin concentration is influenced by other factors, such as the presence of inflammation or cancer (Fodinger and Sunder-Plassmann, 1999). Therefore, epidemiological studies on the relationship of serum ferritin and chronic diseases (see later) need to determine parameters for inflammation along with the ferritin values in order to exclude inflammation as a cause of elevated serum ferritin levels. Moreover, serum ferritin showed associations with a variety of parameters (Milman and Kirchhoff, 1999), such as serum fibrinogen and albumin (Danesh *et al*, 1998). The correlation between serum ferritin and alcohol consumption may partly be due to higher iron absorption, liver damage (Milman and Kirchhoff, 1996) or to an independent effect of alcohol (Meyer *et al*, 1984). Significant positive correlations have been reported between serum ferritin and a number of cardiovascular risk factors including body mass index, serum triglycerides and systolic and diastolic blood pressures; there was no association between serum ferritin and physical activity, serum total cholesterol or serum HDL cholesterol, but a negative association with cigarette smoking (Halle *et al*, 1997; Milman and Kirchhof, 1999). A negative association reported between serum ferritin and aspirin consumption may have arisen from increased occult blood losses and/or suppression of cytokine-mediated inflammation (Fleming *et al*, 2001a). There are also correlations between serum ferritin and high income and nature of employment, but not with social class (Milman and Kirchhof, 1996 and 1999). In light of the above there is a need for caution in the interpretation of associations of ferritin with chronic disease.

The serum concentration of the transferrin receptor (TfR) represents the extent to which this receptor is expressed in different parts of the body; approximately 80% of serum TfR derives from the erythroid marrow (Cook, 1999). Elevated serum TfR is directly proportional to the severity of iron storage depletion, as determined by serial phlebotomies (Skikne *et al*, 1990).

2.3. Requirements and recommended intakes

The recommended daily intakes for different groups of the population are based on the amount of ingested iron necessary for absorption of the estimated average amounts of iron lost each day.

During the first year of life the body requires approximately 260 mg of iron for metabolism and growth, i.e. 0.6-0.8 mg Fe/day, which corresponds to a dietary intake of 6-8 mg Fe/day, assuming 10% absorption. These data are the rationale for the recommendation of 1 mg Fe/kg body weight per day for children between the 4th month and the 3rd year of life (Oski, 1993). The Scientific Committee on Food recommended daily intakes of 6 mg and 4 mg for infants aged 0.5-1 year and 1-3 years respectively, assuming 15% absorption of the daily intake (SCF, 1993).

An adult male loses approx. 1 mg Fe/day, mostly from the intestine (Green *et al*, 1968), and a daily intake of approx. 10 mg Fe is needed to replace these basal losses, assuming 10% absorption, and the recommended dietary iron intake has been estimated as between 8 and 10 mg Fe/day (SCF, 1993; FNB, 2001; Arbeitsgruppe Referenzwerte für Nährstoffzufuhr, 2000).

Menstrual iron losses are below 1.6 mg Fe/day in 95% of women, which leads to an average total loss of approx. 2.5 mg Fe/day (Baynes and Bothwell, 1990). Assuming 10-20% absorption in iron

deficiency, an intake of 15-20 mg Fe/day is recommended for women of reproductive age (SCF, 1993; FNB, 2001; Arbeitsgruppe Referenzwerte für Nährstoffzufuhr, 2000).

During pregnancy, 450 mg Fe is needed to allow increased erythropoiesis, while 270-300 mg and 50-90 mg are transferred to the foetus and placenta, which gives a total extra demand of 770-840 mg. This demand corresponds to approx. 3 mg Fe/day and will be provided by an intake of 30 mg Fe/day and is the rationale for the recommended higher iron intake in pregnancy (FNB, 2001; Arbeitsgruppe Referenzwerte für Nährstoffzufuhr, 2000).

2.4. Food sources and intake

The iron content of food varies greatly, and factors such as the soil, climate conditions and processing can influence the iron content of similar foods. The results can also be affected by differences in analytical methods (Becker 1996). Foods rich in total iron include liver and offal, game and beef; cereals, cereal products and pulses also contain moderate to high levels. Poor sources of iron include milk and dairy products, whereas pork, poultry, and green vegetables contain intermediate concentrations.

Average iron intakes for populations in 8 European countries are given in Table 1. The recommended intakes for males are met by the mean values and exceeded more than two-fold at the 97.5th percentile.

3. HAZARD IDENTIFICATION

3.1. In vitro and animal toxicity data

3.1.1. In vitro data

Ferrous lactate, ferric pyrophosphate, ferric orthophosphate and sodium ferric pyrophosphate were negative in *Saccharomyces cerevisiae* strain D4 and *Salmonella typhimurium* strains TA1535, TA1537 and TA1538 without metabolic activation in plate- and suspension-tests. Ferrous sulphate was active in suspension tests with metabolic activation and ferrous gluconate was mutagenic for the indicator strain TA1538 in activation tests with primate liver preparations, but was inactive in other tests (Litton Bionetics, 1975, 1976 a and b). Iron in the presence of haemoglobin induces DNA strand breaks and oxidised bases in the human colon tumor cell line HT 29, clone 19A, as determined by single cell microelectrophoresis (comet assay) (Glei *et al*, 2002). The concentrations used in this study were similar to those found in human faeces after oral supplementation of 19 mg Fe/day (Lund *et al*, 1999).

Table 1. The daily intakes of iron in EU countries (mg/day)

	Population	N	Method	Supplements	Mean	97.5%
Austria[a]	Individual (M,F)	2488	24 h recall	Not defined	13	25
Germany[b]	Individual (M) Individual (F)	1763 2267	Computer-assisted dietary interview	Not defined	14-19 12-14	20-27 16-20
Ireland[c]	Individual (M) Individual (F) Individual (M) Individual (F)	662 717 662 717	7-day record	- - + +	14 10 14 14	26 19 29 72
Italy[d]	Household	2734	7-day record	+	13	22
Netherlands[e]	Individual (M,F)	5958	2-day record	-	11	19
Norway[f]	Individual (M) Individual (F) Individual (M) Individual (F)	1298 1374 1298 1374	Quantitative food frequency questionnaire	- - + +	12 10 13 12	23 17 27 27
Sweden[g]	Individual (M) Individual (F) Individual (M) Individual (F)	1897 2223 770 1655	8-day record	- - + +	17 13 22 18	29 21 41 37
UK[h]	Individual (M) Individual (F) Individual (M) Individual (F)	833 891 833 891	7-day record	- - + +	13 10 14 12	23 18 28 27

[a] Elmadfa *et al* (1998)
[b] Mensink *et al* (2002) - values are 90th percentile.
[c] IUNA (2001)
[d] Turrini (INRAN)
[e] Hulshof and Kruizinga (1999)
[f] Johansson and Solvoll (1999)
[g] Elmstahl *et al* (1994) - values are the median and 95th percentile. These data relate to a period when sifted flour was fortified with iron; this ceased in 1995, since when intakes from food have been on average 2-3mg/day lower.
[h] Henderson *et al* (2003) - values are the mean for total iron

3.1.2. Animal toxicity data

A problem with the use of animal data for the hazard characterisation of iron is that there are large species and strain differences in response to dietary iron overload, e.g. between rats and mice and between different mouse strains (Whittaker *et al*, 1997; Lebeau *et al*, 2002). Animal models can be useful in some circumstances, for example hepatic iron accumulation is significantly increased in HFE knockout mice (a model for hereditary haemochromatosis) (Fleming *et al*, 2001b).

Acute toxicity occurred in mice after oral doses of ferrous compounds in the range 200-650 mg Fe/kg body weight, with ferrous sulphate being the most toxic and ferrous fumarate the least toxic. Administration of 50 and 100 mg Fe/kg body weight/day for 12 weeks decreased growth rates in male rats with potency in the order ferrous sulphate > succinate > fumarate > gluconate. An emetic effect was found in cats and an irritant effect on the gastric mucosa in rabbits (Berenbaum *et al*, 1960).

The presence of iron in the lumen of the colon may act as a catalyst for the production of free radicals by bacteria (Babbs, 1990). Feeding diets containing 29 mg or 102 mg Fe/kg dry weight of the diet increased free radical generation and lipid peroxidation in rat colon and caecum, and administration of 100 mg Fe/kg diet caused a small but significant increase in cell proliferation in the rat colon (Lund *et al*, 1998). Iron acts as a tumour promotor in the mouse colon after exposure to the genotoxic carcinogens dimethylhydralazine or azoxymethane (Siegers *et al*, 1988). Dietary phytate, which forms a stable ligand complex with iron, prevented the promotion of colon carcinogenesis in experimental animals (Nelson *et al*, 1989), and protects the pig colon from lipid peroxidation caused by high iron intakes (Porres *et al*, 1999).

Oxidative damage of DNA is believed to be the cause of renal adenocarcinoma in rats treated with an intraperitoneal dose of the iron chelate of nitrilotriacetic acid (Ebina *et al*, 1986; Hamazaki *et al*, 1989a). The induction of renal cancer was associated with lipid peroxidation (Hamazaki *et al*, 1989b) and this could be reduced significantly by vitamin E administration (Hamazaki *et al*, 1988). The relevance of these observations to normal dietary forms of iron is unclear, but the observations indicate that administration of synthetic chemical forms of iron may lead to redox-related damage.

3.2. Human toxicity

3.2.1. Acute effects of iron

Acute poisoning with oral pharmaceutical-like iron preparations causes mucosal erosion in the stomach and intestine, with young children particularly at risk (Anderson, 1994). Side effects of oral iron preparations and acute iron toxicity may be mediated directly by the presence of high concentrations of 'free iron' in the gastrointestinal mucosa (Engle *et al*, 1987). Absorption of high quantities of iron causes shock symptoms due to vasodilatation, capillary leakage and heart failure, and can result in CNS and kidney damage (Anderson, 1994), and hepatocellular necrosis with bleeding disorders and hepatic failure (Engle *et al*, 1987).

Side effects of oral iron preparations at therapeutic dose levels of 50-220 mg Fe/day include nausea, vomiting, heartburn, epigastric discomfort, diarrhoea and intractable constipation (Blot *et al*, 1981; Brock *et al*, 1985; Coplin *et al*, 1991; Frykman *et al*, 1994; Ganzoni *et al*, 1974; Hallberg *et al*, 1966; Liguori, 1993; Reddaiah *et al*, 1989). The side effects in the upper gastrointestinal tract depend on the local iron concentrations and are due to irritation of the mucosa, alteration of gastrointestinal motility and/or rapid transfer of iron into the circulation (Cook *et al*, 1990).

3.2.2. Chronic effects of iron

Information about the chronic effects of iron predominantly found in adults can be divided into:

i. adverse effects reported in subjects with iron overload, and

ii. associations reported between increased iron intakes or elevated markers of body stores and specific diseases.

3.2.2.1. Adverse effects reported in subjects with iron overload

The main causes of chronic iron overload are:

i. genetic defects in the mechanisms involved in iron homeostasis, of which the most important is hereditary haemochromatosis;

ii. excessive dietary intake associated with ethanol ingestion; and

iii. iatrogenic excessive intake, for example for the treatment of haemoglobinopathies.

3.2.2.1.1. Hereditary haemochromatosis

a. Homozygotes for hereditary haemochromatosis

Homozygotes for hereditary haemochromatosis are a sub-population that is highly sensitive to iron overload. This disease is due to a defect in the HFE (formerly HLA) gene that results in increased iron absorption, and has been reported to occur in 0.3-0.5% of the Caucasian population (Powell *et al*, 1994). Recent studies on the genetic basis of haemochromatosis have identified five iron-overload conditions that are described as haemochromatosis (OMIM, 2004). There are three important HFE mutations, C282Y, H63D and S65C, of which the H63D variant is more common than the C282Y mutation in New Zealand and the USA (Burt *et al*, 1998; Steinberg *et al*, 2001). The distributions of these two mutations differ within Europe, C282Y being limited to those of north-western European ancestry, and H63D being found at allele frequencies of more than 5% in countries bordering the Mediterranean, in the Middle East, and in the Indian subcontinent (Rochette *et al*, 1999). Milman and Pedersen (2003) linked the distribution of the C282Y mutation to the spread of the Vikings, since the highest frequencies (5.1 to 9.7%) are in populations in the northern part of Europe, such as Denmark, Norway, Sweden, Faroe Islands and Iceland, intermediate allele frequencies (3.1 to 4.8%) are found in central Europe, and low allele frequencies (0 to 3.1%) occur in populations in southern Europe and the Mediterranean.

The male/female ratio among homozygotes is 1:1, but males are at greater risk of serious manifestations (Olynyk and Bacon, 1994), because women have higher iron losses via menstruation. Disorders associated with chronic iron overload include hepatomegaly, joint inflammation, diabetes mellitus, cardiomyopathy and hepatoma. Although there have been conflicting reports, recent data show that subjects homozygous for C282Y show increased incidences of arthritis and liver fibrosis (Bulaj *et al*, 2000; Ajioka and Kushner, 2003), although many homozygous subjects appear asymptomatic (Asberg *et al*, 2001; Beutler, 2003). Mutation S65C appears to result in a mild to moderate influence on iron uptake and accumulation (Holmström *et al*, 2002). The penetrance of the C282Y mutation, which is the most frequent mutation causing hereditary haemochromatosis, has recently been estimated to be in the region of 1% (Beutler *et al*, 2002) to 25% (European Haemochromatosis Consortium, 2002). A recent paper indicates that the apparent low penetrance may be due to the non-specific nature of early symptoms because about 50% of subjects homozygous for C282Y had biochemical indicators of iron overload (as defined by a transferrin saturation >52% combined with a serum ferritin >300 µg/L for men and >200 µg/L for women (Ryan *et al*, 2002).

b. Heterozygotes for hereditary haemochromatosis

The frequency of heterozygotes in Caucasians is estimated to be 13% (9.5-18%) (Nelson *et al*, 2001). In recent screening studies 5-15% were heterozygous for the HFE gene mutation C282Y (Burt *et al*, 1998; Distante *et al*, 1999; Rochette *et al*, 1999; Steinberg *et al*, 2001; Moirand *et al*, 2002; Milman and Pedersen, 2003) and 5% of patients with suspected iron overload had the S65C mutation (Holmström *et al*, 2002). Heterozygous subjects tend to show a greater incidence of biochemical indicators of high body loads of iron, such as serum ferritin, transferrin and transferrin saturation. However, iron absorption does not appear to be altered in heterozygotes (Hunt and Zeng, 2004). Overall there is inconsistent evidence of an increased risk of adverse effects associated with iron overload in heterozygous subjects (see below).

3.2.2.1.2. Secondary haemochromatosis

There have been rare anecdotal cases of patients who developed secondary haemochromatosis and died of cirrhosis, diabetes or cardiac failure after ingesting 160-1200 mg/day of therapeutic doses of iron over more than a decade (see Turnberg, 1965; Johnson, 1968; Green *et al*, 1989). Subjects with hereditary haemochromatosis may have contributed to these early case reports.

3.2.2.1.3. Bantu siderosis

Bantu siderosis is caused by excess oral iron intake with home-brewed beer fermented in iron drums in sub-Saharan Africa. Regular consumption of such beer leads to hepatic cirrhosis (Isaacson *et al*, 1961) and diabetes (Seftel *et al*, 1961). The bioavailability of such iron is high, but the alcohol intake with the beer may have contributed to the development of cirrhosis (Tsukamoto *et al*, 1995). A genetic difference, distinct from HFE-linked haemochromatosis, has been hypothesised to increase intestinal iron absorption in affected Bantu subjects (Gordeuk *et al* 1992; Mc Namara *et al*, 1998). This possibility was supported by analyses using transferrin saturation (Moyo *et al*, 1998), although the blood samples were taken from non-fasting individuals at different times of the day which makes transferrin saturation values unreliable. Iron absorption in affected Bantu subjects, who had high iron stores, was lower than that in healthy white volunteers, which argues against a genetic alteration of iron absorption and its regulation (Bothwell *et al*, 1964). Despite the presence of possible genetic differences and alcohol intake as confounders, iron is clearly the underlying causative agent of liver cirrhosis in Bantu siderosis.

3.2.2.1.4. Serum ferritin, liver iron and risk of liver fibrosis

There is a significant positive correlation between serum ferritin and liver iron concentrations (Asberg *et al*, 2001) and biochemical indicators of iron overload (a serum ferritin >300 µg/L for men and >200 µg/L for women) are equivalent to liver iron concentrations of about 158 µmol/g protein, although there was very wide variability in the relationship. Severe hepatic fibrosis (cirrhosis) is associated with hepatic iron concentrations above 400 µmol Fe/g (22.3 mg per g) dry weight (Bassett *et al*, 1986). It is difficult to relate serum ferritin to the risk of liver disease. Different degrees of fibrosis were found in 32 patients in a survey of 120 patients with haemochromatosis (Bell *et al*, 2000); the median serum ferritin levels in patients with grade 1 (n=8), grade 2 (n=12), grade 3 (n=8) and grade 4 (cirrhosis; n=5) liver disease were 858, 1441, 2764 and 4094 µg/L respectively, although there was considerable overlap between the groups, so that a clear cut-off cannot be defined.

3.2.2.2. Associations reported between increased iron intakes or elevated markers of body stores and specific diseases

3.2.2.2.1. Iron and cancer risk

Because of its potential pro-oxidant effects, there has been extensive research into possible links between iron and cancer development. Homozygotes for hereditary haemochromatosis with hepatic cirrhosis have an increased risk for hepatocellular carcinoma (Powell, 1970), and this association is supported by an increased risk in Black Africans with dietary iron-overload, after controlling for alcohol, hepatitis B and C and exposure to aflatoxin B1 (Mandishona *et al*, 1998). Evidence for a possibile increased incidence of extrahepatic malignancies in hereditary haemochromatosis is inconsistent (see Niederau *et al*, 1985; Hsing *et al*, 1995; Fracanzani *et al*, 2001).

The incidences of tumours in the upper digestive tract (Freng *et al*, 1998; Zhang *et al*, 1997) have been reported to be negatively associated with dietary iron intake.

Evidence supporting a link between high iron stores/intakes and cancer of the lower bowel includes a significant association between the recurrence of colorectal adenoma after their removal and serum ferritin concentrations, dietary iron intake and meat intake (Tseng *et al*, 2000). A case-control study from Switzerland showed that the risk of colorectal cancer was positively associated with iron intake (Levi *et al*, 2000). In agreement with these studies a nested case-control study on 105 cases with colorectal cancer and 523 controls with a follow up of 4.7 years showed a significantly increased risk for colorectal cancer associated with higher total iron intake (Kato *et al*, 1999). In another case-control study with 433 cases of rectal adenocarcinoma, iron intake normalised for meat consumption was associated with an added risk ratio of 3.2 (Deneo-Pellegrini *et al*, 1999).

A number of studies have indicated an association between markers of body iron stores and cancer of the lower bowel. In the general population Stevens *et al* (1988 and 1994) found an increased frequency of cancer in the oesophagus, the bladder, and in the colorectal area at transferrin saturation levels above 40%. In one study, the risk of colorectal cancer correlated with increasing iron stores (Knekt *et*

al, 1994), while in a different study serum iron concentration was significantly increased in individuals with colorectal cancer (Wurzelmann *et al*, 1996), although the differences were small. A case-control study of 264 men and 98 women, which was controlled for smoking, gender and alcohol consumption, showed an association between serum ferritin concentration and adenoma of the colon (Nelson *et al*, 1994). The relative risk for colorectal cancer, colonic adenoma and haematological malignancies was increased in heterozygotes for hereditary haemochromatosis; the risk for stomach cancer was also increased in this study (Nelson *et al*, 1995), which is inconsistent with an association with low iron stores in other studies (Knekt *et al*, 1994).

Hepatocellular carcinoma is a recognised risk for subjects homozygous for hereditary haemochromatosis, and is responsible for about one-third of deaths in affected homozygotes (OMIM, 2004). Hepatocellular carcinoma occurs in other situations associated with hepatic degenerative and fibrotic changes, such as alcohol-induced hepatic cirrhosis.

Overall, these results indicate the possibility of a role of luminal exposure to excessive iron in the development of colon carcinoma, but the evidence is limited and not convincing. There are few data available for other cancers and the evidence is not convincing.

3.2.2.2.2. Iron and cardiovascular risk

a. Iron stores and cardiovascular risk

In a study on risk factors for ischemic heart disease, in which 51 out of 1931 men experienced myocardial infarction (AMI), a serum ferritin concentration >200 µg/L was associated with a 2.2 fold higher risk for AMI, after adjusting for cigarette smoking, systolic blood pressure, blood glucose, HDL-cholesterol, apolipoprotein, triglycerides and leukocyte (Salonen *et al*, 1992 and 1994), with the strongest association in those with high LDL cholesterol. The study was criticised because there was not adequate control of effects of inflammation on serum ferritin and because the relationship should have been analysed in tertiles and not with a cut-off value of 200 µg/L ferritin which is in the high range of normal values (FNB, 2001; Vera-Schriftenreihe, 1995; Claeys *et al*, 2002).

Following this study, a considerable number of epidemiological investigations were performed between 1994 and 1997, involving a total of 7800 cases of coronary heart disease (CHD). A meta-analysis undertaken by Danesh and Appleby (1999) reported a combined risk ratio of 1.0 for the five studies that compared ferritin values below and above 200 µg/L in 570 CHD cases. A combined relative risk ratio of 0.9 was reported for the comparison between top and bottom tertiles for transferrin saturation, which included 6194 cases of CHD in 5 studies. The combined risk ratios were 1.0, 0.8, and 0.8 for total iron-binding capacity, serum iron and total dietary iron intake. All of these findings show the absence of a relationship between iron stores and cardiovascular risk, although the control for confounders, such as liver disease, inflammation and cancer, was insufficient in some of the studies (Sullivan, 1999; Kiechel *et al*, 1997).

The data from the NHANES II study (1976-1980) have been analysed for the risk for AMI, with an average follow-up of about 14 years, in relation to iron status as determined by serum-ferritin concentrations (Sempos *et al*, 2000). Cholesterol, blood pressure, BMI, diabetes and smoking were included as confounders, but the analysis did not control for inflammation. There was a U-shaped distribution of risk increment, with increased risk for white women and black males with the lowest and highest iron status, but the numbers in these groups were too low and statistical significance was not reached.

A recent prospective study with 177 AMI cases and 89 controls from Switzerland (Claeys *et al*, 2002) showed an increased risk for AMI in subjects with serum ferritin concentrations over 300 µg/L, but statistical significance was lost when the data were adjusted for smoking, hypertension, diabetes, BMI and total cholesterol by multiple regression analysis. The authors concluded that high serum ferritin concentrations appear to be a weak risk factor, but that the major risk factors may correlate with high iron stores thereby complicating the analysis of dependent co-variables.

A nested case-control study with 60 AMI cases and 112 controls (Klipstein-Grobusch *et al*, 1999) analysed subjects more than 55 years of age, with a mean follow up of 4 years. The cohort was chosen to assess risk factors for disabling cardiovascular, neurodegenerative and locomotor diseases. In this study the possible effects of inflammation were controlled using blood sedimentation rate and serum C-reactive protein, while iron status was assessed by serum ferritin, serum iron, serum transferrin concentration and total iron intake. Serum ferritin was not associated with a statistically significant increased risk of AMI, or with the other measures of iron status. Smoking, hypercholesterolaemia and

diabetes were confounders that increased AMI risk, while no association was found for sex, age, BMI and systolic blood pressure.

A prospective, nested, case-control study analysed the risk for heart attacks during 1982-1992 (Toumainen *et al*, 1998). The average follow-up period on 99 male cases of AMI and 98 age-matched controls was 6.4 years, and the study controlled for inflammation and liver diseases by determination of serum C-reactive protein and γ-glutamyl-transferase activity. The relative risks for AMI in the highest and middle tertiles were significantly increased compared to the lowest tertile of iron status, as determined by the TfR/ferritin ratio.

Cardiovascular disease is not part of the recognised syndrome of hereditary haemochromatosis and epidemiological studies investigating the possibility of an increased risk of cardiovascular disease in heterozygous subjects have yielded inconsistent results. Nassar *et al* (1998) reported that higher concentrations of plasma ferritin were associated with a higher risk for early-onset acute myocardial infarction (AMI), but the increased risk for heterozygous subjects was not statistically significant. A prospective cohort study from Finland, which controlled for 13 confounders (Tuomainen *et al*, 1999), reported that the risk of AMI in male heterozygotes for haemochromatosis was nearly twice as high as in controls, and that smoking increased the risk significantly. The findings of a retrospective study (Franco *et al*, 1998) did not support the hypothesis of increased cardiovascular risk because the fraction of heterozygotes with late-onset atherosclerosis was lower than in corresponding controls; however the cases had been selected on the basis of pre-existing atherosclerosis, and the results may have been influenced by a survival bias. Roest *et al* (1999) found nearly twice as many heterozygous women among cardiovascular cases than among controls (7.2% vs. 4.1%), but the difference was most apparent for cerebrovascular death rather than for AMI. A more recent case-control study in middle aged men and women involving 243 cases and 535 controls found a slightly increased risk of coronary heart disease in heterozygotes for C282Y mutation (RR: 1.6; 95% CI, 0.9-2.9). The association was statistically significant (RR 2.70, 95% CI: 1.2-6.1) after adjustment for age, gender, smoking, diabetes, hypertension, LDL and HDL cholesterol and triglycerides although the estimates were based on small numbers subjects in some comparisons (Rasmussen *et al*, 2001). In contrast no association was found between the severity of coronary atherosclerosis and the presence of C282Y and H63D mutations in heterozygotes in a prospective case-control study on 174 patients with angiographically documented stenosis or history of AMI, 187 controls and 142 blood donors (Battiloro *et al*, 2000). Although H63D carriers, but not C282Y carriers, were more numerous among subjects with atherosclerotic plaques in the AXA study (Hetet *et al*, 2001), analysis of the ECTIM and GENIC studies found no significant impact of H63D or C282Y mutations on the risk of myocardial and brain infarction. A prospective case control study from Switzerland with 177 patients surviving AMI found no direct association between haemochromatosis mutations and AMI events (Claeys *et al*, 2002). A recent study investigated ferritin levels and C282Y mutations in 546 patients with angiographically confirmed severe coronary atherosclerotic disease and 303 individuals mostly with valvular disease, who were shown angiographically to be free of coronary atherosclerosis (Bozzini *et al*, 2002). There was no difference in serum ferritin levels after adjustment for C-reactive protein and sex, and the incidences of heterozygotes for C282Y were 4.8% in those with severe coronary atherosclerotic disease and 6.6% in those without coronary atherosclerosis. The authors concluded that their data do not support a role for iron stores as predictors of coronary atherosclerotic disease.

Most epidemiological studies have correlated the iron-related health risk directly to the cumulative iron stores, the determination of which relies on proxy parameters. Some of these parameters, such as transferrin saturation and serum iron, represent the turnover of the haemoglobin iron pool rather than the size of iron stores (Bothwell *et al*, 1979). These parameters together with the TIBC showed a low correlation to cardiovascular risk (Klipstein-Grobusch *et al*, 1999). Serum ferritin levels represent iron stores more directly, but this parameter may be confounded by inflammatory processes and other influences. Therefore, the better controlled epidemiological studies used parameters, such as serum C-reactive protein, blood sedimentation rate or liver enzymes, to control for inflammation (Klipstein-Grobusch *et al*, 1999). Serum TfR is less confounded by inflammation, and although this parameter responds better to deficiency states than to overload, it can be used to control increased ferritin values for iron deficiency (see Tuomainen *et al*, 1998). The studies of Tuomainen *et al* (1998) and Klipstein-Grobusch *et al* (1999) appear to be the best controlled, and these do not show a clear or consistent relationship between iron status and cardiovascular risk.

Overall, epidemiological data on associations between iron status and increased risks of cardiovascular disease are contradictory and unconvincing at the present time.

b. Iron intake and cardiovascular risk

Data on correlations between dietary iron intake and the risk of AMI are difficult to interpret. In dietary studies it is difficult to define the contribution of iron intake *per se* to any reported associations, because iron intake, especially haem iron, usually correlates with the increased intakes of other important dietary risk factors.

Dietary iron intake, estimated by a 4-day food recording, was associated with a 5-8% increase in the risk of AMI for each milligram of daily iron intake (Salonen *et al*, 1992; Tuomainen *et al*, 1998), but there must be doubts about the validity of a short-term diary to represent long-term intake and the contributions of confounding dietary factors. Assessment of total habitual dietary iron intake during the previous year showed no significant variations in coronary mortality in quintiles of dietary iron intake (Reunanen *et al*, 1995). There was no association between total iron and the risk for AMI in a study that used a semiquantitative food questionnaire to assess habitual food intake over the past year, although haem intake, estimated as 40% of total iron intake, was significantly associated with myocardial risk (Klipstein-Grobusch *et al*, 1999). Results from a food frequency questionnaire in a US study showed a decreased risk for AMI at high total iron intake, but a significantly increased the risk of AMI at high haem iron intakes (Ascherio *et al*, 1994). Such studies do not provide convincing evidence of a causal relationship between iron intake and risk of AMI.

If iron-related oxidative stress is a contributory factor for increased cardiovascular risk, the overall risk will depend also on other pro-oxidative factors and the strength of antioxidative defence mechanisms. Variations in such interfering factors may have contributed to the inconsistent outcomes of the different epidemiological trials on iron and cardiovascular risk, and could either have masked a real effect or generated a spurious apparent effect. Overall the epidemiological data on iron and cardiovascular risk remain controversial (Schümann *et al*, 2002) and there is no consistent evidence on this topic.

3.2.2.2.3. Iron and risk of type II diabetes mellitus

Recent studies have reported a positive association between serum ferritin concentration, haem iron intake from red meat sources and the risk of developing type II diabetes mellitus (Jiang *et al*, 2004a and 2004b). One study was a prospective nested case-control study within the Nurses' Health Study. From the cohort of 32826 women, 698 developed diabetes mellitus during 10 years of follow-up (Jiang *et al*, 2004a). A logistic regression analysis of the data corrected for potential confounding factors showed that there was an increasing risk across the quintiles of ferritin concentration, which was not altered by correction for markers of inflammation. The second study was a prospective cohort study among American health professionals (40-75 years old males; n=38.394) (Jiang *et al*, 2004b). After adjustment for age, BMI and other diabetes risk factors, intake of haem iron from red meat sources, but not total iron intake, was found to be associated with increased diabetes risk across the quintiles of haem iron intake (RR=1.63; P for trend <0.001). These studies do not provide convincing evidence of a causal relationship between iron intake or stores and type II diabetes.

4. DOSE-RESPONSE ASSESSMENT

4.1. Acute oral iron intoxication

There have been numerous reports of accidental poisoning with medicinal iron, especially in young children (FNB, 2001). An acute oral dose of 60 mg Fe/kg body weight can be lethal; characteristically poisoned subjects initially show nausea, vomiting and lethargy or coma, then an asymptomatic period for up to 24 hours, which is followed by gastrointestinal perforation, coma, convulsions, cardiovascular collapse and hepatic and renal failure (Merrill *et al*, 2001). Oral doses below about 10-20 mg Fe/kg body weight do not cause acute systemic toxicity (FNB, 2001).

4.2. Gastrointestinal effects of oral iron preparations

The side effects of oral iron preparations increase with increase in dosage, but there are fewer side effects with slow delivery systems or if the iron is taken with food (Brock *et al*, 1985; Reddaiah *et al*, 1989). The adverse gastrointestinal effects are related to the concentration of iron in the intestinal lumen (Cook *et al*, 1990).

A daily dose of 50 mg of iron produced a higher incidence of gastrointestinal effects in subjects given conventional ferrous sulphate compared with subjects given the same amount in a wax-matrix (Brock *et al*, 1985), and also in subjects given ferrous sulphate compared with subjects given the same amount

of iron as bis-glycino iron (Coplin *et al*, 1991). Neither of these studies included a placebo group, and therefore the association with iron is based on different responses to different preparations. A higher incidence of side effects was reported in subjects given 60 mg of iron as iron fumarate daily compared with placebo; daily doses of 120 mg of iron as fumarate for 8 weeks given to 19 young women in a double blind cross-over study resulted in gastrointestinal effects in 5 subjects while receiving iron, compared with 2 while taking placebo (Frykman *et al*, 1994).

Overall a dose level of 50 mg Fe/day (Brock *et al*, 1985) or 60 mg Fe/day (Frykman *et al*, 1994) appears to be the LOAEL for transient gastrointestinal side effects in humans. This hazard applies to supplemental forms of non-haem iron.

4.3. Iron overload

Iron overload with clinical symptoms has been found in adult subjects homozygous for hereditary haemochromatosis, those under long-term, high-dose medical treatment with iron, and those given repeated blood transfusions.

A prolonged intake of 160-1200 mg Fe/day in the form of a highly absorbable therapeutic iron preparation (Green *et al*, 1989) has been shown to cause secondary haemochromatosis and may result in death. The reports are scarce and anecdotal, and the possible contribution of hereditary haemochromatosis was not excluded in the older reports.

The point at which an elevated serum ferritin level becomes associated with an increased risk of adverse effects is not known, although serum ferritin values above 200 µg/L for women and 300 µg/L for men have been considered as indicative of biochemical iron overload (Holmström *et al*, 2002). Data for a small number of patients with haemochromatosis suggest that grade 1 hepatic fibrosis may develop at ferritin levels of above approximately 500 µg/L (Bell *et al*, 2000). Although a proportion of the population have serum ferritin levels indicative of biochemical iron overload, the risk of symptomatic clinical iron overload in the general population, including those heterozygous for hereditary haemochromatosis, is considered to be low.

Serum ferritin levels were not increased by fortification giving an additional intake of 7.5 mg Fe/day for 2 years in iron-replete male subjects (Ballot *et al*, 1989) or following the addition of 10 mg Fe/day as ferrous sulphate to the food of a healthy male subject for 500 days (Sayers *et al*, 1994). These studies indicate no effects of 7.5-10 mg Fe/day in addition to normal dietary iron intake of approximately 10 mg Fe/day. Serum ferritin was increased in the elderly after intake of an additional 30 mg Fe/day or more as supplements (Fleming *et al*, 2002). Theoretical calculations of the accumulation of iron in a fertile woman given different daily intakes (Borch-Iohnsen and Petersson Grawe, 1995) indicated that a daily intake of 60 mg for 5 years could lead to a serum ferritin value close to that seen in iron overload. Similar calculations were not made for iron replete males.

Chronic excess intake of highly available iron with beer was observed in Bantu siderosis, with intake levels between 50-100 mg Fe/day associated with liver cirrhosis and diabetes (Bothwell *et al*, 1964). There is little doubt that iron intake was an essential causative factor for this disease. A threshold of 5 mg Fe/g wet weight of liver was established for the development of cirrhosis (Bothwell and Bradlow, 1960), which is almost identical to the threshold for iron-induced cirrhosis in hereditary haemochromatosis (Basset *et al*, 1986). The hepatic and pancreatic damage in Bantu siderosis may be confounded by chronic alcohol intake and possibly by a genetic disorder (see above), and these observations cannot be used as a basis for the derivation of an UL.

Overall there is a poor correlation between iron intake and biochemical indicators of iron status, between biochemical indicators and actual body stores, or between body stores and adverse clinical effects. In consequence, the dose-response relationship between iron intake and the adverse effects of iron accumulation has not been defined adequately.

4.4. Cancer

Studies have reported associations between iron intake, or iron stores, and cancer of the gastrointestinal tract, especially the lower bowel. However, the reported associations do not provide a consistent body of evidence, and do not demonstrate causality between high iron intakes and cancer development.

4.5. Cardiovascular disease and diabetes mellitus

Epidemiological data on associations between iron status and increased risks of cardiovascular disease are contradictory and unconvincing at the present time. Two epidemiology studies have reported an association between indicators of body iron intake or stores and the incidence of diabetes mellitus. These studies do not provide convincing evidence of a causal relationship between iron intake or stores and type II diabetes.

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The Panel considered that the adverse GI effects which have been reported after short-term oral dosage at 50-60 mg daily of supplemental non-haem iron preparations are not a suitable basis to establish an UL for iron from all sources.
An UL cannot be established for iron based on iron overload due to a poor correlation between iron intake and biochemical indicators of iron status, between biochemical indicators and actual body stores, or between body stores and adverse effects.

An UL cannot be established for iron (including haem iron) based on increased risk of chronic diseases such as cardiovascular disease, diabetes and cancer, due to the lack of convincing evidence of a causal relationship between iron intake or stores and chronic diseases.

2. RISK CHARACTERISATION

Adverse gastrointestinal effects (i.e. nausea, epigastric discomfort, constipation) have been reported after short-term oral dosage at 50-60 mg daily of supplemental non-haem iron preparations, particularly if taken without food.

The limited data indicate that supplemental intakes of non-haem iron at levels of 30 mg/day or more (in addition to iron intake from food) can be associated with indicators of high iron stores (e.g elevated serum ferritin) in older adults. However, the point at which an elevated serum ferritin level becomes associated with an increased risk of adverse effects (such as liver fibrosis) is not known. Furthermore, epidemiological associations between high iron intake and/or stores and increased risk of chronic diseases such as cardiovascular disease, type II diabetes and cancer of the gastrointestinal tract are conflicting and do not provide convincing evidence of a causal relationship between iron intake or stores and such chronic diseases.

Based on estimates of current iron intakes in European countries, the risk of adverse effects from high iron intake from food sources, including fortified foods in some countries, but excluding supplements, is considered to be low for the population as a whole. However, intake from food supplements in men and postmenopausal women may increase the proportion of the population likely to develop biochemical indicators of high iron stores. Some groups at special risk for poor iron status, such as menstruating women or children, could benefit from additional iron intake and/or improved availability of dietary iron.

A particularly sensitive subpopulation (up to 0.5% of the population) are homozygotes for hereditary haemochromatosis, who are susceptible to iron overload even at normal dietary iron intakes. Such individuals should avoid iron-supplements and highly iron-fortified foods. The majority of homozygotes are not diagnosed or identified, and they are not aware of their greater susceptibility until sufficient iron has accumulated to produce adverse effects.

3. RECOMMENDATIONS FOR FURTHER WORK

1. Despite the numerous studies that have used biochemical indicators of iron status, there remains a need for data on the relationship between dietary intake of iron, as haem iron, non-haem iron and supplements, and iron status and also between iron status and iron stores in different age groups.

2. The available data on associations between iron status and disease are inconsistent and complex. There is a need for studies defining the relationships between dietary iron intake, as haem iron, non-haem iron and supplements, and diseases such as cancer and cardiovascular disease.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF NICKEL

(REQUEST N° EFSA-Q-2003-018)

(ADOPTED ON 25 JANUARY 2005 BY WRITTEN PROCEDURE)

SUMMARY

The European Food Safety Authority is asked to derive an upper level for the intake of nickel from food that is unlikely to pose a risk of adverse health effects.

Nickel has not been shown to be essential for humans. Orally ingested nickel salts can cause adverse effects on kidneys, spleen, lungs and the myeloid system in experimental animals. Furthermore, perinatal mortality was reported to be increased in the offspring of female rats ingesting nickel salts, even at the lowest administered dose (1.3 mg nickel/kg body weight/day). While there is evidence that inhaled nickel salts are carcinogenic in rodents and humans, orally ingested nickel salts have not been shown to be carcinogenic; however the data presently available are very limited.

Individuals sensitised to nickel through dermal contact and who have allergic contact dermatitis (estimated to be up to 15% of women but frequently undiagnosed) develop hand eczema from oral, as well as dermal, exposure to nickel salts. Oral intakes of nickel as low as about 500 μg/day (about 8 μg/kg body weight/day) have been reported to aggravate hand eczema in nickel sensitised subjects.

In the absence of adequate dose-response data for these effects, it is not possible to establish a tolerable upper intake level.

The intake of nickel from the average diet is estimated to be about 150 μg/day (about 2.5 μg/kg body weight/day), but may reach 900 μg/day (about 15 μg/kg body weight/day) or more, when large amounts of food items with high nickel contents are consumed. In addition, first-run drinking water, which may contain up to 1000 μg/L, and leaching from kitchen utensils into food may also contribute to nickel intake. Intakes of 150 and 900 μg/day are about 500 and 90-fold lower, respectively, than the lowest dose reported to cause adverse effects in rats. Average intakes from food are about one third of the lowest intake reported to aggravate hand eczema in nickel sensitised subjects.

KEY WORDS

Nickel, tolerable upper intake level, food safety.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to

1 - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html)

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Nickel occurs naturally in soil, water, plants and animals. In its compounds, it has normally the valency state of +2, but valency states of 0, +1, +3, and +4 also exist.

2. NUTRITIONAL BACKGROUND

2.1. Food levels and dietary intake

The nickel concentration is highest in cocoa (8.2-12 mg/kg), soya beans (4.7-5.9 mg/kg), oatmeal (0.33-4.8 mg/kg), hazelnuts (0.66-2.3 mg/kg), almonds (1.2-1.3 mg/kg) and legumes. The intake from a Danish average diet was estimated to be 150 μg/person/day, but may reach 900 μg/person/day or more, when large amounts of food items with high nickel contents not included in the Danish average diet are consumed (Flyvholm *et al*, 1984). The nickel content of a typical Swedish diet assessed by analysis of market baskets was on average 82 μg/day (Becker and Kumpulainen, 1991), while it was 115 μg/day (range 70-170 μg/day) in duplicate 7-day diets collected from 15 women in the Stockholm area (Jorhem *et al*, 1998). Other estimates of 100-146 μg/day (Pennington and Jones, 1987) and 20-406 μg/day (Dabeka and McKenzie, 1995) for the average nickel intake by adults are in the same range.

The maximum nickel content in running drinking water from different water works in the greater Copenhagen area was 35 μg/L (Andersen *et al*, 1983). Similarly, maximum concentrations in drinking and mineral water found in Germany were 34 μg/L and 31 μg/L, respectively (Scheller *et al*, 1988). After remaining in the tap for 8 hours or overnight, however, levels of 490 μg/L (Andersen *et al*, 1983) and 1000 μg/L (WHO, 1996) have been reported, respectively.

Release from kitchen utensils can increase the nickel content of food. The average contribution of this source to the oral intake of nickel is unknown, but could augment dietary exposure by as much as 1 mg/day (Grandjean *et al*, 1989, quoted by IARC, 1990).

2.2. Nutritional requirements and recommendations

Nutritional requirements or recommended dietary allowances for nickel have not been established. The SCF stated explicitly that the data were not sufficiently conclusive to justify setting any recommended intakes (SCF, 1993).

2.3. Deficiency

In some species, signs of deficiency have been observed. In rats, nickel deficiency was found to be associated with growth retardation, impaired reproduction function and lower haemoglobin levels. In humans, however, nickel deficiency has not been demonstrated.

3. BIOLOGICAL CONSIDERATIONS

3.1. Function

Nickel is essential for the catalytic activity of some plant and bacterial enzymes. It is said to influence iron absorption and metabolism and the haemopoietic process. However, biochemical functions of nickel have not been demonstrated in humans and higher animals.

3.2. Absorption, distribution, metabolism and excretion

The rate of absorption of nickel salts can be quite high in the fasting state, but is reduced significantly in the presence of food, such as milk, coffee, tea and orange juice (Solomons *et al*, 1982). After an overnight fast, the absorbed nickel averaged 27±17% of a dose of nickel sulphate ingested in water versus 0.7±0.4% of the same dose ingested in food (Sunderman *et al*, 1989). In a study with four healthy adults who were given after an overnight fast a dose of 10 μg/kg body weight of the stable isotope ^{62}Ni, and using ICP-MS, the absorbed amount was 29 to 40% (mean 33.4) of the dose. Plasma nickel levels rose rapidly within 1.5 to 2.5 hours to peak levels of 15 to 20 μg/L and declined by a factor of >10 during the next 3-4 days. Between 51 and 82% of the absorbed amount was excreted in urine over five days, but 34.8 ± 13.4% of the absorbed amount was retained after five days (Patriarca *et al*, 1997). Nickel binds to albumin, histidine and α_2-macroglobulin and is widely distributed in the organism. Transplacental transfer has been demonstrated in rodents. Absorbed nickel is mainly excreted in the urine, but to a minor extent also in bile and sweat. It is secreted into human milk (Heseker, 2000).

3.3. Normal levels in human tissues and fluid

The total nickel content of the human body is estimated to be 0.5 mg (Heseker, 2000). Highest levels in human tissues occur in lungs, thyroid, adrenals, and kidneys with concentrations of 173, 141, 132 and 62 μg/kg dry weight, respectively (Rezuke *et al*, 1987). In non-occupationally exposed men, the mean concentration of nickel in whole blood and serum is in the range of 1-5 μg/L and in urine less than 10 μg/L (ECETOC, 1989).

4. HAZARD IDENTIFICATION

This section focuses on the oral toxicity of nickel compounds and on studies particularly important for the risk assessment of nickel in food. It does not consider data for other routes with the exception of carcinogenicity. Data on hazards of inhalation and dermal exposure including aspects of occupational medicine have been reviewed elsewhere (Coogan *et al*, 1989; ECETOC, 1989; IARC, 1990; WHO, 1991; WHO, 1996).

4.1. Acute toxicity

The acute oral toxicity of nickel compounds depends on their solubility. The soluble nickel chloride and nickel sulphate were found to have LD_{50} values in rats, equivalent to 42-129 mg nickel/kg body weight (ECETOC, 1989).

4.2. Subacute/subchronic toxicity

30 male rats were administered by gavage 25 mg nickel sulphate/kg body weight/day equivalent to 9.5 mg nickel/kg body weight/day over 120 days (10 males as controls). Testis, livers and kidneys were examined histologically and histochemically. In treated animals severe lesions in the germ cells

particularly in spermiogenesis were observed. Changes in liver and kidneys were detected only rarely (Waltschewa *et al*, 1972).

According to Fairhurst and Illing (1987) quoted and reviewed by ECETOC (1989), a number of oral subacute and subchronic studies have been performed with nickel carbonate, chloride, and sulphate mainly in rats. In the most relevant of these studies the following effects were found in rats: (a) decreased body weight gain and slightly lowered haemoglobin levels (nickel chloride in the diet, equivalent to 0 and 20 mg nickel/kg diet, given for 42 days), (b) reduced body weight gain and elevated serum glucose at all dose levels (nickel chloride in drinking water, equivalent to 0, 2.5, 5 und 10 mg nickel/L, given for 28 days) and (c) lack of weight gain and extensive proliferation of lymphoid cells and histiocytes as well as micronecrosis in the intestine at the highest dose level (nickel sulphate given by gavage at doses equivalent to 0, 0.0005, 0.005, 0.05, 0.5 and 5 mg nickel/kg body weight/day for seven months).

Groups of 7 female B6C3F1 mice were exposed to 0, 1, 5, and 10 g nickel sulphate/L drinking water. Calculated from the water consumption, these concentrations were equivalent to about 0, 116, 286, and 396 mg nickel sulphate/kg body weight/day or 0, 44, 109, and 151 mg nickel/kg body weight/day. Water consumption, blood and tissues nickel concentration, body and organ weights, histopathology, immune responses, bone marrow cellularity and proliferation as well as cellular enzyme activities were evaluated. Absolute liver weight was significantly decreased in dosed animals and both the absolute and relative thymus weight was significantly and dose-related reduced, even at the lowest dose. The kidney was the major organ of nickel accumulation. The primary toxic effects were expressed in the myeloid system. There were dose-related decreases in bone marrow cellularity, and in granulocyte macrophage and pluripotent stem-cells proliferative responses (Dieter *et al,* 1988).

Groups of 30 male and 30 female CD rats were administered nickel chloride by gavage at doses of 0, 5, 35, and 100 mg/kg body weight/day for 90 days. In the mid and high dose group, clinical signs of toxicity were seen, body weights and weights of kidney, liver and spleen were reduced and mortality increased. No adverse effects were seen at the dose of 5 mg/kg body weight/day (ABC, 1988).

Groups of 10 Wistar rats of each sex received nickel sulphate in a concentration of 100 mg nickel/L drinking water for 3 and 6 months. The average oral intake was calculated on the basis of drinking water consumption to be 6.9 (males) and 7.6 (females) mg nickel/kg body weight/day. Urinary albumin levels were significantly increased in females exposed for 6 months. The increase observed in males was not significant due to outliers in the controls. No effect was seen on urinary levels of β2-microglobulin or total protein. Kidney weights were significantly increased in the exposed animals (Vyskocil *et al*, 1994).

Groups of 8 male Sprague Dawley rats were given nickel sulphate hexahydrate in drinking water in concentrations of 0, 0.02, 0.05, and 0.1%, i.e. 0, 44.7, 111.75, and 223.5 mg nickel/L for 13 weeks. At the highest dose, final body weight, plasma total proteins, albumin, globulins, glutamic pyruvic transaminase activity and the urine volume were significantly decreased and the lymphocyte subpopulations (T and B cells) suppressed. At the lower dose levels, T and B cells were induced. No gross or microscopic changes were seen in any of the various tissues examined. However, the relative liver weights were significantly decreased in the mid and high dose groups and the relative spleen weights significantly increased in all treated groups as well as the relative kidney weights at both the lowest and highest dose and the relative lung weight at the highest dose. Alkaline phosphatase activity in the bronchoalveolar lavage fluid was significantly decreased at any dose level, indicating a significant decrease in the activity of type II cells in the alveolar space and some early damage to the rat lung (Obone *et al*, 1999).

4.3. Chronic toxicity/Carcinogenicity

The carcinogenicity of nickel and nickel compounds has been assessed by several organisations including IARC (1990, 1999), WHO (1991) and EC (2004).

4.3.1. Animal studies

The data on the carcinogenicity of nickel compounds in experimental animals following oral exposure are very limited. The chronic toxicity and carcinogenicity of nickel sulphate by oral route has been studied in rats and dogs (Ambrose *et al*, 1976). Wistar rats (25 males and 25 females per group) were exposed to 0, 100, 1000 or 2500 mg/kg nickel in feed for 2 years. Growth was significantly depressed at 1000 and 2500 mg/kg; increased heart and decreased liver weights were observed only in females at 1000 and 2500 mg/kg. No neoplasms or other lesions were observed. Beagle dogs (3 males and 3 females per group) were exposed to 0, 100, 1000 or 2500 mg/kg nickel in feed for two years. Growth was significantly depressed and lung lesions were observed at 2500 mg/kg. No neoplasms were observed. Both studies have strong limitations because of the low number of animals (rats and dogs), the high mortality in rats and the limited reporting of the study design and results. The carcinogenicity of nickel acetate has been tested in three drinking water studies with rats and mice (Schroeder *et al*, 1964 and 1974; Schroeder and Mitchener, 1975), receiving 0 or 5 mg/L nickel as nickel acetate from the time of weaning until death. Histological examinations were limited to the lungs, heart, liver, kidneys and spleen. No increased incidence of neoplasms was observed in either rats or mice. Also these studies are strongly limited in the design and in the reported results.

Overall, the available data are too limited for an evaluation of the carcinogenic potential of nickel compounds in rodents following oral administration. According to information supplied by industry (NiPERA) to EC (2004), a two-year oral carcinogenicity study with nickel sulphate is planned to be ready in 2005. No data regarding the carcinogenicity of nickel chloride, nickel nitrate, nickel carbonate and nickel metal by oral route have been found.

Much attention has been directed towards a series of long-term inhalation NTP studies (1996 a, b, c,). Rats and mice were exposed to aerosols of nickel subsulfide, nickel oxide or nickel hexahydrate for 2 years. It was concluded that there was clear evidence of carcinogenic activity of nickel subsulfide based on increased incidences of tumours in the lung and in the adrenal medulla. Another inhalation study (78 weeks) of nickel subsulfide in rats, also reported an increase in the incidence of lung tumours (Ottolenghi *et al*, 1974, quoted by IARC, 1990). No carcinogenic activity was seen in mice. With regard to nickel oxide there was some evidence of carcinogenicity in rats based on increased incidences of tumours in the lung and in the adrenal medulla, with equivocal results in female mice. No carcinogenic activity was seen in rats or mice exposed to nickel sulphate. No studies regarding carcinogenicity of nickel chloride, nickel nitrate and nickel carbonate following inhalation exposure or intratracheal instillation in experimental animals have been located. Injection of various nickel compounds in different ways all have caused malignant tumours, usually sarcomas but also other types, at the site of application (IARC, 1990).

Injection of nickel produces distant tumours of the liver in some strains of mice (IARC, 1990). Intraplacental exposure to nickel acetate followed by exposure of the offspring to the promoter barbital in the drinking water produced renal cortical and pelvic tumours (Diwan *et al*, 1992). Additionally, pituitary tumours (combined adenomas and carcinomas) were significantly increased in offspring of both sexes given prenatal nickel acetate only. The results of this study indicate that nickel acetate is a transplacental initiator of kidney tumours and a complete transplacental carcinogen of pituitary tumours. A variety of carcinogenicity studies indicate that metallic nickel can produce tumours when given by intratracheal instillation, subcutaneous, intramuscular or intraperitoneal injection in rats and hamsters (IARC, 1990).

According to IARC (1999), there is also sufficient evidence in experimental animals for the carcinogenicity of implants of metallic nickel and for nickel alloy powder containing approximately 66-67% nickel, 13-16% chromium and 7% iron.

Three studies in experimental animals indicate a possible promoting effect of nickel sulphate, when applied locally to the nasopharynx or the oral cavity, or by the feed to pups; however,

the indications are rather weak (IARC, 1990; WHO, 1991). In a two-stage carcinogenesis assay, orally administered nickel chloride in drinking water (600 mg/L) for 25 weeks enhanced the renal carcinogenicity of N-ethyl-N-hydroxyethylnitrosamine in rats, but not the hepatocarcinogenicity in rats after initiation with N-nitrosodiethylamine, the gastric carcinogenicity in rats after initiation with N-methyl-N-nitro-N-nitrosoguanidine, the pancreatic carcinogenicity in Syrian golden hamsters following initiation with N-nitroso-bis (2-oxopropyl)-amine, or the skin carcinogenesis in mice initiated with 7, 12-dimethylbenzanthracene (IARC, 1990; WHO, 1991). Nickel metal weakly enhanced the lung carcinogenicity of 20-methylcholanthrene in rats treated by intratracheal instillation (IARC, 1990). A two-stage carcinogenesis study was carried out in which nickel acetate tetrahydrate (single i.p. injection of 5.3 mg/kg body weight) was tested as a tumour initiator in male rats using sodium barbital (500 mg/L in drinking water) as the promoter (Kasprzak *et al*, 1990). Increased incidences of renal cortical adenomas and combined adenomas and adenocarcinomas were observed.

In conclusion, the available data indicate that nickel sulphate, nickel chloride and nickel metal may have a promoting effect in combination with selected initiators. There is also some evidence, again limited, that soluble nickel compounds may act as promoters also by the oral route.

4.3.2. Human data

Several cohort studies of workers exposed by inhalation to various nickel compounds showed an increased risk of lung and nasal cancer (IARC, 1990). Although the precise compound responsible of the carcinogenic effects in humans was not always clear, studies indicated that nickel sulphate and combinations of nickel sulphides and oxides encountered in the nickel refining industry were responsible for cancer in humans. An additional study had shown that exposure of nickel refinery workers to soluble nickel compounds alone or in combination with other forms of nickel caused significant excess risks for lung and nasal cancer and that smoking and nickel exposure had a multiplicative effect (Andersen *et al*, 1996). Nickel exposure in mild-steel welders has been associated with tumours (carcinomas) of the trachea, bronchus and lung in some cases (Simonato, 1991), although subjects were exposed also to chromium, which complicated the results.

4.3.3. Overall conclusion

IARC (1990) made an overall evaluation of nickel compounds as a group (Group 1: Human carcinogens), based on sufficient evidence of epidemiological information, sufficient evidence in experimental animals and on indications from mechanistic and animal studies that the event responsible for inducing cancer is generation of ionic nickel at target sites.

Recently, the European Commission Working Group of Specialized Experts in the fields of carcinogenicity and mutagenicity, has concluded that nickel sulphate, nickel chloride, nickel nitrate and nickel carbonate, should be considered as human carcinogens by inhalation (Carc. Cat.1, with the risk phrase R49 “May cause cancer by inhalation”) (EC, 2004).

The experimental evidence for carcinogenicity of nickel compounds or metallic nickel following oral exposure is lacking; however, the data presently available are very limited. A long-term study with nickel sulphate by oral route, currently being carried out, will provide additional information on which to evaluate this effect. There is also some evidence, although again limited, that soluble nickel compounds may act as promoters by oral route.

4.4. Genotoxicity

The genotoxicity of nickel and nickel compounds has been reviewed by several organisations including ECETOC (1989), IARC (1990), WHO (1991), ATSDR (1997) and EC (2004).

4.4.1. *In vitro studies*

4.4.1.1. *Gene mutation*

Most of the presently available data comes from studies in bacteria with nickel chloride. In general, the nickel compounds tested gave negative results in *Salmonella enterica var.* Typhimurium (nickel chloride, nickel sulphate and nickel nitrate) and *Escherichia coli* (nickel chloride and nickel sulphate). Only a fluctuation test gave a weak positive result with nickel chloride in *Salmonella enterica var.* Typhimurium. The overall evidence indicates that nickel compounds are not mutagenic in bacteria. Both nickel chloride and nickel sulphate have been tested in gene mutation tests with different mammalian cells, many of which with weakly positive results (e.g. in mouse lymphoma and V79 cells). In at least some of these assays, the positive results were likely due to genetic events other than gene mutations (e.g. chromosomal aberrations and DNA methylation). For instance, it has been shown that the increase in mutation frequency at the *gpt* gene of V79 cells (Christie *et al*, 1992) were due to changes in DNA methylation (Klein, 1994). DNA methylation seems to be related to the inhibition of tumour suppressor genes (Costa and Klein, 1999).

4.4.1.2. *Chromosomal effects*

Chromosomal aberrations (CA) have been extensively studied with nickel chloride and nickel sulphate in cultured mammalian cells (IARC, 1990; WHO, 1991). Positive results, although weak, were seen in almost all studies in the range of 0.59-59 mg nickel/L. A weak (1.5 to 2 fold) increase in sister chromatide exchange (SCE) was also detected at concentrations of 14 and 19 mg/L. Positive results were also seen with nickel carbonate (CA and SCE in CHO cells). Induction of chromosomal aberrations were observed also in cultured human lymphocytes with Ni_3S_2 (7.3-73 mg/L), $NiCO_3$ (0.59-59 mg/L) and $NiSO_4$ (1.1 mg/L). Most aberrations were gaps rather than breaks or fragments. Disturbance of spindle function was also seen in rat embryo cells (nickel chloride) and human peripheral lymphocytes (nickel sulphate), with also weak positive results in the micronucleus test (kinetochore stained) in human diploid fibroblasts, suggesting that numerical chromosome changes (e.g. aneuploidies) might occur.

4.4.1.3. *DNA damage and repair*

Most data come from studies with nickel chloride and nickel sulphate (IARC, 1990; WHO, 1991). Both soluble salts induced mitotic gene conversion in yeast, DNA single-strand breaks (SSB) and DNA-protein cross-links (DPC) in cultured mammalian cells. The formation of DPC may involve non-covalent association of DNA and chromatin proteins. SSB are repaired quickly while DPC appear to persist. Nickel ion binds to chromatin more strongly than to DNA. Nickel sulphate induced inhibition of DNA synthesis/repair.

4.4.1.4. *Cell transformation*

Nickel sulphate and nickel chloride have been shown to induce cell transformation in Syrian hamster cells (SHE), BALB 3T3 and C3H10T1/2 mouse cells (IARC, 1990; WHO, 1991).

Soluble nickel (Ni^{2+}) can act as both an initiator and promoter in SHE cells, although it appears to be a more potent promoter than initiator. Nickel chloride (1 mg Ni/L) produced a ten-fold increase in morphological transformation in BALB 3T3 mouse cells. Nickel subsulphide (0.1-7 mg/L), nickel sulphide (0.3-3 mg/L) and nickel oxide (3-23 mg/L) caused dose-dependent increases in the frequency of transformation in C3H10T1/2 mouse cells, while nickel sulphate and nickel chloride (0.03-6 mg/L) were negative. Several studies have measured the transforming potential (as anchorage independance) in human foreskin cells showing positive effects.

Transformation assays do not directly measure genotoxicity *per se*, but it is generally believed that cell transformation involves some form of genotoxicity, including alterations in DNA sequence and expression.

4.4.2. *In vivo studies*

4.4.2.1. Gene mutations

Weakly positive effects have been seen in one study in Drosophila (Wing spot mutation) with nickel chloride (Ogawa *et al*, 1994).

No significant increase in mutation frequency was found in the nasal mucosa or lung cells of transgenic LacZCD2F1 mice or lacI F344 rats following *in vivo* inhalation exposure for two hours to nickel subsulphide (Ni_3S_2) at dose levels close to the MTD (Mayer *et al*, 1998). The results do not support any conclusion regarding the ability of this compound to induce gene mutations *in vivo*, due to the short exposure time, as this model needs 4-6 weeks of exposure for maximal expression of mutations.

4.4.2.2. Chromosomal effects

Most of the studies were carried out with nickel sulphate, chloride and nitrate. Chromosomal aberrations were induced at high doses in bone marrow cells of mice, rats and hamster via oral, intratracheal or intraperitoneal administration (IARC, 1990; IPCS 1991). No increases in SCE were found. The data from micronucleus are conflicting, with negative results (Deknudt and Leonard, 1982; Covance, 2003) and positive results in Indian studies by i.p. (Dhir *et al*, 1991) or by oral gavage (Sharma *et al*, 1987; Sobti and Gill, 1989). Nickel metal was positive in rat bone marrow cells by intratracheal administration (Zhong *et al*, 1990). No significant increase of dominant lethal mutations were reported (Deknudt and Leonard, 1982; Saichenko, 1985). A significant increase in sperm abnormalities was shown by Sobti and Gill (1989).

4.4.2.3. DNA damage and repair

There is evidence that both soluble and insoluble nickel compounds can produce DNA single strand breaks and DNA-protein cross-links *in vivo* in rat liver cells (IARC, 1990). An inhalation study in mice and in rats by Benson *et al* (2002) has shown DNA single strand breaks in the comet assay after high doses of nickel sulphate and nickel subsulfide. There was no indication of oxidative damage, although inflammation was evident. The DNA damage therefore most likely was related to inflammation and/or apoptosis. Nickel subsulfide but not nickel sulphate was able to induce cell proliferation.

4.4.2.4. Human data

The frequency of chromosomal gaps was significantly increased (3-5-fold) in peripheral T-cells of workers exposed to nickel (Boysen *et al*, 1980; Waksvik and Boysen, 1982; Waksvik *et al*, 1981 a and b). By contrast, no significant increase in the frequency of chromosomal breaks or SCE was found. Cytogenetic studies conducted in retired nickel workers 4-15 years after employment revealed increases both in gaps (1.4-fold) and breaks (8-fold) in peripheral T-cells. No difference in the frequency of SCE was observed (Waksvik *et al*, 1984 a and b). In another bio-monitoring study CAs were measured in peripheral lymphocytes of workers occupationally exposed to oxidic nickel and nickel sulphate in a Czech Republic chemical plant. A significant although small increase (1.6-fold) in the mean value of CAs (gaps, chromatid and chromosome breaks) was found in the combined exposed group compared to the control (Senft *et al*, 1992). Possible confounding factors were not discussed. A cytogenetic study in peripheral lymphocytes of workers occupationally exposed to nickel carbonyl did not reveal significant increases of chromosome breaks or gaps (Decheng *et al*, 1987).

4.4.3. *Mechanisms of genotoxicity*

The mechanism of the genetic activity of nickel compounds is not clearly defined. Results of *in vitro* and *in vivo* studies have shown that nickel compounds produce DNA single-strand breaks either directly or indirectly. The DNA breaks are the most logic candidates for the initial DNA lesions responsible of the various effects at chromosome level. However, the mechanisms by which DNA breaks are induced are not clear. One hypothesis involves the generation of oxygen free radicals in a process analogous to the Fenton reaction. However, oxidative damage by reactive oxygen species is unlikely to play a major role, due to the observed genetic profile of nickel compounds, with negative results in tests for *hprt*

mutations and for oxidative damage of DNA. Inhibition of DNA synthesis seems to be more likely, which is consistent with the negativity in bacteria. Another possible mechanism may involve inihibition of DNA repair. Impaired repair function has been seen *in vitro* at low non-cytotoxic nickel concentrations (Hartwig and Schwerdtle, 2002). Clearly, more research into the mechanisms of nickel mutagenesis and carcinogenesis are needed.

4.4.4. Comment

There is considerable evidence for the *in vitro* genotoxicity of soluble nickel compounds; the database for nickel carbonate and metallic nickel is much more limited or inadequate. Positive effects are generally seen in studies of chromosomal effects (CA and SCE), DNA damage and repair (SSB and DPC) and cell transformation.

The ability of nickel compounds to induce gene mutations is less clear. The weight of evidence suggests that nickel compounds are unable to efficiently induce point mutations. The few positive results reported for gene locus mutations (*hprt* gene) consist of small effects at high, toxic doses and where the mutations were not characterized at a molecular level. These results are likely to be due to other genetic events than points mutations (frame-shift or base substitution type mutations), e.g. chromosomal aberrations and DNA methylation.

Interpretation of the results of *in vivo* studies is more complicated. The *in vivo* clastogenicity of nickel chloride is the more convincing; however, when taken together all the data presently available for the three soluble compounds (nickel chloride, sulphate and nitrate), there is evidence of *in vivo* genotoxicity at chromosome level in somatic cells, although this is manifested at high, toxic doses. There is also supportive evidence from studies on workers exposed by inhalation to nickel compounds, showing increased frequencies of chromosomal gaps or aberrations.

Evidence for any possible effect on germ cells is particularly limited. There is evidence that the nickel ion reaches the testis in rodents after i.p. administration, but there are few data on possible effects.

Recently (EC, 2004) the European Commission Working Group of Specialized Experts in the fields of carcinogenicity and mutagenicity has proposed the following classification of nickel sulphate, nickel chloride and nickel nitrate: Mutag. Cat. 3, with the risk phrase R68 “Possible risk of irreversible effect”. This conclusion was based on evidence of *in vivo* genotoxicity in somatic cells, after systemic exposure. Hence, the possibility that germ cells are affected could not be excluded. However further testing of effects on germ cells was not considered practicable. There was insufficient evidence for classification of the mutagenicity of nickel carbonate.

4.5. Reproductive and developmental toxicity

In a 3 generation study, groups of Wistar rats were fed diets containing 0, 250, 500 and 1000 mg/kg nickel as nickel sulphate hexahydrate, equivalent to about 0, 12.5, 25 or 50 mg nickel/kg body weight/day. In all groups, 20 rats of each sex were mated. In the first generation, the number of stillborn rats was increased at all dietary levels. The number of siblings per litter and siblings weaned decreased with increasing doses. At the highest dose, body weights of weanlings were markedly reduced. No adverse effects were noted on fertility, gestation, viability and lactation indices. Teratogenic effects were not observed (Ambrose *et al*, 1976).

In a 2 generation study, nickel chloride was administered in drinking water to groups of 30 CD rats of each sex at dose levels of 0, 50, 250 and 500 mg/L, equivalent to 0, 7.3, 30.8 and 51.6 mg/kg body weight/day, for 90 days before breeding. At the highest dose, there was a significant decrease in the maternal body weight, along with absolute and relative liver weights. In the F_{1a} generation, at this dose the number of live pups/litter was significantly decreased, pup mortality significantly increased

and average pups body weight significantly decreased. In the F_{1b} litters, increased pup mortality and decreased live litter size was also observed in the lower dose groups. These effects are questionable, however, because the room temperature was higher than normal at certain times along with lower levels of humidity (RTI, 1987).

Groups of 34 female Long-Evans rats received nickel chloride in drinking water for 11 weeks prior to breeding and during two successive gestation and lactation periods at doses of 0, 10, 50, and 250 mg nickel/L equivalent to 1.33, 6.8 or 31.8 mg nickel/kg body weight/day. At the highest dose in the first generation and at all doses in the second generation, a dose-related increase in pups born dead or dying shortly after birth was observed. Body weight gain was reduced in dams of the mid and top dose groups (Smith *et al*, 1993).

4.6. Human data

Twenty of 32 electroplating workers, who inadvertently drank water contaminated with nickel sulphate and chloride, developed symptoms, such as nausea, vomiting, diarrhoea, giddiness, lassitude, headache, cough and shortness of breath. Laboratory tests showed elevated levels of blood reticulocytes, urine albumin, and serum bilirubin. The nickel doses that caused these symptoms were estimated to be in the range of 7.1-35.7 mg/kg body weight (Sunderman *et al*, 1988).

Nickel salts are potent skin sensitisers in humans, causing allergic contact dermatitis. Nickel ions bind to cellular and matrix proteins of the skin and induce a cellular immune response (type IV hypersensitivity reaction) (Büdinger *et al*, 2000). The prevalence of nickel sensitivity in the population is about 8-14.5% for adult women and about 1% for men (WHO, 1996). In sensitised individuals, not only dermal exposure, but also oral intake of low doses can provoke eczema.

In a number of oral challenge studies, single oral doses of a few mg nickel provoked dermal reactions in nickel-sensitised subjects (Christensen and Möller, 1975; Kaaber *et al*, 1978; Gawkrodger *et al*, 1986; Menne and Maibach, 1991). The lowest oral doses, given to nickel sensitive subjects and reported to exacerbate hand eczema, were 0.49 mg/day in a high nickel diet (Nielsen *et al*, 1990), equivalent to about 8 μg nickel/kg body weight/day, and 12 μg/kg body weight/day given in drinking water on an empty stomach (Nielsen *et al*, 1999).

CONCLUSIONS AND RECOMMENDATIONS

1. DOSE RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

In studies on subchronic toxicity, the main targets for the toxicity of orally ingested nickel salts are kidneys, spleen, lungs, and the myeloid system. In addition, perinatal mortality has been reported to increase in rats, even at the lowest administered dose of 1.3 mg nickel/kg body weight/day. The available studies do not allow the establishment of a NOAEL.

There is evidence that nickel salts are carcinogenic in rodents and humans by inhalation. The evidence for carcinogenicity following oral exposure is lacking, however the data presently available are very limited. The Panel notes that a long-term study with nickel sulphate in rats by the oral route, which will improve the presently limited data-base, is on-going.

The genotoxicity of nickel salts, observed at chromosome level at high, toxic doses is likely due to indirect mechanisms.

It is not possible to derive a threshold for provoking dermal reactions in nickel-sensitised subjects. Although only dermal exposure to nickel can lead to sensitisation, oral doses of nickel have been shown

to exacerbate hand eczema in nickel-sensitised individuals. In some studies, as little as 8 and 12 μg nickel/kg body weight provoked such reactions.

In the absence of adequate dose-response data for these effects, it is not possible to establish a tolerable upper intake level.

2. RISK CHARACTERIZATION

Nickel has not been demonstrated to be essential for humans.

Estimates of nickel intake from the average diet range from 80 to 150 μg/person/day, but may reach 900 μg/person/day or more, when large amounts of food items with high nickel contents are consumed. An intake of 900 μg nickel/person/day, equivalent to 15 μg/kg body weight/day, would be 90-fold lower than the lowest dose of 1.33 mg nickel/kg body weight/day reported to increase the perinatal mortality of rats. In the worst case, however, the first-run drinking water, which remained in the tap overnight, would be consumed and the release of nickel from kitchen utensils would cause an additional alimentary exposure. In that case, the margin between the dietary intake and toxic doses would be considerably lower.

In the group of nickel-sensitised persons, the margin of exposure is even lower. An intake of 150 μg nickel/person/day from the average diet, equivalent to 2.5 μg/kg body weight/day in a 60 kg adult, is about one third of the lowest reported dose of 8 μg nickel/kg body weight (490 μg/day) able to cause flare-ups of hand eczema in sensitised subjects. Consumption of food with high nickel content and additional exposure from first-run drinking water and kitchen utensils could result in an intake higher than the critical dose.

Any additional nickel intake from supplements would further increase the risk. In this context, the Panel draws attention to the high prevalence of nickel sensitisation in the population and to the fact, that many individuals may not be aware that they are sensitised.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF FLUORIDE

(REQUEST N° EFSA-Q-2003-018)
(ADOPTED ON 22 FEBRUARY 2005)

SUMMARY

Fluoride is not essential for human growth and development but is beneficial in the prevention of dental caries (tooth decay) when ingested in amounts of about 0.05 mg/kg body weight per day and when applied topically with dental products such as toothpaste. Dental enamel which contains fluoride is less likely to develop caries, because of greater resistance to ingested acids or to acids generated from ingested sugars by the oral bacteria. In addition, fluoride inhibits sugar metabolism by oral bacteria.

Fluoride content of the body is not under physiological control. Absorbed fluoride is partly retained in bone and partly excreted, predominantly via the kidney. In infants retention in bone can be as high as 90% of the absorbed amount, whereas in adults retention is 50% or less. Fluoride is also incorporated into dental enamel during tooth formation.

Excessive intake of fluoride during enamel maturation before tooth eruption from birth to eight years of age, when enamel formation is complete, can lead to reduced mineral content of enamel and to dental fluorosis of deciduous but predominantly of permanent teeth. The incidence and severity of dental fluorosis is dose-dependent. Mild dental fluorosis is not readily apparent and is associated with increased resistance to caries. The Panel considered moderate dental fluorosis, which is characterised by staining and minute pitting of teeth, to be an adverse effect. On the basis that the prevalence of moderate dental fluorosis of permanent teeth is less than 5% in populations ingesting 0.08-0.12 mg fluoride/kg body weight/day, the Panel considered that the upper level (UL) for fluoride is 0.1 mg fluoride/kg/day in children aged 1-8 years. This is equivalent to 1.4 and 2.2 mg fluoride per day in children aged 1-3 years and 4-8 years, respectively.

Fluoride accretion in bone increases bone density but excessive long term intake reduces bone strength and increases risk of fracture and skeletal fluorosis (stiffness of joints, skeletal deformities). Studies with therapeutic oral administration of fluoride in amounts of 0.6 mg/kg body weight/day in postmenopausal women over several years increased the risk for non-vertebral bone fractures significantly. The Panel applied an uncertainty factor of 5 to derive an UL of 0.12 mg/kg body weight/day. This is equivalent to an UL of 5 mg/day in children aged 9-14 years and 7 mg/day for age 15 years and older, including pregnant and lactating women.

The UL for fluoride applies to intake from water, beverages, foodstuffs, including fluoridated salt, dental health products and fluoride tablets for caries prevention.

Children aged 1-8 years have fluoride intakes from food and water well below the UL provided the fluoride content of their drinking water is not higher than 1.0 mg/L. An increase in the prevalence of mild dental fluorosis observed in some countries has been attributed to the inappropriate use of dental care products, particularly of fluoridated toothpaste.

The Panel did not establish an UL for infants. Breast-fed infants have very low fluoride intakes from human milk (2-40 μg/day) and are not at risk of developing enamel fluorosis even when given fluoride supplements of 0.25 mg/day. The Panel notes that the Scientific Committee on Food has recommended a maximum fluoride level of 0.6-0.7 mg/L in infant formula and follow on formula, equivalent to an intake of about 0.1 mg/kg body weight per day in infants during the first six months of life (body weight 5 kg). For powdered formula, this maximum will be exceeded if water containing more than 0.7 mg/L is used for its preparation.

For children older than eight years and adults the probability of exceeding the UL of 5/7 mg fluoride/day on a normal diet is generally estimated to be low. However, consumption of water with a high fluoride content, e.g. more than 2-3 mg/L, predisposes to exceeding the UL.

KEY WORDS

Fluorine, fluoride, fluorosis, bone, teeth, drinking water, food, supplement, dental product.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested the inclusion of boron, nickel, silicon, vanadium and tin in the proposal. The Commission did not accept the Parliament's request in the absence of a positive safety evaluation by the SCF. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Fluorine is a gaseous halogen with an atomic mass of 18.998. It is the most electronegative and reactive of all elements, therefore it occurs naturally only in ionic forms, fluorides, after reaction with metallic elements or with hydrogen. Fluorides are ubiquitous in air, water and the lithosphere, where they are seventeenth in the order of frequency of occurrence (0.06-0.09% of the earth's crust) (WHO, 1994). Fluorides occur in rocks and soil as fluorspar (CaF_2), cryolite ($3NaFxAlF_3$) or apatite ($3Ca_3(PO4)$ $2xCa(F,OH,Cl)_2$, in mica, hornblende, or as pegmatites like topaz and tourmaline. Cryolite used for the production of aluminium and rock phosphates used for the production of fertilisers can have fluoride contents up to 4.2%. Most of this fluoride is firmly bound and not biologically available. Availability of fluoride from soil depends on the solubility of the fluoride compound, the acidity of the soil and the presence of water.

All water contains fluorides, sea water between 1.2 and 1.5 mg/L. Waters with high fluoride content are usually found at the foot of high mountains. Ground water with fluoride concentrations as high as 25 mg/L have been found. Surface water usually has lower fluoride content below 0.5 mg/L, but very high fluoride levels have been found in lakes in Tanzania (95 mg/L) and Kenya (2800 mg/L) (WHO, 2000).

Fluoride in air exists in gaseous or particulate forms and arises from fluoride containing soils, industry, coal fires and especially volcanoes. In non-industrial areas it ranges between 0.05-1.9 $\mu g/m^3$. Hydrogen fluoride, a highly corrosive gas or liquid at room temperature is used extensively by industry. It readily dissolves in water to hydrofluoric acid, which though a weak acid, etches glass and because of its industrial use is the most important atmosphere contaminant. It is rapidly converted to fluoride salts.

1. Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

The most important fluorides for human use are sodium and potassium fluoride, which are highly soluble in water. They are used for addition to foods (e.g. salt), dental products and fluoridation of water. They are permitted for use in foods for particular nutritional uses (FPNU) and food supplements (Commission Directive 2001/15/EC; Directive 2002/46/EC).

In Annex III part 1 of the amended Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products, 20 fluoride compounds are listed which may be used in oral hygiene products up to a maximum concentration in the finished products of 0.15% (1500 ppm), calculated as fluorine.

Fluorosilicic acid or hydrofluorosilicic acid (H_2SiF_6) or sodiumhexafluorosilicate (Na_2SiF_6) are used for drinking water fluoridation.

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

2.1. Function of fluoride

There is insufficient evidence for the indispensability of fluoride for human health. Because of the ubiquity of fluoride it is virtually impossible to create an experimental situation free of fluoride.

Schwarz and Milne (1972) reared several generations of F344 rats in isolators on a fluoride-deficient diet (0.002-0.023 mg/kg/day). Rats on this diet showed decreased gain in weight and bleached incisors. Weight gain was improved by fluoride supplementation of the diet (2.5 mg/kg), tooth pigmentation was not. Rats in both the group on the fluoride-deficient and the fluoride-supplemented diet had shaggy fur, loss of hair and seborrhoea, indicative of a probable deficiency of other nutrients in the synthetic diet as well.

In a cohort study of 109 infants exclusively breast-fed for at least four months (fluoride in breast-milk 0.003 mg/L) and living in an area with low fluoride content of the drinking water (0.018-0.166 mg/L), those receiving a fluoride supplement from the 6th day of life onwards in addition to their fluoride intake of less than 0.003 mg/day from human milk, showed a significantly greater increase in length and weight, especially when the mother had taken fluoride supplements during pregnancy, and a significantly (by 12 days) earlier eruption of the first tooth in boys, than those who did not receive a fluoride supplement during the first six months of life (Bergmann, 1994). Although suggestive, these results do not prove an essential role of fluoride in human development and growth.

In vitro, fluoride (0.02-0.1 mg/L) addition to a supersaturated solution of calcium phosphate initiates the formation of hydroxylapatite ($Ca_3(PO_4)_2 \cdot Ca(OH)_2$) which is the mineral substance of bone and teeth. With increasing fluoride concentrations fluoroapatite ($Ca_3(PO_4)_2 \cdot CaF_2$) is formed and results in more regular and bigger apatite crystals which are less acid soluble (Featherstone *et al*, 1983; Newesely, 1961; Okazaki *et al*, 1985).

Fluoride in the body is mainly associated with calcified tissue (bone and teeth) due to its high affinity for calcium. In bone the substitution of fluoride for hydroxyl groups in apatite alters the mineral structure of the bone. This is electrostatically more stable and more compact and results in increased density and hardness, but not increased mechanical strength in rabbit (Chachra *et al*, 1999). Both in rats and in humans there is evidence for a biphasic effect of fluoride on bone strength, with increases in both bone strength and bone fluoride content at moderately high fluoride intake (16 mg/L in drinking water of rats during 16 weeks) leading to a bone fluoride content of up to 1200 mg/kg and a decrease with higher fluoride intake (up to 128 mg/L in drinking water) and bone fluoride content up to 10,000 mg/kg (Turner *et al*, 1992).

Besides the physicochemical effects of fluoride on the bone, fluoride in high doses (0.02-0.2 mg/L) was found to be mitogenic in osteoblasts and inhibitory to osteoclasts of chicken embryos *in vitro* (Farley *et al*, 1983; 1988; Gruber and Baylink, 1991). The mitogenic effect is restricted to osteoblastic precursors (Bonjour *et al*, 1993) and the same fluoride dose can be toxic to individual osteoblasts (Chachra *et al*, 1999). Fluoride can activate thyroid adenylate cyclase (ATP pyrophosphate-lyase (cyclizing)) *in vitro* at very high concentrations (10 mg/L or 190 mg/L) (Goldhammer and Wolff, 1982).

Fluoride has a cariostatic effect on erupted teeth of both children and adults. A pre-eruptive effect of fluoride through increasing fluoridation of the developing enamel is supported by evidence (Groeneveld *et al*, 1990; Murray, 1993), but difficult to differentiate from the cariostatic effect of fluoride on erupted teeth.

The prevalence of dental caries in a population was not inversely related to the concentration of fluoride in enamel (Clarkson *et al*, 1996), which apart from the outmost surface is accumulated through pre-eruptive enamel development (Richards *et al*, 1992; Weatherall and Robinson, 1988). Fluoridated enamel is less acid soluble (Beltran and Burt, 1988). It was also demonstrated that the positive effect on reduction of caries in both deciduous and permanent teeth was more marked the earlier children were exposed to fluoridated water or fluoride supplements (Groeneveld *et al*, 1990; Stephen *et al*, 1987). Comparisons of caries prevalence between two communities in England with water fluoride concentrations of 0.2 and 1.5-2.0 mg/L showed that in all age groups (from 15 to >44 years) caries experience of all teeth was significantly lower in the community with high fluoride water concentration (44% less in persons over 45 years) (Jackson *et al*, 1973). A similar study in Sweden compared caries prevalence in 30 to 40-years old life-long residents of Uppsala (n=260; water fluoride concentration 1.0 mg/L) with those of Enköping (n=236; fluoride in water 0.3 mg/L) and found 21% units less decayed and filled surfaces in Uppsala. Caries prevalence in that study was not influenced by other topical fluoride sources (Wiktorsson *et al*, 1992).

The cariostatic effect of fluoride in saliva or plaque on erupted teeth is due to an inhibition of the demineralisation of sound enamel by ingested acid foods or acid formed by cariogenic bacteria in the dental plaque and by enhancing remineralisation of demineralised enamel. Demineralised enamel takes up more fluoride than sound enamel and the resultant structure is more acid resistant and contains more fluoride (Featherstone, 1999; White and Nancollas, 1990). Moreover, fluoride affects the metabolism of carbohydrates and the production of adhesive polysaccharides by cariogenic bacteria (Hamilton, 1990). However, caries is not a fluoride deficiency disease and no specific fluoride deficiency syndrome has been found.

2.2. Fluoride homeostasis

Ninety-nine percent of the total fluoride content of the body is concentrated in calcified tissue. Body fluid and soft tissue fluoride concentrations are not under homeostatic control and reflect the recent intake (Ekstrand *et al*, 1977). In blood the fluoride ion concentration in plasma is twice that in blood cells (Whitford, 1996). Via the plasma fluoride is distributed to all tissues. The ratio fluoride in soft tissue to fluoride in plasma is between 0.4 and 0.9. Exceptions are the kidney, pineal gland, brain and adipose tissue. The kidney can accumulate fluoride to higher concentrations than in plasma (Taves *et al*, 1983). Experiments with radioactive fluoride have shown that it is not actively transported into the thyroid gland of humans or rats. Nonetheless, after long-term exposure to a high fluoride content in feed or water, the thyroid glands of some animals (cows and rats) have been found to contain increased fluoride levels compared to their non-exposed controls (Bürgi *et al*, 1984).

2.2.1. Intestinal fluoride absorption

Inhalation of fluoride from the air, as a rule, does not contribute more than 0.01 mg/day to the total intake, except in occupational settings where intake by that route can be several milligrams (Hodge and Smith, 1977). For the purpose of setting an UL for oral exposure to fluoride, exposure via inhalation is not relevant and shall not be taken into account.

Readily soluble fluorides (sodium, hydrogen, fluorosilicic, sodium monophosphate) are rapidly almost completely absorbed with a plasma peak level occurring after 30 minutes (70, 130, 300, 450 μg/L after single doses of 1.5, 3, 6, 10 mg of fluoride as the sodium salt, respectively), in contrast to the low-soluble fluoride compounds calcium fluoride, magnesium fluoride and aluminium fluoride. Fluoride from toothpaste is also absorbed. Sodium monofluorophosphate from toothpaste needs dephosphorylation before absorption in the lower intestine. There is variability in the bioavailability of fluoride from different foods (Trautner and Siebert, 1983).

Most of fluoride is absorbed as undissociated hydrogen fluoride and absorption occurs by passive diffusion in both the stomach and the small intestine. Higher acidity of the stomach increases absorption. The presence of calcium, magnesium, phosphorus and aluminium decreases the absorption of fluoride (Cerklewski, 1997; Harrison *et al*, 1984; Kuhr *et al*, 1987; McClure *et al*, 1945; Spencer *et al*, 1981). In the case of calcium the inhibitory effect depends on the presence of food. Sodium fluoride tablets given in water on an empty stomach were almost 100% absorbed. The same doses given together with milk were 70% absorbed, and were 60% absorbed when given with a meal (Ekstrand and Ehrnebo, 1979; Shulman and Vallejo, 1990; Trautner and Einwag, 1987). Consecutively faecal fluoride excretion is increased.

2.2.2. Fluoride distribution and storage in the body

Absorbed fluoride is rapidly distributed by the circulation to the intracellular and extracellular fluid but is retained only in calcified tissues. The fluoride plasma concentration is dependent on the fluoride dose ingested, dose frequency and the plasma half-life, which was determined to be 3-9 hours after giving doses of 3 to 10 mg as tablets orally. The plasma clearance of fluoride ranged between 0.12 and 0.2 L/kg/h independent on the dose (Ekstrand *et al*, 1977). Plasma fluoride occurs in both ionic and non-ionic forms. The non-ionic fluoride in plasma consists mostly of fat-soluble fluorocompounds. Ionic fluoride is not bound to plasma proteins or other compounds. Its level (μmol) reflects the recent fluoride intake and the fluoride content of drinking water (in mg/L) when water is the predominant fluoride source (WHO, 1994). Plasma fluoride levels increase with age and with increasing fluoride content of bone, and as a consequence of renal insufficiency (Ekstrand and Whitford, 1988; Ekstrand *et al*, 1978; Singer and Ophaug, 1979).

Fluoride concentrations in plasma ranging from 0.4-2.4 μmol/L (7.6-45.6 μg/L) have been reported in healthy adults (IPCS, 2002). Concentrations are lower (<10μg/L) in persons living in areas with a low fluoride content in the drinking water (<0.2 mg/L) and the diet (Ekstrand *et al*, 1977; Fuchs *et al*, 1975; Schiffl and Binswanger, 1980), somewhat higher (13 μg/L) in those whose drinking water is fluoridated (1 mg/L) (Taves, 1966), and can be twenty-fold elevated in patients with both skeletal and dental fluorosis due to high fluoride levels in drinking water (>8 mg/L) (Jha *et al*, 1982). Circulating fluoride passes the placenta and reaches the fetus. The level of fluoride in cord blood is about 75% of the level in maternal blood. The fluoride concentration in the placenta can be higher than in maternal blood. Use of 1.5 mg fluoride supplements during pregnancy markedly increased placental fluoride levels and to a lesser extent fetal blood levels (Caldera *et al*, 1988; Shen and Taves, 1974).

Fluoride concentrations in ductal and glandular saliva closely follow the plasma concentration but at a lower level (about two-thirds of the plasma level (Ekstrand, 1977; Whitford *et al*, 1999a). Apart from the intake via water and diet the fluoride concentration in saliva and dental plaque is dependent on topical fluoride application via dental care products (Oliveby *et al*, 1990; Ekstrand, 1997; Ekstrand, 1977; Ekstrand *et al*, 1977; Featherstone, 1999; Hetzer, 1997; Sjögren *et al*, 1993; Twetman *et al*, 1998). Children with no caries experience were found to have higher salivary fluoride concentrations than children highly affected by caries (40 versus 20 μg/L) (Shields *et al*, 1987).

Fluoride retention in bone (and dentine) is proportional to the long-term fluoride exposure and, moreover, dependent on the turnover rate of bone, on age, gender and the type of bone (Caraccio *et al*, 1983). Infants and young children will retain up to 75% of the absorbed dose in skeletal tissue. Exclusively breast-fed infants not receiving a fluoride supplement showed negative fluoride balances up to the age of four months and excreted more fluoride than they ingested (Bergmann, 1994).

Fluoride is primarily taken up on the surface of bone crystallites via isoionic and heteroionic exchange. It is later incorporated into the crystal lattice structure of teeth and bone by replacing hydroxyl ions and producing partially fluoridated hydroxyapatite (WHO, 1994).

Fluoride is not irreversibly bound to bone, as has been demonstrated in persons who after having lived in areas with a high fluoride concentration in drinking water moved to an area with low fluoride levels in water. Their urinary fluoride excretion fell slowly over many years and their plasma fluoride levels remained high, indicating release of fluoride from remodelling of bone (WHO, 1994; Khandare *et al*, 2004).

A linear relationship between the fluoride content of drinking water and bone fluoride content was reported by Zipkin (1958). Fluoride increases with age in bone, more rapidly in women than in men and preferably in cancelleous bone (Alhava *et al*, 1980; Eble *et al*, 1992). The fluoride concentration in bone ash from 28 stillborn infants and of infants dying during the first days of life was around 70 mg/kg and not related to gestational age, weight or length (Bergmann, 1994).

In contrast to skeletal bone and dentine which accumulate fluoride throughout life and in proportion to the absorbed dose of fluoride, enamel of teeth reflects the biologically available fluoride at the time of tooth formation. Enamel maturation of deciduous teeth is completed between the age of 2 to 12 months. In permanent teeth enamel maturation is completed at the age of 7-8 years, except in the third molars, in which it continues until the age of 12-16 years. Post-eruptive fluoride uptake of enamel is expressed only in the outer layer and depends on fluoride in saliva, food, dental plaque and dental products (WHO, 1994). In areas with low fluoride concentrations in drinking water (≤0.1 mg/L) the fluoride concentration at 2 micrometer depth of enamel averages 1700 mg/kg, with fluoride concentrations in water of 1 mg/L it is

2200-3200 mg/kg. When water contains 5-7 mg/L of fluoride the concentration in enamel has been 4800 mg/kg. Such concentrations usually are accompanied by dental fluorosis (NRC, 1993).

2.2.3. Excretion of fluoride

Absorbed fluoride which is not deposited in calcified tissue is excreted almost exclusively via the kidney. The percentage of absorbed fluoride excreted via the kidney is about 50% in healthy young and middle-aged adults, in young infants and children it can be only 10-20%, in elderly persons higher than 50%. Fluoride is filtered in the renal glomeruli and reabsorbed in the renal tubuli (10-90%), dependent on the pH of the tubular fluid. The renal clearance of fluoride is 30-50 mL/min in adults (Ekstrand *et al*, 1982; Schiffl and Binswanger, 1982). Fluoride excretion is reduced with impaired renal function (Schiffl and Binswanger, 1980; Spak *et al*, 1985; Torra *et al*, 1998).

About 10-25% of the daily intake of fluoride is excreted via the faeces (WHO, 1994).

Fluoride concentration in human milk is reported to range between 2 and 95 μg/L (IPCS, 2002), which wide range is probably due to analytical difficulties. Whereas Spak *et al* (1983) found no correlation between the fluoride content of drinking water (0.2 to 1 mg/L) and fluoride content of human milk (7.6 μg/L), Dabeka *et al* (1986) could show a relationship: 32 mothers in an area with fluoride in drinking water of <0.16 mg/L secreted milk with an average fluoride concentration of 4.4 μg/L, while 112 mothers in an area with drinking water fluoride concentrations of 1 mg/L had fluoride concentrations in their milk of 9.1 μg/L. Ekstrand *et al* (1981) have shown that fluoride supplements of 1.5 mg given to the mothers did not increase the fluoride concentration in milk. Very variable fluoride concentrations in human milk were reported also from Finland (1.9-51.3 μg/L) (Esala *et al*, 1982) and very low concentrations from Germany 3-4 μg/L in areas with low fluoride in drinking water (<0.2 mg/L). There was no change in the fluoride concentration with progression of lactation (Bergmann, 1994).

2.2.4. Biomarkers for fluoride exposure and status

The determination of the fluoride concentration in body fluids (urine, plasma, saliva) gives some indication of recent fluoride intake and does not well reflect the fluoride body burden. Renal fluoride excretion varies, moreover, with urinary flow and pH. There is no clear-cut relationship between fluoride content in bone and extracellular fluids. The concentration of fluoride in nails (50% higher in finger than in toenails) and hair appears to be proportional to the exposure over longer periods of time taking into account their growth rate (Czarnowski and Krechniak, 1990; Schamschula *et al*, 1985; Kono *et al*, 1990; Whitford *et al*, 1999b). An additional daily intake of 3.0 mg fluoride over 30 days resulted immediately in a 90% increase of the basal urinary fluoride excretion and three months later in an increase of the fluoride content of fingernails (Whitford *et al*, 1999b). Subjects living in areas with high fluoride content in water (1.6-3.1 mg/L) had 1.8 and 2.9 times higher fluoride contents in fingernails than subjects from areas with intermediate (0.5-1.1 mg/L) and low (<0.11 mg/L) fluoride content in the water, respectively (Schamschula *et al*, 1985).

Fluoride concentrations in calcified tissues reflect the historical body burden. This concerns especially the skeleton, taking into account that fluoride is not evenly distributed and is for example higher in cancellous than in cortical bone (Alhava *et al*, 1980). The fluoride content in enamel is indicative of the amount taken up during tooth formation, whereas the surface layers of enamel of erupted teeth is affected by the fluoride concentrations in the mouth. The fluoride content in enamel biopsies from 137 children aged 14 years at 0.44-0.48 μm and 2.4-2.6 μm depth was proportional to the fluoride content of the drinking water (0.09 versus 1.9 mg/L: 1549 and 641 versus 3790 and 2110 mg/kg, respectively) (Schamschula *et al*, 1985). Dentine, which like bone slowly increases in fluoride content throughout life and, unlike bone, does not undergo resorption, is probably the most suitable indicator of chronic fluoride intake.

The incidence of dental fluorosis in a population is related to the concentration of fluoride in drinking water (Dean, 1942) and from food (Liang *et al*, 1997). It can be considered as a biomarker for total exposure during the time of life when enamel is formed (up to age 7-8 years) (WHO, 1994).

2.3. Recommended dietary intakes for functional effects and typical intakes

2.3.1. Adequate intakes

The SCF did not define adequate or recommended fluoride intakes (SCF, 1993). Other bodies defined adequate fluoride intakes on the basis of the negative relationship between caries prevalence and fluoride intake (FNB, 1997; D-A-CH, 2000).

There is no convincing evidence that health and development of humans depend on the intake of fluoride, however, due to the ubiquitous presence of fluoride in the environment a zero exposure is not possible under normal circumstances.

Based on epidemiological studies of the inverse relationship between dental caries and the concentration of fluoride in drinking water in the 1940s it was concluded that fluoride has a beneficial effect in increasing the resistance to dental caries in children (Dean *et al*, 1942) and at all ages (Russell and Elvove, 1951). In communities with water fluoride concentrations (0.7 to 1.2 mg/L, depending on the average regional temperature) the caries prevalence was 40-60% lower than in communities with low water fluoride concentrations. The studies of Dean (1942) had also shown that a positive relationship existed between water fluoride concentration and the prevalence of dental fluorosis. A concentration of about 1 mg fluoride/L in drinking water was identified as being "optimal" both in reducing caries prevalence and keeping dental fluorosis prevalence below 10% in the population. This fluorosis was of the mild to very mild type (see Annex 2) and practically none of the moderate to severe type.

From this "optimal" water fluoride concentration derives the estimated adequate fluoride intake of infants and children above the age of 6 months of 0.05 mg/kg body weight/day (Burt, 1992; Singer and Ophaug, 1979): age 7-12 months 0.5 mg/day; age 1-3 years 0.7 mg/day; age 4-8 years 1 mg/day; age 9-13 years 2 mg/day; age 14-18 years 3 mg/day; for females and males of 19 years and above 3 and 4 mg/day, respectively (FNB, 1997). The guidance reference values of the Austrian, German and Swiss Nutritional societies are based on the same calculation (D-A-CH, 2000).There is a difference in the adequate intake or guidance value for fluoride below the age of six months defined by the FNB and by D-A-CH. The very low fluoride intake of breast-fed infants which is about 0.01 mg/day is defined as the adequate intake for age 0-6 months by the FNB. Assuming an average body weight of 5 kg for an infant of that age group and a guidance value of 0.05 mg/kg body weight/day a guidance value of 0.25 mg fluoride/day has been calculated (D-A-CH, 2000).

2.3.2. Fluoride intake (exposure)

Fluoride exposure via inhalation and the skin will not be considered, because in normal circumstances they contribute little to the total intake. However, the fluoride content of food dried over high-fluoride coal fires can increase considerably (from 5- to 50-fold) and be a significant source of oral ingestion, as shown in China (Liang *et al*, 1997).

Exposure by oral ingestion of fluoride is by water, food (including fluoridated salt available in Austria, Belgium, Czech Republic, France, Germany, Spain and Switzerland), cosmetic dental products and fluoride supplements. Fluoride supplements are considered to be drugs in most countries of the European Community.

2.3.2.1. Water

Among the main sources of total fluoride intake in Europe are drinking and mineral waters with more than 0.3 mg/L of fluoride. From U.S. and Canadian studies the total fluoride intake of adults in areas with different fluoride content of drinking water was estimated: 0.3-1 mg/day, 1.4-3.4 mg/day with water fluoride content <0.3 mg/L and 1.0 mg/L, respectively (FNB, 1997).

Fluoride concentrations in drinking water in Europe differ between countries and within countries dependent on natural circumstances and on water fluoridation (United Kingdom, Ireland, Spain). In Ireland, the recommended fluoride content of public drinking water was recently reduced from 0.8-1.0 mg/L to 0.6-to 0.8 mg/L (Government of Ireland, 2002). Water fluoridation which had been practiced in Basel, Switzerland since 1962 (0.7-0.9 mg/L) was terminated in 2003 and fluoride content in water has returned to its natural low level of 0.1-0.2 mg/L (KL BS, 2003).

In Germany the fluoride concentration in groundwater is generally low. A survey based on 1040 sample points measured a mean fluoride concentration of 0.1 mg/L with a minimum of less than 0.1 mg/L, and a maximum value of 1.1 mg/L (Schleyer and Kerndorff, 1992).

Fluoride concentrations in drinking water collected during 1985 from public water plants in the Netherlands was 0.04-0.23 mg/L (Sloof *et al*, 1989). The range of fluoride concentrations in 5900 groundwater samples from Finland was reported to be <0.1-3.0 mg/L (Lahermo *et al*, 1990). Fluoride concentration in 4000 drinking water samples from 36 districts in the Czech Republic ranged between 0.05 and 3.0 mg/L (NIPH, 1996) and it was 0.02-3.0 mg/L in drinking water from 94 locations in Poland (Czarnowski *et al*, 1996). The highest fluoride content in drinking water of the canton Valois, Switzerland was found to be 0.9 mg/L,

whereas about half of the cantonal area was served with drinking water containing less than 0.1 mg/L (Rapport Annuel, 1999).

Total tap water intake of adolescents in the UK and in Germany was 676 g/day and 718 g/day, respectively (Sichert-Hellert *et al*, 2001; Zohouri *et al*, 2004). Total fluoride intake from all kind of drinks in British adolescents was estimated to be 0.47 mg/day.

Drinking tap water, however, is increasingly replaced by the use of bottled water. Whereas drinking water for human consumption according to Council Directive 98/83/EC, following the advice of the Scientific Committee on Food (SCF, 1998), may not contain more than 1.5 mg fluoride/L, bottled natural mineral waters can have higher fluoride levels. Natural mineral waters which contain more than 1 mg fluoride/L can be labelled as "contains fluoride". According to Council Directive 88/777/EEC on the approximation of the laws of the Member States relating to the exploitation and marketing of natural mineral waters, Member States can make national provisions for labelling a natural mineral water as suitable for the use in infant nutrition. According to Directive 2003/40/EEC the fluoride content of natural mineral waters must be not more than 5 mg/L by 1 January 2008. Mineral waters exceeding 1.5 mg fluoride/L shall bear on the label the words "contains more than 1.5 mg/L of fluoride: not suitable for regular consumption by infants and children under 7 years of age" and shall indicate the actual fluoride content.

A survey of 150 mineral and table waters from the German market measured an average fluoride concentration of 0.58 ± 0.71 mg/L: 24% had a fluoride concentration below 0.1 mg/L, 43% equal to or below 0.3 mg/L, 31% between 0.3 and 0.6 mg/L, and 8 (5%) waters had a fluoride concentration above 1.5 mg/L with a maximum value of 4.5 mg/L. The average consumption of bottled water in Germany at the time of the survey was estimated to be 104 L per year (Schulte *et al*, 1996). In a similar survey of 33 bottled waters from the Swedish market a median fluoride concentration of 0.19 mg/L with a range of 0-3.05 mg/L was determined (Rosborg, 2002). The fluoride concentration in 25 commercial brands of bottled water (spring, mineral or distilled) available in the UK was 0.08 (± 0.08) mg/L with a range from 0.01-0.37 mg/L. The average bottled water intake was estimated to be 108 mL/day in adults (Zohouri *et al*, 2003) and only 20 mL/day in adolescents (Zohouri *et al*, 2004). Twenty-four mineral waters available in Belgium had fluoride concentrations below 1 mg/L in 16 cases, but the highest value found was 5.5 mg/L. A case of dental fluorosis in an eight-year old girl was attributed to the preparation of her infant formula with mineral water containing 1.2 mg fluoride/L. Her fluoride intake from age three months to age 12 months was well above 0.1 mg/kg body weight/day (Bottenberg, 2004)

2.3.2.2 Food

Fluoride intake from food is generally low except when food is prepared with fluoridated water. Exceptions are tea which can contain considerable amounts of fluoride (0.34-5.2 mg/L) (Schmidt and Funke, 1984; Wei *et al*, 1989; Chan and Koh, 1996), dependent on type, brewing and fluoride content of water. Some brands of instant teas were reported to be another significant source of fluoride intake (up to 6.5 mg/L when prepared with distilled water) (Whyte *et al*, 2005).

Vegetables and fruit, except when grown near fluoride emitting industrial plants, contain between 0.02 and 0.2 mg/kg fresh weight, milk and milk products 0.05-0.15 mg/kg, bread, cereals and meals 0.1-0.29 mg/kg, meat and meat products 0.15-0.29 mg/kg, eggs 0.18 mg/kg, fish and fish sticks 0.48-1.91 mg/kg (Bergmann, 1994; EGVM, 2001). The fluoride content of both fish and meat depends on the care taken with deboning, and can be as high as 5 mg/kg. (Bergmann, 1994). Dried herbs contain up to 2.0 mg/kg fluoride. Table 1 summarises the fluoride content in various types of foods from various parts of the world compiled by IPCS (2002) as well as Chinese data on corn and vegetables dried naturally or over high-fluoride coal fires (Liang *et al*, 1997).

The fluoride content of the water used in industrial production and home cooking affects the fluoride content of the prepared food. The use of water containing 1 mg/L has been estimated to increase the fluoride content of the food by 0.5 mg/kg compared to low-fluoride water (Becker and Bruce, 1981; Marier and Rose, 1966).

Breast-fed infants receive very little fluoride, because human milk contains between 2-10 μg/L. An intake of 800 mL human milk corresponds to 1.6-8 μg/day or approximately 0.3-1.6 μg/kg/day (Bergmann, 1994; Fomon *et al*, 2000). Infant formula, with the exception of soy protein based formula, has a low fluoride content when the powder is prepared with distilled water (0.01 to 0.05 mg/L). If these formulas were prepared with water containing 0.3 mg fluoride/L and a 5-kg infant drinks 800 mL, fluoride intakes of 60

μg fluoride/kg body weight/day or less would result. The use of fluoridated drinking water (1 mg/L) would considerably increase the fluoride intake threefold (Bergmann, 1994; Kramb *et al*, 2001).

Table 1. Fluoride contents in some food categories (from IPCS, 2002)

Food	Fluoride (mg/kg)	Country of origin
Milk and milk products	0.01 - 0.8	Canada, Hungary, Germany
Meat and poultry	0.01 - 1.7	Canada, Hungary, Germany
Fish	0.06 - 4.57	Canada, USA
Soups	0.41 - 0.94	Canada, Hungary
Baked goods and cereals	0.04 - 1.85	Canada, China, Hungary, Germany
Vegetables	0.01 - 1.34	Canada, China, Hungary, Germany
Fruits and fruit juices	0.01 - 2.8	Canada, Hungary, Germany, USA
Fats and oils	0.05 - 0.13	Canada
Sugars and candies	0.05 - 0.13	Canada
Beverages	0.003 - 1.28	Canada, Hungary, Germany, USA
Tea leaves	82 - 371	China, Hungary, Hong Kong
brewed	0.05 - 4.97	Canada, Germany
Corn, dried naturally	0.55 - 5.48	China
Corn, dried over coal fire	3.25 - 246.1	China
Vegetables, fresh	0.31 - 9.25	China
Vegetables, dried over coal fire	8.0 - 52.0	China

Similar differences in fluoride content of infant formulas prepared with low-fluoride (0.2 mg/L) and high-fluoride (1 mg/L) water and in intakes from such formulas were calculated by Fomon *et al* (2000). With increasing percentages of the population receiving fluoridated drinking water in the United States a parallel increase of the percentage of infants receiving more than 70 μg fluoride/kg body weight/day has been reported. Not all of this increase in fluoride intake was due to the increase in drinking water fluoridation, but to fluoride supplements (Fomon *et al*, 2000). Since 1979, liquid ready-to-feed infant formulas in the United States and Canada contain 200 μg fluoride/L.

In a recent study from the United States a mathematical model to estimate the average daily fluoride intake from all dietary sources was applied. The average or central tendency exposure (CTE) and the high-end or reasonable maximum exposure (RME) of infants in areas without fluoridation of the drinking water was 0.074 and 0.11 mg/kg/day, respectively, whereas in areas with fluoridated drinking water the CTE and RME were 0.11 and 0.21 mg/kg/day. For children between the age of three and five years the same model calculations estimated the CTE and RME in areas without fluoridation to be 0.025 and 0.04 mg/kg/day, while in areas with fluoridation of the drinking water the values were 0.05 and 0.09 mg/kg/day, respectively (Erdal and Buchanan, 2005).

The fluoride intake of German children between 1 and 14.9 years of age and of adults was estimated from analysed fluoride concentrations in food and consumption data (Bergmann, 1994) (Table 2).

This model calculation demonstrates the importance of the fluoride content of drinking water for the total dietary fluoride intake and permits to estimate the effect of any additional intake of fluoride from supplements and drugs.

The average total dietary fluoride intake, including tea but excluding drinking water, of the adult population in the UK was estimated from the 1977 Total Diet Study to be 1.2 mg/day (EGVM, 2001). In Sweden the fluoride intake from food and drink of adults in areas with low fluoride levels in drinking water (<0.4 mg/L) has been estimated to be 0.4-1.0 mg/day, while in areas with fluoride concentrations in the water of 1 mg/L the mean intake was estimated to be 2.1-4.4 mg/day (Becker and Bruce, 1981).

Table 2. Estimated fluoride intake of young children, adolescents and adults

Fluoride intake (mg/day)	age 1-1.9 years	age 12-14.9 years	adults
(1) Milk, meat, fish, eggs, cereals, vegetables, potatoes, fruit	0.042	0.114	0.120
(2) Fruit juice, soft drinks, mineral water, tea (adults)	0.011	0.065	0.259
(3) Sum fluoride from food and beverages (1)+(2)	**0.052**	**0.191**	**0.379**
(4) Drinking water (0.013 mg fluoride /L)	0.060	0.073	0.065
(5) Total fluoride intake ((3)+(4)	**0.112**	**0.264**	**0.444**
(6) Drinking water (1.0 mg fluoride /L)	0.458	0.560	0.500
(7) Total fluoride intake (3)+(6)	**0.510**	**0.751**	**0.879**
(8) Drinking water (2.0 mg/l)	0.916	1.120	1.000
(9) Total fluoride intake (3)+(8)	**0.968**	**1.311**	**1.379**
(10) Fluoridated salt, 3 g/day, 250 mg fluoride/kg		0.750	0.750
(11) Total fluoride intake (5)+(10)		**1.014**	**1.194**
(12) Total fluoride intake (7)+(10)		**1.501**	**1.629**
(13) Total fluoride intake (9)+(10)		**2.061**	**2.129**

Another dietary source of fluoride is fluoridated salt which contains 200-250 mg fluoride/kg of salt, mostly in the form of potassium fluoride. One gram of salt provides 0.2 to 0.25 mg of fluoride. The use of fluoridated salt may be restricted to use at home, like in Germany, where 75% of such salt is fluoridated, or it can be used in the preparation/production of meals and foods as well (Switzerland, France). The amount of fluoridated salt ingested per person per day is estimated to be 3 g in France, were 35% of salt is fluoridated, and 2 g in Germany corresponding to an additional fluoride intake of 0.50-0.75 mg/day (AFSSA, 2003). Fluoride from salt is well absorbed as demonstrated by Marthaler *et al* (1995).

2.3.2.3. Fluoride-containing dental products

Dental products (toothpaste, rinses and gels) which contain fluoride can, especially when inappropriately used, increase the total intake of fluoride considerably (Burt, 1992). This happens particularly in young children below the age of 7 years who swallow between ten to nearly 100% of the toothpaste (Barnhart *et al*, 1974; Hargreaves *et al*, 1972; Naccache *et al*, 1990, 1992; Salama *et al*, 1989; Simard *et al*, 1989). Depending on the amount of toothpaste used per brushing and on the fluoride content significant amounts of fluoride are swallowed and absorbed (up to 0.3 mg per brushing), as demonstrated by peak increases of fluoride in plasma of 3-4 year old children within thirty minutes after brushing with 0.6 g each of a toothpaste with 1000 mg fluoride/kg. The observed peak plasma level almost reached the same height as after the ingestion of a 0.5 mg fluoride tablet (75 to 85 μg/L) (Ekstrand *et al*, 1983). Fluoride from toothpaste swallowed by a four-year old child was found to contribute up to one third to one half of total daily fluoride intakes of 3.6 and 2.3 mg, respectively (Richards and Banting, 1996). In the European Communities about 90% of all toothpastes are fluoridated with a maximum level of 1500 mg/kg.

The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP, 2003) states that the amount of toothpaste applied to the toothbrush of a child below the age of 6 years can vary between 0.05 and 0.8 g. The recommended “pea size” amount is taken to be 0.25 g. In a model calculation with amounts of toothpaste of either 0.1 or 0.25 g which would correspond to fluoride doses between 0.1 and 0.37 mg if the toothpaste contained 1000 or 1500 mg/kg, swallowing of either 20% or 40% of the toothpaste was assumed and absorption of either 80 or 100% of the fluoride dose. The amount of absorbed fluoride would then range between 0.016 mg and 0.15 mg fluoride. The SCCNFP considered this amount as the sole source of fluoride exposure - even if applied three times per day - to not pose a safety concern when used by children under the age of six years and not likely to cause fluorosis. If compared to the “adequate” fluoride intake (FNB, 1997; D-A-CH, 2000) of children at that age (0.7-1 mg/day), fluoride ingestion from such toothpastes could amount to up to 50% of that amount.

In the model calculation for 3-5 year old children in the USA the fluoride intake from ingested toothpaste was estimated to be 30-60% of the dietary CTE and to be higher than the dietary RME (Erdal and Buchanan, 2005).

2.3.2.4. Fluoride supplements

Fluoride supplements are recommended by medical societies in some countries (e.g. DAKJ, 2000) for caries prevention, especially if the fluoride concentration from drinking water is low. The various recommended regimens differ considerably with regard to the starting time (birth or 6 months of age), amounts in relation to age, and restrictions in the presence of salt fluoridation or in dependence on the fluoride concentration in drinking water (D-A-CH, 2000; FNB 1997). However, these recommendations are formulated as a public health measure and the supplements are regulated as drugs and available on prescription. The EGVM has concluded that comments on fluoride with regard to food fortification, therefore, are inappropriate (EGVM, 2003). The assessment of the need or the usefulness and safety of fluoride containing drugs is not in the terms of reference of the Panel. Their potential contribution to the total daily intake, however, has to be taken into account in the risk assessment of fluoride. This contribution can amount up to 70% of the estimated reasonable maximum dietary exposure value in both infants and young children (Erdal and Buchanan, 2005).

2.3.2.5. Summary

The total daily intake of fluoride from all sources can range from the low intake of 0.5 mg/day from solid foods, milk, beverages and low-fluoride water reported for Germany (Bergmann, 1994), when no fluoridated salt is used, no fluoride containing dentifrice is used and no supplements are taken, to the moderate amount of 1.2 mg/day reported for the United Kingdom (EGVM, 2001). If fluoridated salt would be used 0.5-0.75 mg fluoride would be added, if fluoridated water was drunk (1 mg/L) and used for the preparation of food and tea (1-2 L of water/day; 500 mL of tea with a fluoride concentration of 5 mg/L) 3.5 to 4.0 mg fluoride would be added. The sum could be 6.0 mg fluoride per day, without fluoride from toothpaste taken into account. Even more extreme scenarios are possible and not completely unrealistic, when fluoridated drinking water is replaced by the regular use of mineral water with fluoride concentrations above 1 mg/L.

For infants and children between the age of three and five years in the USA total daily intakes from all sources (drinking water, beverages, infant formula, cows' milk, food, soil, supplements and toothpaste) have been estimated using defined assumptions for intake, concentration in source, absorption and body weight. Cumulative CTE and RME for infants in non-fluoridated areas were 0.08 and 0.11 mg/kg/day, respectively, and 0.11 and 0.2 mg/kg/day, respectively for fluoridated areas. For young children the CTE and RME for non-fluoridated areas were 0.06 and 0.21 mg/kg/day and for fluoridated areas 0.06 and 0.23 mg/kg/day (Erdal and Buchanan, 2005). The assumptions used in that study are perhaps not applicable for all European countries, but the results illustrate well the range of potential exposure to fluoride via oral ingestion in infants and young children under variable conditions.

3. HAZARD IDENTIFICATION

3.1. In vitro and animal toxicity

Animal studies are considered in the risk assessment of fluoride insofar they support the multitude of human studies investigating both toxic effects and beneficial effects of fluoride in varying doses.

3.1.1. Acute toxicity

The LD_{50} for oral administration of sodium fluoride, sodiummonofluorophosphate and stannous fluoride in rats was reported to be 31-101, 75-102 and 45.7 mg fluoride/kg body weight, respectively (ATSDR, 1993; IARC, 1982). The LD_{50} for the same fluoride compounds in mice was found to be 44.3 and 58 mg fluoride/kg body weight, 54 and 94 mg fluoride/kg body weight and 25.5 and 31.2 mg fluoride/kg body weight, respectively (IARC, 1982; Whitford, 1990).

Symptoms of acute oral exposure included salivation, lacrimation, vomiting, diarrhoea, respiratory arrest and cardiac depression. Depending on the age of the animals nephrotoxic effects were observed. Gastric mucosal changes following the administration of acutely toxic doses of sodium fluoride by gavage to Holtzman rats (17.8 mg fluoride/kg body weight) occurred within 30 minutes of exposure and showed signs of recovery after 48 hours (Easmann *et al*, 1985).

3.1.2. Short- and medium-term toxicity

3.1.2.1. Short-term studies

In a 14-day study five weeks old male and female F344/N rats and B6C3F$_1$ mice received a low-fluoride semisynthetic diet and drinking water ad libitum. Fluoride concentration in drinking water was zero, 22.5, 45, 90, 180 or 360 mg/L (as sodium fluoride). All rats on drinking water with a fluoride level of 360 mg/L died by day seven (male) and day ten (female). All rats receiving 180 or 360 mg/Lppm fluoride in drinking water showed dehydration and lethargy and reduced water consumption. There were no gross lesions seen on necropsy after 14 days.

Mice on the same regimen survived the 14-day study period except for two male animals in the highest dose group. Weight losses occurred only in the highest dose group. There were no gross abnormalities on necropsy (NTP, 1990).

Male Holtzman rats which received drinking water with a fluoride content of either 38 or 85.5 mg/L during 21 days showed an increase in cortical and trabecular bone with the lower fluoride dose and an inhibition of endosteal bone formation and reductions of cancelleous bone volume with the higher dose (Turner *et al*, 1989). Uslu (1983) observed a delay in fracture healing and a reduced collagen synthesis in male albino rats receiving 14 mg fluoride/kg body weight/day over 30 days.

Female Wistar rats administered fluoridated drinking water (113.5 or 136.2 mg fluoride/L) over five weeks showed signs of reduced trabecular bone mineralisation, particularly if the feed was deficient in calcium (Harrison *et al*, 1984).

An increase of dermatan sulphate and chondroitin-6-sulphate in the tibia of male Sprague-Dawley rats which were dosed with 17.5 mg fluoride/kg body weight per day during one to two months was observed (Prince and Navia, 1983). An increase in bone matrix formation (by 20%) was also observed in male C57BL/6 mice receiving only 0.8 mg fluoride/kg body weight/day over a period of four weeks (Marie and Hott, 1986).

Male Swiss mice administered orally 5.2 mg fluoride/kg body weight/day over 35 days were reported to have reduced erythrocyte and lymphocyte numbers in blood and increases in monocytes, eosinophils and basophils when compared to controls (Pillai *et al*, 1988).

3.1.2.2. Medium-term studies

In a 90-day study with female Wistar rats which received drinking water with either 100 or 150 mg fluoride/L vertebral bone quality, as measured by compression resistance related to ash content, was reduced (Søgaard *et al*, 1995).

Whereas adult rats receiving drinking water with 16 mg fluoride/L over a period of 16 weeks showed an increase in femoral bone bending strength (by 38%), there was a decrease (by 20%) in rats with drinking water containing 64-128 mg fluoride/L for the same period (Turner *et al*, 1992).

In a six-month study male and female four to six-week old F344/N rats and B6C3F$_1$ mice on a low-fluoride semisynthetic diet were administered water without fluoride or water containing 4.5, 13.5, 45 or 135 mg fluoride/L (rats) or 4.5, 22.5, 45, 90, 135 or 270 mg fluoride/L (mice). Body weight reduction and dental fluorosis occurred in the high-dose animals. The fluoride content of bone increased in bone in relation to fluoride content of drinking water.

Nine female mice in the high-fluoride (270 mg/L) group and one male in the 135 mg fluoride/L-group and four males in the highest-fluoride dose group died. Histological changes were identified in the kidney, liver, testes and myocardium of spontaneously dying mice. There was acute nephrosis with multifocal degeneration and tubular necrosis. Multifocal myocardial degeneration and scattered accumulation of mineral was seen. Livers showed sparse enlarged multinucleated cells. Changes indicative of altered rates of bone deposition and remodelling were seen especially in the femur of nearly all mice receiving water with and above 45 mg fluoride/L and in half of male mice receiving water with 22.5 mg fluoride/L (NTP, 1990).

The administration of 13.6 mg fluoride/kg body weight/day in distilled water by gavage over ten weeks in C57BL/6N mice increased T-cell mitogenesis (by 84%) and reduced B-cell activity (antibody production, by 10%) (Sein, 1988). Antibody production was also inhibited in female rabbits which received over 6-9 months 4.5 mg fluoride/kg body weight/day (Jain and Susheela, 1987b).

Fluoride was reported to affect negatively some endocrine organs, particularly the thyroid, in animal studies (ATSDR, 2001). Rats administered 0.5 mg fluoride/kg/day via drinking water during two months showed decreased thyroxine levels and an increased T_3-resin uptake ratio (Bobek *et al*, 1976). However, when three-months old iodine depleted Wistar rats were administered fluoride in drinking water (60 and 200 mg fluoride/L) during a six-day repletion period with ^{125}I-labelled iodine, no antithyroid effect of fluoride was observed. Neither organification of iodine nor any subsequent step of thyroid hormone biosynthesis were affected. Fluoride had no effect on thyroglobulin content of the thyroid gland or on the degree of iodination of thyroglobulin (Siebenhüner *et al*, 1984).

Male Kunmin mice divided into nine groups which received for 150 days drinking waters deficient, normal or excessive (2.5 mg iodine/L and/or 30 mg fluoride/L) in iodine and fluoride showed goiter induced by both iodine deficiency and iodine excess. Fluoride excess induced dental fluorosis and increased fluoride content in bone. Fluoride excess also affected the thyroid changes due to both iodine deficiency and excess, After 100 days the effect of excess fluoride on the thyroid (weight, colloid goiter, T_3 and T_4 levels) was stimulatory in iodine deficiency and it was inhibitory in iodine excess, while after 150 days of fluoride excess these changes reversed or were no longer influenced by fluoride. Radioiodine uptake was inhibited by fluoride excess both in iodine deficiency and iodine sufficiency, while no such effect of fluoride could be observed in iodine excess (Zhao *et al*, 1998).

3.1.3. Long-term toxicity

3.1.3.1. Growth, survival, effects on bone and teeth and other organs

Several comprehensive studies of the carcinogenicity of sodium fluoride were conducted over a period of two years in male and female F344/N rats, Sprague-Dawley rats and B6C3F$_1$ and CD-1 mice (NTP, 1990: Maurer *et al*, 1990; Maurer *et al*, 1993; NRC, 1993). Sodium fluoride was administered either in drinking water *ad libitum* or in feed. The fluoride doses [mg /kg body weight/day] in rats were 0.1 and 0.2 (controls); 0.8; 1.8; 2.5 or 2.7; 4.1 or 4.5; 11.3; the fluoride doses in mice were 0.6 (control); 1.7 to 1.9; 4.5to 5.7; 8.1 or 9.1; and 11.3.

The administration of sodium fluoride, with the exception of the highest dose in rats, had no effect on organ and body weights compared to controls in both rats and mice, no effect on feed and water consumption and no effect on survival. White discoloration of teeth occurred in all groups to a certain extent, but its incidence was higher and it occurred earlier in the highest dose groups (80-100% of animals). Fluoride content of bone was age and dose related.

Rats which had received 4.5 or 11.3 mg fluoride/kg/day had an increased incidence of hyperostosis in the skull and showed hyperkeratosis and acanthosis of the stomach mucosa when compared to the control group with 0.1 mg fluoride/kg body weight/day.

Bone matrix synthesis and mineralisation was inhibited in male and female rats which received drinking water with sodium fluoride in concentrations of 22.7 and 36.3 mg fluoride/L for 250 days (Quiu *et al*, 1987). Sprague-Dawley rats which had been administered drinking water with 50 mg fluoride/L during 18 months showed reduced femoral bone strength. Regression analysis indicated that older rats lost 36% of femoral bone strength when bone fluoride content increased from zero to 10,000 ppm (Turner *et al*, 1995).

Rabbits which received daily single oral doses of 4.5 mg fluoride/kg body weight/day for six to 24 months showed in comparison to controls a multitude of changes in blood chemistry, composition of bone, morphology of organs and signs of a disturbed collagen biosynthesis (Bhatnagar and Susheela, 1998; Jain and Susheela, 1987a, 1987b; Jha *et al*, 1982; Sharma and Susheela, 1988a; Sharma and Susheela, 1988b; Sharma, 1982; Susheela and Das, 1988; Susheela and Jain, 1983; Susheela and Kharb, 1990; Susheela and Sharma, 1982).

Alterations in trabecular and cortical bone remodelling (both resorptive and formative) were also observed in growing pigs receiving 2 mg fluoride/kg body weight/day (as sodium fluoride) orally during six months. The animals remained healthy and gained weight like control pigs. There was an increase in bone density (by 17%) and in ash weight density (by 3%) of vertebral trabecular bone, however the maximum compressive strength normalised for ash density was decreased (Kragstrup *et al*, 1989; Mosekilde *et al*, 1987). Beagle dogs ingesting 0.32 mg fluoride/kg body weight/day from drinking water over periods of six months remained healthy and showed increased trabecular bone remodelling activity, but also evidence of disturbed bone cell differentiation (Snow and Anderson, 1986).

3.1.3.2. Carcinogenicity

In male F344/N rats receiving 0.2 (control), 0.8, 2.5 or 4.1 mg fluoride/kg body weight/day in drinking water the incidence of osteosarcoma (three in the vertebra and one in the humerus) was 0/80 in the control group and 0/51, 1/50 and 3/80 in the low-, medium- and high-fluoride groups, respectively. Another osteosarcoma of subcutaneous origin occurred in a fourth high-dose male rat. No osteosarcomas were observed in female rats. The historical incidence of osteosarcomas in control male rats from dosed feed or water studies was 10/2,106 (0.47%) and 37/6,131 (0.6%) in male control rats from studies including all routes of administration. The four osteosarcomas of bone occurred with a statistically significant dose-response trend by the logistic regression test (p=0.027). The pair wise comparison of the incidence in the high-dose group versus that in controls was not statistically significant (p=0.099) and remained so when the subcutaneous osteosarcoma was included (p=0.057).

Other types of tumours, namely squameous papillomas or squameous cell carcinomas of the oral cavity, thyroid gland follicular cell tumours (adenomas and carcinomas) did not show differences in incidence in relation to the fluoride intake (NTP, 1990).

A total of three osteosarcomas and one osteoma occurred in male and female B6C3F_1 mice receiving 0.6 (control), 1.7, 4.9 or 8.1 mg fluoride/kg body weight/day (male) and 0.6, 1.9, 5.7 and 9.1 mg fluoride/kg body weight/day (female). An osteosarcoma occurred in one low-dose male mouse, in one low-dose female mouse and one osteosarcoma and one osteoma were observed in female control mice. No osteosarcoma occurred in the medium- or high-dose mice. The incidence of hepatic neoplasms (adenoma, carcinoma, hepatoblastoma) was similar in male and female mice of control and fluoride exposed groups. The incidence of malignant lymphoma in female mice was 11/80, 5/52, 11/50 and 19/80, respectively (NTP, 1990).

On the basis of these studies NTP concluded that there was “equivocal evidence of carcinogenic activity of sodium fluoride in male F344/N rats”.

In the carcinogenicity study with Sprague-Dawley rats receiving 0.1, 1.8, 4.5 or 11.3 mg fluoride/kg/day in their feed the incidence of bone tumours was 0/70, 0/58, 2/70 (one chordoma and one chondroma) and 1/70 (fibroblastic sarcoma) in male rats and 0/70, 2/52 (one osteosarcoma and one chondroma), 0/70 and 0/70 in female rats. From this study fluoride was considered to be not carcinogenic for rats. In contrast to the NTP (1990) study not all bones were investigated microscopically in this study. It should be noted that the bone ash concentration of fluoride in the NTP study with the highest fluoride dose administered was approximately one third of that observed in the study with Sprague-Dawley rats (Maurer *et al*, 1990).

In a carcinogenicity bioassay with male and female CD-1 mice over a period of 95 and 97 weeks, respectively, which were administered 1.8, 4.5 or 11.3 mg fluoride/kg body weight/day in the feed in groups of 60 animals per gender and dose, the incidence of osteomas in male control and dosed mice was 1/50, 0/42, 2/44 and 13/50, whereas it was 2/50, 4/42, 2/44 and 13/50 in female mice. These animals were infected with a Type C retrovirus; moreover, there is controversy if these types of tumour should be classified as neoplasms (Maurer *et al*, 1993; NRC, 1993). In this context it should be noted that fluoride concentration in bone ash of the mice in the highest dose group of the NTP (1990) study was less than 50% of the fluoride concentration measured in the highest dose group of this study (NRC, 1993).

Overall, based on the results of the most adequate long-term carcinogenicity studies, there is equivocal evidence of carcinogenicity in male rats and no evidence of carcinogenicity in mice.

3.1.4. Genotoxicity

3.1.4.1. In vitro studies

In general fluoride is not mutagenic in prokaryotic cells. Sodium fluoride did not induce gene mutations in Salmonella typhimurium at doses of 100 to 10,000 μg/plate in strains TA98, TA100, TA1535, TA 1537, TA 1538 and TA1597 and when tested with and without Aroclor1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Martin *et al*, 1979; Haworth *et al*, 1983). However, fluoride is not taken up significantly by strain TA98 cells (Ahn and Jeffery, 1994).

Both sodium and potassium fluoride (500-700 μg/mL) increased the frequency of mutations at the thymidine kinase locus in cultured mouse lymphoma and human lymphoblastoid cells (Caspary *et al*, 1987; Cole *et al*, 1986; Crespi *et al*, 1990). At these fluoride levels in the medium growth and survival of

cells were also reduced. Sodium fluoride (200-500 μg/mL) did not increase the frequency of mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in various cell systems exposed under neutral or acidic conditions (Oberly *et al*, 1990; Slamenova *et al*, 1992; 1996).

The frequency of chromosomal aberrations in many *in-vitro* assays was increased following exposure to sodium fluoride when compared to unexposed cells including human leukocytes, human peripheral blood lymphocytes, human fibroblasts, human amnion cells, human lymphoid cells and human keratinocytes (IPCS, 2002). The chromosomal aberrations consisted mostly of breaks/deletions and gaps with very few exchanges. Below sodium fluoride concentrations of 10 mg/L (4.52 mg fluoride/L) there were no significant increases in chromosome aberrations observed in human fibroblasts, Chinese hamster ovary cells or human diploid lung cells nor in Chinese hamster lung cells at concentrations at or below 500 mg/L (226 mg fluoride/L). The pattern observed was considered to be caused by effects of fluoride upon the synthesis of proteins involved in DNA synthesis and/or repair (IPCS, 2002). An increase in sister chromatid exchange (SCEs) was reported in Chinese hamster ovary cells at doses of 66.7 and 75 mg sodium fluoride/L without S9 and at doses greater than 1200 mg/L with S9 when harvesting time was extended (NTP, 1990).

Both negative and positive results on cytogenetic changes - mostly chromosomal aberrations - have been reported with sodium monofluorophosphate in human lymphocytes and leukocytes (Zeiger *et al*, 1993).

3.1.4.2. In vivo studies

Positive genotoxicity findings occurred at doses which were highly toxic to cells and whole animals while lower doses were generally negative for genotoxicity.

Increases in the occurrence of chromosome aberrations were reported in Swiss mice bone marrow cells following acute oral, intraperitoneal or subcutaneous exposure to sodium fluoride (4.5-18 mg fluoride/kg body weight), with an increase in micronuclei after intraperitoneal administration only (Pati and Bunya, 1987). In other studies with Swiss Webster mice from colonies with oral exposure to sodium fluoride via water (50 mg fluoride/L) or feed (up to 50 mg fluoride/kg) for at least seven generations no difference in the occurrence of chromosome aberrations in bone marrow or testis cells was observed in comparison to animals from colonies maintained at low fluoride exposure (<0.05 mg/kg or L in feed or water) (Kram *et al*, 1978; Martin *et al*, 1979) and no change in SCEs occurrence was seen in bone marrow cells from mouse or Chinese hamster orally exposed to sodium fluoride (Kram *et al*, 1978) or from Chinese hamsters orally exposed during 21 weeks to sodium fluoride in drinking water (1, 10, 50 or 75 mg/L) (Li *et al*, 1989).

Chromosome aberrations were induced in a dose-dependent manner in spermatocytes from BALB/c mice given drinking water with 0, 1, 5, 10, 50, 100 or 200 mg fluoride/L for 3-6 weeks in the highest exposed animals (Mohamed and Chandler, 1982). Swiss Webster mice, on the contrary, who received fluoride in drinking water (1-100 mg/L) for six months or were maintained for several generations on drinking water with 50 mg fluoride/L did not develop chromosomal aberrations in mitotic or meiotic cells of testes (Martin *et al*, 1979).

Sprague-Dawley rats which were fed either a normal diet low in fluoride (<0.12 mg/kg) or the same diet deficient in calcium (0.25 and 0.125%) or a low-fluoride diet containing 20% or 10% of protein or the 20%-protein diet in restricted amounts (deficiency in total nutrient and energy intake) over 48 weeks and which were further divided into groups administered deionised water with no fluoride added or with fluoride concentrations of 5, 15 or 50 mg/L did not demonstrate changes in the occurrence of SCEs in bone marrow cells that could be attributed to fluoride. Malnourished energy-restricted rats showed an increase in SCE frequency compared to sufficiently nourished rats irrespective of the fluoride content of their drinking water (Dunipace *et al*, 1998).

Sperm head morphologic abnormalities increased in Swiss mice which received doses of 10-40 mg sodium fluoride/kg body weight intraperitoneally over five days and were sampled 35 days later (Pati and Bhunya, 1987). No morphological abnormalities of sperm and no increase in the frequency of micronuclei occurrence were observed in mice which drank fluoridated water (up to 75 mg/L corresponding to 23 mg fluoride/kg body weight) during 21 weeks (Dunipace *et al*, 1989) or were given fluoride up to 32 mg/kg by gavage over five days and killed after a further 30 days (Li *et al*, 1987).

In summary, fluoride was unable to induce gene mutations in bacterial cells and in Chinese hamster cells. It was positive in the mouse lymphoma *in vitro* assay and in several cultured mammalian cells

at chromosome level only at cytotoxic concentrations, probably by indirect mechanisms (e.g. effects on DNA synthesis/repair). Conflicting results were reported on the *in vivo* induction of chromosomal damage at highly toxic concentrations.

3.1.5. Reproductive toxicity

In a multigeneration study female Swiss Webster mice received a low-fluoride diet and drinking water with either zero, 50, 100 or 200 mg fluoride/L. Litter production, infertility proportions, age at delivery of first litter, time interval between litters and frequency of conception were comparable in the control group and in mice receiving water with up to 50 mg fluoride/L. At higher doses maternal toxicity and decreased reproduction were observed (Messer *et al*, 1973). However, the feed in this study was marginal in iron content. Reproduction rate, litter size and weight were comparable in female Webster mice administered diets with less than 0.5 or 2 or 100 mg fluoride/kg for up to three generations (Tao and Suttie, 1976). However, mice which were administered ≥5.2 mg fluoride/kg body weight/day on days 6-15 after mating showed no signs of pregnancy or of implantation of embryos within the uterus (Pillai *et al*, 1989). Reductions in fertility have been observed in male mice administered 4.5 mg fluoride/kg body weight/day and in male rabbits given 9.1 or 18.1 mg fluoride/kg body weight per day over 30 days (Chinoy *et al*, 1991; Chinoy and Sharma, 1998).

Sperm motility and viability were reduced in both rats and mice after 30 days of oral administration of 4.5 or 9 mg fluoride/kg body weight/day, resulting in loss of fertility (Chinoy *et al*, 1995; Chinoy and Sharma, 1998). Reversible histopathological and biochemical changes were observed in the testes of male mice administered 4.5 or 9 mg fluoride/kg body weight/day orally for 30 days (Chinoy and Sequeira, 1989a and b) and in the testes of male rabbits after the administration of 4.5 mg fluoride/kg body weight/day over 18-29 months (Susheela and Kumar, 1991).

Serum testosterone was found to increase in rats after drinking water with a fluoride content of 45 and 90 mg/L for two weeks. Thereafter, levels decreased and were not different from controls (0.3 mg/L) after six weeks (Zhao *et al*, 1995). In rats which had received drinking water with either zero, 11.3, 45.2, 79.1 or 90.4 mg fluoride/L for 14 weeks no effects were observed on sperm count, testes weight, histopathology of testes, serum testosterone, luteinising hormone and follicle stimulating hormone nor in the F_1 offspring exposed *in utero* and after birth to fluoride (Sprando *et al*, 1997 and 1998).

No adverse effects on foetal development were found in Charles River rats when the dams ingested about 25 mg fluoride/kg body weight/day from drinking water on days 0-20 of gestation, despite signs of maternal toxicity (decreased fluid and feed consumption and reduced body weight) (Collins *et al*, 1995). Foetal development was not impaired when pregnant CD rats and New Zealand White rabbits were administered approximately 13.2 and 13.7 mg fluoride/kg body weight/day from both feed and water during days 6 through 15 and 6 through 19 of gestation, respectively. The NOAEL for maternal toxicity from fluoride in drinking water was 8.1 mg/kg/day for both rats and rabbits. The NOAEL for developmental toxicity from fluoride in drinking water administered during organogenesis was 12.2 and 13.1 mg/kg body weight/day for rats and rabbits, respectively (Heindel *et al*, 1996).

3.1.6. Interactions

In a study with male Sprague-Dawley rats lasting 48 weeks with half of the animals of each study group killed after 16 weeks the effects of nutritionally deficient diets (calcium, or protein or energy and total nutrients) on the manifestation of toxic fluoride effects outside the skeleton were investigated. All diets were low in fluoride (<0.12 mg/kg). The fluoride content of the drinking water was varied between zero, 5, 15 and 50 mg/L as sodium fluoride, to achieve plasma levels of fluoride comparable to humans with drinking water with fluoride contents of 1, 3 and 10 mg/L. There were 16-20 animals per group.

Average faecal fluoride excretion decreased with decreasing calcium content of the diet. Calcium deficient rats excreted more fluoride in their urine and calcium deficient rats retained significantly more fluoride (plasma, kidney, liver, femur, vertebra) when exposed to water containing 15 or 50 mg fluoride/L. On a body weight basis malnourished rats consumed and retained significantly more fluoride than rats fed *ad libitum* in proportion to fluoride intake. Fluoride bioavailability was influenced by diet: absorption was 92-94%, 76-78% and 58-64% with a calcium content of 0.125%, 0.25% and 0.5%, respectively. The protein content of the diet did not influence the percentage of fluoride absorbed (44-56%). Absorption in the malnourished rats was 73% of fluoride intake. The results of this study confirm the suggestion that nutritional deficiencies have an effect on both the metabolism of fluoride and on resulting tissue fluoride levels (Dunipace *et al*, 1998).

3.2. Human toxicity

3.2.1. Acute toxicity

Acute high oral exposure to fluoride may lead to nausea, vomiting, abdominal pain, diarrhoea, drowsiness, headaches, polyuria and polydipsia, coma, convulsions cardiac arrest and death. Most cases resulted from accidental or suicidal ingestion of fluoride containing insecticides or dental products. Some occurred in consequence to improperly fluoridated drinking water.

The lethal dose for humans is reported to be 40-80 mg/kg bodyweight or 5-10 g of sodium fluoride. One thirteen month-old boy died from cardiac arrest within five hours after ingestion of fluoride with severe hypocalcaemia (Boink *et al*, 1994). One three-year old child died who had swallowed sodium fluoride tablets amounting to 16 mg fluoride/kg bodyweight (Eichler *et al*, 1982).

The minimum acute dose leading to gastrointestinal effects was described to be 0.4 to 5 mg/kg body weight (Eichler *et al*, 1982; Whitford, 1996). The acute toxicity dose is lower for the more soluble salts of fluorine, which may be present in dental care products. The gastrointestinal effects arise from the action of hydrofluoric acid which is produced from fluoride salts in the stomach (Spak *et al*, 1990).

Augenstein *et al* (1991) reported on 87 cases of fluoride ingestion in children below the age of 12 years. Sixty-seven of these had ingested sodium fluoride tablets, fourteen fluoride containing drops, solutions or mouth rinses. Thirty percent of the children became symptomatic, most of them within one hour after ingestion, all of them within six hours. Eight children from 36 with a fluoride intake below 1 mg/kg body weight, 50% with an intake between 3 and 4 mg/kg and 100% with intakes above 4 mg/kg developed symptoms.

Several incidences of fluoride poisoning caused by accidentally overfluoridation of public water systems have been reported. In one incidence 200 pupils and 12 adults became ill with nausea and vomiting within minutes after ingestion of orange juice with a fluoride concentration of 270 mg/L (Infante, 1974).

Eight patients with renal insufficiency were dialysed with accidentally over fluoridated water (dose of 1 g fluoride) and became symptomatic because of virtually absent renal elimination of fluoride. One patient died from cardiac arrest. Postmortal fluoride concentration in blood was 4.9 mg/L (McIvor *et al*, 1983; Waldbott, 1981).

3.2.2. Chronic toxicity

3.2.2.1. Epidemiological studies

3.2.2.1.1. Skeletal fluorosis

Skeletal fluorosis may arise from long-term excessive exposure to fluoride both by oral ingestion and by inhalation. In the preclinical stage of fluorosis the patient may be asymptomatic and only have an increase in bone density on radiography. With increasing fluoride incorporation into bone clinical stage I and II with pain and stiffness of joints, osteosclerosis of both cortical and cancelleous bone, osteophytes and calcification of ligaments develop. Crippling skeletal fluorosis (clinical stage III) may be associated with movement restriction of joints, skeletal deformities, severe calcification of ligaments, muscle wasting and neurological symptoms. All stages are accompanied by disturbed or deficient mineralisation of the bone, and osteomalacia may be present, particularly when calcium intake is insufficient. Crippling fluorosis is rare in non-tropical countries without occupational exposure to high airborne fluoride concentrations. A fluoride intake of at least 15-20 mg/day for periods of 20 years has been reported from epidemiological studies in these patients, via consumption of drinking water high in fluoride (>4 mg/L). Only five cases of crippling fluorosis have been reported in the USA during the last 40 years (NRC, 1993). One patient with a fluoride intake of 50 mg/day through drinking water with 25 mg fluoride/L over six years was reported from Canada (Boyle and Chagnon, 1995). Patients with renal insufficiency have an increased risk of developing skeletal fluorosis.

Parallel to higher fluoride concentrations in water and food the prevalence of skeletal fluorosis in the population increases (Liang *et al*, 1997; Xu *et al*, 1997). At fluoride concentrations in water of 4 mg/L and higher, and a daily total fluoride intake of more than 14.0 mg/day, the prevalence of skeletal fluorosis in individuals with normal nutritional intake was 44%, and in individuals with deficient nutrition 69% and was associated with an even higher rate of dental fluorosis (88.3 and 95.0%, respectively) (Liang *et al*, 1997). Skeletal fluorosis of stage I to III was associated with dental fluorosis in nine patients aged between 17

and 30 years living in Indian communities with fluoride concentrations of the drinking water of 8.1 to 8.6 mg/L (Jha *et al*, 1982).

In the preclinical stage of skeletal fluorosis the fluoride concentration in bone ash is 3500-5500 mg/kg. In clinical stage I the fluoride content in bone ash is usually between 6000 and 7000 mg/kg; in stages II and III it exceeds 7500-8000 mg/kg (Hodge and Smith, 1977). Skeletal fluorosis, especially of trabecular bone, may be reversible to a certain degree when fluoride exposure is ended and fluoride balance becomes negative, dependent on the extent of bone remodelling (Grandjean and Thomsen, 1983).

Symptomatic skeletal fluorosis was chosen by the FNB (1997) as the critical endpoint for fluoride toxicity. The data base consisted of radiographic studies performed in children and adults in the 1950s and a study on bone mass measured by single photon absorptiometry in women. The results in communities with different fluoride concentrations in their drinking water were compared. The relevant studies are briefly described below.

McCauley and McClure (1954) found no differences in calcification of carpal bones of 2050 children 7 to 14 years of age living in Cumberland (Maryland) with a fluoride content of the drinking water of 0.12 mg/L (n=769), in Amarillo (Texas) with water fluoride of 3.3-6.2 mg/L (n=591) or in Lubbock (Texas) with water fluoride of 3.5-4.4 mg/L (n= 690). In the two high-fluoride communities enamel fluorosis (from very mild and worse, according to Dean, see Annex 2) occurred in 90.3 and 97.8% of children, respectively.

Schlesinger *et al* (1956) reported radiographic findings from 1528 children first investigated at age 0-9 years in 1943 and from 905 of these children re-investigated in 1954/55. Children were either living in Newburgh with water fluoridation (1.2 mg/L) since 1940 or in Kingston with fluoride-"free" water. No differences in bone density and bone maturation were found.

A 10-year radiographic follow-up study of residents (>15 years; mean 38.2 and 36.7 years) was started in 1943 in two communities: Bartlett with water naturally containing 7.6-8 mg fluoride/L (n=116) and Cameron where water contained 0.4 mg fluoride/L (n=121). Apart from a higher incidence of dental fluorosis, coarse trabeculation of bone (5.4%), hypertrophic bone changes (10.8%) and fractures (15%) were more frequent in Bartlett than in Cameron (2.5%, 7.4%, 7.6%, respectively). However, these differences were statistically not significant. The authors concluded that roentgenographic evidence of bone changes can be produced by excessive fluoride in water, but in "only a select few (10 to 15% of those exposed)". These skeletal changes were not associated with other physical findings, even though the fluoride content in bone could be six times "normal" (Leone *et al*, 1954 and 1955).

Stevenson and Watson (1957) reviewed 170 000 roentgenographs obtained in one hospital between 1943 and 1953 and identified 23 cases of osteosclerosis. These cases were life-long residents (aged 44-85 years) in areas with a fluoride content of the drinking water of >4 to 8 mg/L. However, even severe roentgenographic changes were not accompanied by clinical symptoms.

Sowers *et al* (1986) investigated bone mass at mid-radius by single-photon absorptiometry and fracture rate in 827 women (age 20-80 years) from three communities with either water naturally high in fluoride (4 mg/L) or with fluoridated water (1 mg/L). They found a non-significant lower bone mass in participants older than 55 years from the high-fluoride community and an increased fracture incidence (p=0.0001). Estimated mean fluoride content from water was 5±2.1 mg/day in the high-fluoride community.

On the basis of these studies FNB (1997) identified a fluoride intake of 10 mg/day as likely not to cause skeletal fluorosis and therefore as NOAEL for North America. An uncertainty factor of 1 was chosen to define the UL, because the NOAEL was based on human studies and because the observed skeletal changes were non-symptomatic.

3.2.2.1.2. Dental fluorosis

Dental fluorosis is caused by excessive fluoride incorporation into dental enamel before eruption of teeth. Susceptibility to dental fluorosis ends around the age of eight years, when enamel maturation of permanent teeth is completed except for the third molars (see Annex 1 for timetable of dentition). Dental fluorosis is the result of hypomineralisation of the developing tooth with a disturbance of the normal loss of early-secreted matrix proteins and their excessive retention in the developing enamel in the presence of high fluoride concentrations. The most sensitive period for this adverse effect of fluoride is the pre-eruptive maturation stage of enamel development. For the maxillar central incisors, for example, the

most critical phase of exposure to fluoride in drinking water was found to start at the age of 22 months and to last for about four months thereafter (Evans and Stamm, 1991). Hypomineralisation of both the surface and subsurface of the enamel means greater porosity. Increased porosity of the enamel makes it more vulnerable to mechanical stress and more accessible to fluoride. Therefore, fluorotic teeth have higher fluoride contents than normal teeth. The staining of fluorotic teeth in the more severe forms of dental fluorosis develops after tooth eruption. In general human dental fluorosis is more severe in teeth that mineralise later in life than in those mineralising early, and, therefore, it is primarily a condition of permanent teeth and in these increases in severity from the anterior to the posterior teeth (Thylstrup and Fejerskov, 1978). Extensive fluorosis of primary teeth, however, can be observed in areas of the world with high fluoride exposure through e.g. water (Thylstrup, 1978).

Dental fluorosis can be difficult to discriminate from other conditions in which amelogenesis in humans can be disturbed, such as calcium deficiency and generalised malnutrition. The likelihood of dental fluorosis increases in disorders of acid-base balance with reduction of the renal clearance of fluoride.

Milder forms of dental fluorosis, characterised by white spots and opaque striations on the surface of teeth are a cosmetic effect and do not impair function. On the contrary, it is associated with increased resistance against caries. Different classification or scoring systems have been developed for dental fluorosis. Three of the most commonly used systems are set out in Annex 2.

The scores from Dean's index are based on the two worst-affected teeth in the mouth and are derived from inspection of the non-dried whole tooth. Dean's index has been criticised for low sensitivity at both ends of the scale. Its category "severe" cannot, for example, discriminate between the scores 5 to 9 of the Thylstrup-Fejerskov (TF) index (see below). Dean, using his scoring system, had recorded the occurrence of dental fluorosis in a population as the community index to permit comparison between different populations. This index is calculated as the sum of individual scores in an individual divided by the number of individuals examined. A community fluorosis index of 0.6 in a population was judged to represent a threshold for dental fluorosis as of public health significance. Community indices of 0.6 were observed in communities with fluoride contents of the drinking water between 1.6 and 1.8 mg/L (Dean, 1934; Dean *et al*, 1941 and 1942). Per every increase in fluoride intake of 0.01 mg/kg body weight per day an increase in Dean's fluorosis community index by 0.2 has been predicted (Fejerskov *et al*, 1996a).

The Thylstrup-Fejerskov (TF) index (Thylstrup and Fejerskov, 1978) with a 10-point scale on inspection of dried teeth is more sensitive both at low and high grade fluorosis compared with the Dean scale. It corresponds well with the fluoride content of enamel, except for the first three categories. It has been proposed that the prevalence and severity of dental fluorosis in a population should be presented as a cumulative distribution of severity of scores (ordinate percent of population, abscissa percent of teeth involved per person) (Fejerskov *et al*, 1996b).

The Tooth Surface Index of Fluorosis (TSIF) (Horowitz *et al*, 1984) determines a score on a seven-point scale to each unrestored surface of each non-dried tooth and also provides greater sensitivity than Dean's index. It has been criticised by including staining as a criterium, which is a post-eruptive phenomenon and dependent on a person's dietary and hygiene habits as well as on the degree of enamel porosity.

The application of the above scoring systems leads to results which are not directly comparable. Some investigators tend to modify them further, therefore, the evaluation of studies on the prevalence and severity of dental fluorosis in populations must take account of the chosen methodology.

The development of enamel fluorosis is dose dependent, irrespective of which scoring system is applied (Horowitz *et al*, 1984; Fejerskov *et al*, 1996a; McDonagh *et al*, 2000). Even at low fluoride intakes from water, there will be a certain incidence of dental fluorosis.

From investigations in the 1930s and 1940s on the relationship between fluoride content of drinking water, dental fluorosis and caries occurrence, the dose dependency of occurrence and severity of dental fluorosis was already apparent: fluorosis classified as "moderate to severe" according to Dean appeared at fluoride concentrations in water of 1.9 mg/L (prevalence 2%) and increased in frequency with increasing fluoride content (2.2-2.6 mg/L: 10%; 3.9-4 mg/L: ≅ 40%; 4.4 mg/L and higher: >60%). In communities with a fluoride content in the water of 7.6 mg/L prevalence of fluorosis was 100%, 28% "very mild/mild" and 72% "moderate/severe". From the same investigation it appeared that the reduction in dental caries of children was nearly maximal in communities with a fluoride content of the water supply of 1 mg/L (Dean, 1942; Dean *et al*, 1941 and 1942).

The results of an investigation of 4429 children aged 12 to 14 years from cities with different fluoride contents of drinking water are shown in Table 3. At the time of these examinations fluoride sources were water and food only. Looking at the prevalence of caries in these children it must be born in mind, that there has been a significant decline in caries incidence starting in the 1970s.

To achieve a balance between the water fluoride content that provided best prevention of caries and minimum occurrence of meaningful fluorosis ("mild/very mild" or worse) an "optimal" fluoride content in the water of 0.7-1.2 mg/L (depending on the mean temperature of the region) was established.

Consumption of water with an "optimal" fluoride content as the only source of dietary fluoride amounts to an intake of 0.4 to 1.7 mg fluoride/day in children between one and twelve years of age. On a body weight basis this is about 0.05 mg/kg/day. Later research confirmed that dental fluorosis of the three lowest categories of the TF index occurs even with fluoride intakes of 0.03-0.04 mg/kg/day. Fluorotic enamel of these three TF categories has a normal fluoride content.

Table 3. Incidence and distribution of dental fluorosis in 4429 children aged 12-14 years examined from 13 cities in relation to fluoride content of drinking water (Dean et al, 1942)

	Fluoride content of drinking water (mg/L)											
	≤ 0.2		**0.3-0.4**		**0.6**		**0.9-1.2**		**1.9**		**2.5**	
Number examined	2142		717		614		275		273		404	
Nº fluorosis [n (%)]												
Normal (0)	1912	(89.3)	533	(74)	444	(72)	121	(44)	69	(25)	26	(6.4)
Questionable (0.5)	211	(9.9)	158	(22)	130	(21)	91	(33)	74	(27)	80	(19.8)
Fluorosis [n (%)]		(0.9)		(3.6)		(6.5)		(23)		(48)		(72)
Very mild (1)	19	(0.9)	24	(3.3)	38	(6.2)	58	(21)	110	(40)	170	(42)
Mild (2)	0		2	(0.3)	2	(0.3)	5	(1.8)	17	(6.2)	86	(21)
Moderate (3)	0		0		0		0		3	(1.1)	36	(8.9)
Severe (4)	0		0		0		0		0		0	
Caries in first molars	88%		77%		61%		52%		41%		40%	

In the following Table 4 the results of a cross-sectional survey performed 1980 in Illinois in seven communities with similar demographic characteristics on the incidence and severity of dental fluorosis assessed with both Dean's scoring system and the TSIF in 807 children aged eight to 16 years are presented. The study population was grouped according to the fluoride content of the drinking water which had been used throughout their lives into four groups: "optimal" and two-, three- or four-times "optimal" (1, 2, 3 and 4 mg fluoride /L) (Horowitz *et al*, 1984).

A total prevalence of fluorosis ("very mild" and worse according to Dean) of 48% (95% CI: 40% to 57%) at a water fluoride concentration of 1 mg/L has been estimated in a recent meta-analysis. The prevalence of fluorosis of aesthetic concern ("mild" or worse, according to Dean's classification; TSIF two or more; TF three or more) was 12.5% (95% CI 7% to 21.5%) (McDonagh *et al*, 2000). Sixt-nine per cent of 197 children between the ages of 7 and 11 years who had lived mostly in communities with fluoridated drinking water, demonstrated dental fluorosis, which was "very mild" according to the modified Dean's Index in 39% and "moderate to severe" in 13%. While there was no association between their history of total fluoride exposure and dental fluorosis, there was a significant association with the use of fluoride supplements below the age of three years (Morgan *et al*, 1998).

Only 3% of six to 10-year old children (n=1249) in Germany in a region with less than 0.3 mg fluoride/L in drinking water were found to have dental fluorosis and 8.9% of 10 to 16-year old children (n=1298) living in areas with a fluoride concentration in the drinking water up to 1 mg/L. The fluorosis community index in these latter children was <0.35. The distribution of TF indices in the 6-10 years old children was as follows: TF1 1.2-1.5%; TF2 0.2-0.8%; TF3 0.6-1.2%; TF4 0-0.2%; TF5-9 0-0.3%. In the 10-16 years

old the distribution was: TF1 2.8-3.4%; TF2 3.3-4.6%; TF3 1.1-1.9%; TF4 0.2-0.5%; TF5-9 zero. Data on the total fluoride intake of these children are not available (Hetzer, 1999; Hetzer *et al*, 1997).

Table 4. Incidence and percentage distribution of severity of dental fluorosis assessed both with the TSIF and Dean's index and mean DMF surface scores for dental caries in 807 children aged 8-16 years in relation to the fluoride content of drinking water (Driscoll et al, 1983; Horowitz et al, 1984)

Percentage distribution of TSIF scores										Mean DMF surface scores (n)
Water fluoride level	n	0	1	2	3	4	5	6	7	
optimal	336	84.5	12.4	2.0	1.1	0.0	0.0	0.0	0.0	3.14
2 x optimal	143	58.1	28.4	7.6	5.6	0.1	0.1	0.0	0.0	1.97
3 x optimal	192	50.4	25.7	13.2	9.3	0.4	0.8	0.0	0.2	1.41
4 x optimal	136	31.9	27.0	17.1	20.5	0.4	2.1	0.1	0.8	2.02

Percentage distribution of Dean's score							
		Normal	Questionable	Very mild	Mild	Moderate	Severe
	n	0	0.5	1	2	3	4
optimal	336	56.0	29.5	7.4	4.8	1.8	0.6
2 x optimal	143	18.2	28.7	23.1	16.8	8.4	4.9
3 x optimal	192	22.9	26.0	15.1	19.8	7.8	8.3
4 x optimal	136	12.5	15.4	16.9	25.0	7.4	22.8

For total daily fluoride intakes of 1.7, 3.5 and 14.8 mg/day in well nourished subjects an incidence of dental fluorosis of 6.4, 10.5 and 88.3% was reported in China, whereas for intakes of 1.2, 2.6 and 15.3 mg fluoride/day in malnourished subjects the incidence was 4.8, 24.8 and 95%, respectively (Liang *et al*, 1997).

While the intake of fluoride from water can be estimated with some certainty, e.g., by a formula which includes the variables body weight and average maximum air temperature (water intake [L/kg body weight]= 0.0025+0.0004xmean maximum temperature[°F] (Galagan *et al*, 1957), an estimation of fluoride intake from other sources is prone to the influence of a wide variety in individual habits (see Section 2.3.2). If the fluoride intake from water from Dean's data (Dean, 1942; Dean *et al*, 1941; 1942) is calculated with the above formula on a body weight basis, it appears that an intake of 0.02 mg fluoride/kg/day is associated with a prevalence of dental fluorosis of 40-50% and of 15-25%, when the category "questionable" is excluded. The community index value at that intake is 0.3-0.4. The findings of Dean on the linear relationship between fluoride content of drinking water and the prevalence of dental fluorosis and/or the fluorosis community index were confirmed by two large studies performed in the USA 25 and 40 years later (Richards *et al*, 1967; Butler *et al*, 1985) and no upward shift of the dose-response curve was observed over that period (Fejerskov *et al*, 1996a).

Similar dose-response relationships have been demonstrated between the fluoride intake from fluoride tablets and dental fluorosis. Fejerskov *et al* (1996a) compared the prevalence of dental fluorosis, classified according to Dean, in American and Swedish children, who either lived in areas with fluoridated water (1-1.2 mg/L) or received fluoride tablets (Aasenden and Peebles, 1974; Granath *et al*, 1985). While the prevalence was similar in the two groups of children receiving fluoridated water (total 63-67%; questionable plus very mild 30-31%; mild 8-9%; moderate 2-4%; severe zero), there was a significant difference in prevalence and severity of dental fluorosis between the American and Swedish children who had taken fluoride tablets. The total prevalence in the USA was 84% (questionable 16%; very mild 34%; mild 19%; moderate 14%) and it was 29% in Sweden (questionable 4%; very mild 14%; mild 10%; moderate zero). This difference is explained by the differing dosage regimes: USA 0.5 mg fluoride beginning from birth to 4 months of age until the age of three years and followed by 1 mg/day until the age of six years; Sweden 0.25 mg fluoride from 6 to 18 months and followed by 0.5 mg/ day until the age of six years. On a body weight basis American children received twice as much fluoride in tablet form at ages 6 to 12 months and at ages 3 to 6 years than Swedish children. Moreover, there were methodological differences in

assessment: in Sweden only incisors were recorded, whereas in the USA group the recordings were occasionally based on erupted premolars, which tend to be more severely affected than incisors.

There is no reason to suppose that fluoride available from food, including fluoridated salt and beverages, and from toothpaste has a different effect on maturing enamel than fluoride from water and tablets, although no investigations of this relationship have been available to the Panel.

Also apparent from the studies is the fact that there is no real threshold value for a fluoride intake which is not associated with the occurrence of dental fluorosis in the population.

In summary persons living permanently in communities with water fluoride concentrations of about 1 mg/L had in 10% to 12% mild forms of enamel fluorosis. The fluoride intake of children in these communities was calculated to be 0.02 to 0.1 mg/kg/day. At a fluoride intake from water of 0.08-0.1 mg/kg/day moderate (or worse) fluorosis was recorded in less than 5% of children (Dean, 1942). Very mild and mild forms of dental fluorosis occurred in 48% of children with a calculated fluoride intake from water of 0.043 mg/kg/day (Fejerskov *et al*, 1996a).

3.2.2.1.3. Bone mineral density and fractures

All studies on the relationship between fluoride in drinking water and bone density or risk of bone fracture suffer from imprecise exposure assessment.

Four studies included in a meta-analysis of 18 ecological, cross-sectional and cohort studies on water fluoridation/natural fluoride content of water (up to 4-5 mg/L) and bone fractures reported a significant increase in bone mass with increasing fluoride intake in lumbar spine, a positive change in the femoral neck which was not significant and a negative change for the distal radius, which also was not significant. In this meta-analysis no effect on fracture incidence could be demonstrated (RR=1.02, 95% CI = 0.06-1.09) (Jones *et al*, 1999).

Kröger *et al* (1994) investigated 3222 perimenopausal women for bone density of the spine and found it to be 1% higher in 969 women who had lived for more than ten years in an area with fluoridated water (1.0-1.2 mg/L) than in 2253 women who had used drinking water with less than 0.3 mg fluoride /L, while there was no difference for the bone mineral density of the femoral neck. There was also no difference in self-reported incidence of fractures.

Seven thousand one hundred twenty nine postmenopausal women were investigated for bone density by Phipps *et al* (2000). Women who had lived for more than 20 years in an area with fluoridated water showed 2% higher density in the lumbar spine and femur than women living in an unfluoridated community, but their radius bone density was lower. In this study the risk of incident fracture of the hip and spine was significantly lower among those exposed to fluoridated drinking water than in those not exposed. While there was no difference in risk for fracture of the humerus, the risk for fractures of the wrist was increased for those exposed to fluoridated water.

Karagas *et al* (1996) found no significant difference in risk for hip and ankle fracture in men and women between 65 and 90 years living either in an area with fluoridated water (≥0.7 mg fluoride/L) or in a non-fluoridated area (≤0.3 mg fluoride/L). In men the relative rates of fractures of the proximal humerus and distal forearm were significantly increased (by 23% and 16%, respectively), in the fluoridated area however.

A comparison between a community with drinking water containing 4 mg fluoride/L with two control communities with 1 mg fluoride/L in the water showed that the relative risk of hip, wrist or vertebral fracture was 2.2 (95% CI=1.07-4.69) in women 55-80 years of age. The fluoride intake from beverages only in the high-fluoride community was estimated to be 72 μg/kg body weight/day (Sowers *et al*, 1986).

In a retrospective cohort study involving 144,627 elderly persons who had lived at least 13 years in villages outside the public Finnish water system with fluoride concentrations in well water ranging from less than 0.05 mg/L up to 2.4 mg/L no associations between hip fractures in men or women of all ages and water fluoride content was found. However, in women between the age of 50 and 65 years at the start of the follow-up the relative risk for hip fracture increased with increasing well water fluoride concentrations in comparison with a fluoride concentration of ≤0.1 mg fluoride/L. This relationship was significant for fluoride concentrations of >1.5 mg/L (RR 2.09; 95% CI=1.16-3.76; $p<0.05$) (Kurttio *et al*, 1999).

When fluoride levels in toenails (<2.0, 2-3.35, 3.36-5.5 and >5.5 mg fluoride/kg) collected between 1982 and 1984 were used as markers for chronic fluoride exposure in a case-control study involving 62,641 healthy nurses (53 cases of hip fracture, 188 cases of forearm fracture, 241 matched controls in 1988) a non-significant increase of the risk for forearm fracture (adjusted odds ratio 1.5; 95% CI=0.9-2.7) and a non-significant decreased risk for hip fracture (odds ratio 0.5; 95% CI=0.2-1.5) were calculated for the three highest quartiles of fluoride (Feskanich *et al*, 1998).

Risk factors for fractures were determined in a 5-year prospective follow-up study in 3216 men and women above the age of 65 years and related to fluoride exposure from drinking water supply during ten years. For hip fractures a higher risk could be determined with drinking water fluoride levels of 0.11-0.25 mg/L (odds ratio 3.2) and >0.25 mg/L (odds ratio 2.4) in comparison with fluoride concentrations below 0.11 mg/L. However, no increased risk was estimated for exposure to water with >0.7 or >1.0 mg fluoride/L (odds ratio 0.77; 95% CI=0.37-1.62; odds ratio 0.89; 95% CI=0.21-3.72, respectively) (Jacqmin-Gadda *et al*, 1998).

No relationship between naturally occurring fluoride in drinking water on fractures of the hip could be demonstrated in a population-based case-control study in the United Kingdom. The contribution of drinking water to total fluoride intake in that study was small and probably less than one-third (Hillier *et al*, 2000).

Li *et al* (2001) studied the relationship between hip fracture and other fractures and exposure to fluoride from drinking water in 8266 Chinese men and women from six villages with different fluoride content in water (0.25-0.34, 0.58-0.73, 1.00-1.06, 1.45-2.19, 2.62-3.56, 4.32-7.97 mg/L). Fluoride intake from drinking water was estimated to be the main source of fluoride intake and to be on average 0.73, 1.62, 3.37, 6.54, 7.85 and 14.1 mg/day. The subjects of the study had lived in the same village for more than 25 years and were more than 50 years old when studied. The odds ratios (OR) for all fractures for the different fluoride exposure levels were:

Intake (mg/day)	OR	p Value (relative to the intake of 3.37 mg/day)
0.73	1.50	0.01
1.62	1.25	0.17
3.37	1.0	-
6.54	1.17	0.33
7.85	1.18	0.35
14.1	1.47	0.01

The difference was significant (p=0.01) for fluoride exposure at the lowest and at the highest level (0.73 and 14.13 mg/day) compared with a fluoride exposure of 3.37 mg/day in the village with a fluoride content of the water of 1.00-1.06 mg/L. For fractures of the hip the increase in prevalence with increasing fluoride exposure was significant only for the highest exposure group (14.13 mg/day; OR 3.26; p=0.02) compared to exposure to water of 1.00-1.06 mg fluoride/L. Contrary to fractures of all bones no increase in hip fracture incidence was seen with low-fluoride exposure (<1 mg fluoride /L). This study indicates a bimodal effect of fluoride exposure with an increase in the risk of fractures at all locations both with fluoride intakes lower and higher than about 3.5 mg/day, whereas the risk of fractures of the hip only increased with increasing fluoride exposure.

3.2.2.1.4. Carcinogenicity

In a series of epidemiological studies, both geographic and temporal associations between fluoride in drinking water and risk of cancer mortality were reported (Yiamouyiannis and Burk, 1977). These reports were extensively reviewed both by IARC (1982) and Knox (1985) and criticised because of methodological flaws in adjusting for differences in the age, race and sex of the compared populations.

A number of ecological studies in various countries did not find a consistent relationship between incidence of and mortality from all types of cancer and the consumption of fluoride-containing drinking water (Freni and Gaylor, 1992; Mahoney *et al*, 1991; Yang *et al*, 2000).

Lynch (1984) analysed the relationship between cancer incidence and fluoride in drinking water (both natural and added) in 158 municipalities with a total population of 1,414,878 in 1970. A total of 66,572

cancer cases (bladder, female breast, colon, lung, prostate, rectum and other sites combined) were evaluated for fluoride content in drinking water and duration of exposure in univariate and multivariate cancer-site, sex-specific statistical tests, which included eight sociodemographic variables. The results failed to support a fluoride-cancer association.

A comparison of the annual incidence rates of osteosarcoma for 1970-1988 in Edmonton (Province of Alberta, Canada), where water was first fluoridated in 1967, with rates in Calgary, where fluoridation was started in 1989 showed incidence rates of 0.27 and 0.29 per 100,000 inhabitants in Edmonton and Calgary, based on 26 and 29 cases, respectively, that is no link between fluoridation of water and osteosarcoma (Hrudey *et al*, 1990).

In an update of an earlier analysis of cancer mortality by county in the United States related to drinking water fluoridation, 2,208,000 deaths by cancer in the Caucasian population were analysed, with special emphasis on cancers of bones and joints. The risk of death from cancers of bones and joints after 20-35 years of water fluoridation in both male and females was the same as in the years immediately preceding fluoridation (Hoover *et al*, 1991).

An ecological study in areas of New Jersey observed a higher rate of osteosarcoma in fluoridated communities in 1979-1987 than in non-fluoridated communities with a risk ratio of 3.4 among males under 20 years of age in fluoridated communities. The analysis was based on 12 and eight cases in the fluoridated and non-fluoridated area, respectively (Cohn *et al*, 1992). In a case-control study from New York State the self-reported lifetime intake of fluoride from drinking water and dental care products from 130 patients below the age of 24 years diagnosed to have osteosarcoma between 1978 and 1988 was compared to the lifetime intake of matched controls. Whereas no significant trend for risk was observed on a group basis, there was a decrease in the odds ratios for osteosarcoma with increasing exposure estimates for males, which was statistically significant (p=0.02) (Gelberg *et al*, 1995). Consumption of fluoridated drinking water (>0.7 mg fluoride/L) between 1979 and 1989 was not found to be associated with an increased risk for osteosarcoma (odds ratio 1.0; 95% CI=0.6-1.5) in a case-control study in Wisconsin, USA (Moss *et al*, 1995).

Another hospital-based case-control study, on the contrary, with 22 cases of osteosarcoma and 22 matched controls found that the odds ratio of disease for drinking fluoridated drinking water (>0.7 mg/L) during childhood (birth to 15 years) and during lifetime was 0.33 (95%CI 0.04, 2.50) (McGuire *et al*, 1991).

From the available data no increased risk of developing cancer at the observed fluoride dose levels can be deduced.

3.2.2.1.5. Genotoxic effects

The frequency of SCEs was studied in peripheral lymphocytes obtained from about 700 Chinese adults who had resided for more than 35 years in the same area and consumed drinking water with fluoride concentrations from 0.11-5.03 mg/L. Half of the study population had inadequate nutritional intakes. The fluoride intake from food and water was calculated to range from 20 to 280 μg/kg body weight/day (1.2-15.3 mg/day). The fluoride concentration in plasma in the area with 5.03 mg fluoride/L was 5.56 μmol/L (106 μg/L). Plasma levels of fluoride were higher in persons with inadequate nutrition, and SCE frequencies were higher in such subjects from areas with low fluoride content in water (0.1 and 0.2 mg/L), but there were no significant differences between all the other groups and no differences in micronuclei in blood lymphocytes were observed (Li *et al*, 1995b; Liang *et al*, 1997).

One study investigated adult persons (>50 years) who had resided for more than thirty years in three communities with drinking water fluoride concentrations of 0.2 (n=66), 1.0 (n=63) and 4.0 mg/L (n=70) and who provided samples of the water which had been their main source, urine and blood samples. Mean plasma and urine fluoride concentrations reflected the fluoride content of the water (plasma: 1.1, 1.8 and 4.0 μmol/L; urine: 0.7, 1.1 and 2.8 mg/L). Peripheral blood lymphocytes showed an increased frequency of SCEs in the samples from the 4 mg/L-community (mean 5.9% compared to 5.2% (1 mg fluoride/L) and 5.5% (0.2 mg fluoride/L water) ($p<0.001$). Women showed a significantly higher overall frequency of SCE than males in all three communities ($p<0.05$). However, when 58 residents from the community with a fluoride content of 4 mg/L water were split in those (n=30) who drank this water and those (n=28) who used instead water from wells with a mean fluoride content of 0.3 mg/L, there was no difference in the frequency of SCE in their lymphocytes (Jackson *et al*, 1997).

Increases in the frequency of SCEs and micronuclei in peripheral lymphoblasts have been reported in patients with skeletal fluorosis or residents from fluorosis-endemic areas in comparison to residents from non-fluorosis areas in various countries (China, India). SCE frequency was significantly higher in peripheral blood lymphocytes from 14 inhabitants of a village with fluoride in drinking water of 1.6-2.9 mg/L than in lymphocytes from 14 residents of a village with low-fluoride drinking water (0.6-0.8). However, this was not the case with 28 residents of two other high-fluoride villages. Chromosomal aberrations occurred in higher frequency in blood from all 42 residents of the villages with high-fluoride drinking water (Joseph and Gadhia, 2000). SCE frequency in peripheral blood lymphocytes was significantly increased in 53 patients with skeletal fluorosis aged 16-59 years from a district with drinking water containing 4-15 mg fluoride/L, compared to healthy residents of the same region and to subjects drinking water with a fluoride concentration of less than 1 mg/L. The rate of micronuclei in fluorosis patients was 2 to 3 times that of control subjects and intermediate in healthy fluoride exposed subjects (Wu and Wu, 1995). However, too little details on other life circumstances are given in these studies.

There were no effects on chromosomal aberrations or micronuclei in lymphocytes in seven female osteoporosis patients randomised to treatment with sodium fluoride or monofluorophosphate (fluoride dose 29 mg/day, range 22.6-33.9 mg/day) for an average of 29 (14-49) months when compared to seven matched placebo controls. Serum fluoride concentrations were 0.1-0.2 mg fluoride/L (van Asten *et al*, 1998).

Rapaport (1956) reported an increased frequency of trisomy 21 with increasing fluoride content of drinking water based on information gathered in 1950-1956 on 687 cases admitted to institutions in four American states (Wisconsin, North and South Dakota, Illinois). These findings could not be confirmed in investigations in English cities (Berry, 1958), in Massachusetts (Needleman *et al*, 1974) and in Sweden (Berglund *et al*, 1980). In the last named study the incidence of trisomy 21 was related to the mean fluoride content in the water of the area where the mother lived and to the age of the mother. No influence of fluoride on the incidence of trisomy 21 was seen.

Genotoxic effects associated with a high exposure to fluoride have been observed, predominantly in persons with clinically manifest symptoms of fluoride toxicity (skeletal fluorosis). The data are insufficient for a dose-response assessment.

3.2.2.1.6. Reproductive effects

Chronic occupational exposure to fluoride compounds has been reported to have negative effects on sex hormone levels and menstrual cycle and to increase spontaneous abortion especially in persons with skeletal fluorosis. However, because of exposure to multiple substances, these reports are not conclusive (NRC, 1993).

In India, where fluorosis is endemic in areas with drinking water naturally containing up to 38.5 mg fluoride/L infertility in married men was reported (Neelam *et al*, 1987 cited in Susheela and Kumar, 1991). In an ecological study the total fertility rate of women 10-49 years of age in the period 1970-1988 of states of the USA with at least one community water system providing drinking water with ≥ 3 mg fluoride/L was found to be negatively associated with fluoride content. Because this study used population means and not data on individual women, it remains unresolved if fluoride from drinking water is of influence on human fertility (Freni, 1994).

3.2.2.1.7. Other effects

Nephrotoxic effects of fluoride have not been reported in subjects with skeletal fluorosis due to high fluoride contents in drinking water and in subjects with osteoporosis on long-term treatment with fluoride (section 3.2.2.2).

Fluoride was reported to affect human thyroid function. Increases in serum thyroxine levels without significant changes in T_3 or thyroid stimulating hormone levels were observed in residents of regions in India with high levels of fluoride in the drinking water (up to 6.5 mg/L). Nonetheless fluoride is not considered to be an endocrine disruptor (ATSDR, 2001). In a review of the literature on fluorine and thyroid gland function the authors come to the conclusion that the increase of the metabolic rate observed in men suffering from symptomatic industrial fluorosis was not due to fluoride-induced hyperthyroidism and that the literature on a relationship between fluoride exposure and endemic human goitre neglected to take into account a concomitant iodine deficiency (Bürgi *et al*, 1984). In two studies performed in two regions in China the intelligence of children was measured and related to the fluoride content of drinking water. 907 children (age 8-13 years) from four areas with different degrees of dental

fluorosis prevalence were investigated. Urinary fluoride concentration in these children was 1.02-2.69 mg/L and it was highest in areas with a high incidence of fluorosis. In the non-fluorosis area the mean IQ was 89.9, it was 80.3 in the high-fluorosis area (<0.01). The percentages of children with low or borderline IQs were higher in areas with medium and severe fluorosis and no children with IQs >120 were found in these areas (Li *et al*, 1995a).

The second study in 118 children aged 10-12 years, who were randomly selected from two villages which differed in the fluoride content of the drinking water (3.15 mg/L versus 0.37 mg/L) and the prevalence of dental fluorosis (86% and 14%, respectively) found an average IQ of 103 in the village with low-fluoride drinking water and of 92 in the other village (Lu *et al*, 2000). The significance of these studies is doubtful due to missing data on other factors of relevance.

In a study with 197 children aged 7-11 years, who demonstrated dental fluorosis in 69%, no association between dental fluorosis and behaviour could be demonstrated (Morgan *et al*, 1998).

3.2.2.2. Interventions, clinical studies

Fluoride compounds, mostly sodium fluoride or monofluorophosphate, alone or in combination with calcium and vitamin D, have been used in the prevention and treatment of age-dependent osteoporosis in doses ranging from 4.5 to 57 mg fluoride/day, because fluoride is known to elevate the trabecular volume by increasing both the number of osteoblasts and the formation period of the bone remodelling process. It interacts with bone cell mitogens and increases tyrosine protein phosphorylation. It selectively stimulates the carrier-mediated sodium-dependent transport of anorganic phosphate across the membrane of osteoblast-like cells and the stimulatory effect of insulin and insulin-like growth factor -1 (IGF-1) on phosphate transport in a dose-dependent fashion (Bonjour *et al*, 1993). Although fluoride increases bone mineral density (BMD), there is a corresponding decrease in elasticity and strength of bone tissue (Aaron and de Kanis, 1991).
The evaluation of the effectiveness of fluoride for prevention and treatment is outside the task of this panel, but well-conducted and documented therapeutic trials can help in identifying fluoride doses that lead to adverse effects, although it must be borne in mind that the study subjects are mostly elderly (>50 years), predominantly female and were selected because of already existing changes in bone mass or density, with or without a history of vertebral fractures.

In a meta-analysis of eleven therapeutic studies involving 1429 postmenopausal women (age 50-86 years), with a duration of 2-4 years in ten studies and of 3 months in one study, an analysis of side-effects was included (Haguenauer *et al*, 2000). All trials were randomised and included control groups which received calcium and/or vitamin D in the same dosage as the fluoride intervention group. The increase in lumbar spine BMD was found to be higher in the fluoride group than in the control group. The relative risk (RR) for new vertebral fractures was not significant at two years or at four years. The RR for new non-vertebral fractures was not significant at two years, but was increased at four years in the fluoride treated group (1.2; 95% CI 1.36-2.50) especially if high doses were used. The RR for gastrointestinal side effects was not significant at two years, but was increased at four years (2.18; 95% CI 1.69-4.57), especially if fluoride was used in high doses and in a readily available form. High fluoride doses had no effect on risk of vertebral fractures, but increased the risk of non-vertebral fractures and of gastrointestinal side effects. Table 5 lists these eleven studies and the observed adverse effects in relation to the fluoride dosis.

3.2.2.2.1. Skeletal effects

No differences in the occurrence of adverse skeletal effects (vertebral and non-vertebral fractures and lower-limb pain presumably caused by microfractures) were found in those studies where the fluoride dosis was 4.5 to 26 mg/day (up to 0.4 mg/kg/day) (Hansson and Roos, 1987; Christiansen *et al*, 1980; Grove and Halver, 1981; Gambacciani *et al*, 1995; Sebert *et al*, 1995). In two studies (Reginster *et al*, 1998; Pak *et al*, 1995) there was on the contrary a significantly reduced occurrence of vertebral fractures in the fluoride group compared to the placebo group. Meunier *et al* (1998) reported a significantly higher incidence of lower-limb pain in the group receiving fluoride 20-26 mg/day compared to the placebo group. Some women with lower-extremity pain were roentgenographed and incomplete fractures were identified in most of them at least two weeks after the onset of pain.

Whereas bone mineral density increased in one study in the lumbar spine (+35%) and the femoral neck (+10%) in the fluoride-treated group (0.56 mg fluoride/kg/day) as compared to the placebo group, there was a decrease in the radius (-4%). Vertebral fracture rate did not differ significantly over four years between the treatment and the placebo group. Non-vertebral fractures occurred in the fluoride group

(72 fractures) in higher frequency than in the placebo group (24 fractures); there were 13 hip fractures in the fluoride group and four hip fractures in the placebo group. The odds ratio for non-vertebral fractures was 3.2 (95% CI 1.8-5.6) in the fluoride group compared to the placebo group (Riggs *et al* 1990). Fifty of the 66 women in the fluoride group who completed the four-year trial were treated for an additional two years. Bone mineral density measured at the lumbar spine continued to increase linearly, whereas the rate of decrease in bone mineral density of the radius became less (minus 1.2%/year versus minus 2.2%/year in the four previous years). Vertebral fracture rate decreased somewhat in the additional two years, as did the non-vertebral fracture rate. However the non-vertebral fracture rate remained higher than in the placebo group during the first four years. From multivariate analysis it appeared that the vertebral fracture rate was moderately decreased by sodium fluoride therapy in women whose serum fluoride level and lumbar spine bone mineral density increased, provided that the increase in serum fluoride level did not exceed 8 μM (152 μg/L) and the increase in bone mineral density did not exceed 17% per year (Riggs *et al*, 1994).

3.2.2.2.2. Gastrointestinal effects

Table 5 lists also the frequency of gastrointestinal side effects of fluoride treatment studies. No gastrointestinal effects or the same frequency of nausea and dyspepsia as in the placebo group were observed in postmenopausal women administered 4.5-22 mg fluoride/day (assumed to correspond to 0.13-0.37 mg fluoride/kg body weight/day) over 12 weeks and up to 3 years (Hansson and Roos, 1987; Christiansen *et al*, 1980; Grove and Halver, 1981; Reginster *et al*, 1998; Gambacciani *et al*, 1995; Meunier et al, 1998; Pak *et al*, 1995; Sebert *et al*, 1995).

Nine of 61 postmenopausal women treated with on average 57 mg fluoride/day (as sodium fluoride) over four years complained of severe nausea, vomiting and peptic ulcer or blood loss anaemia. These symptoms did not occur in the control groups without treatment or with calcium and estrogens alone (Riggs *et al*, 1982). Nineteen of 101 postmenopausal women treated with 34 mg fluoride/day during four years had severe gastrointestinal complaints which led to dose reduction, compared to seven of 101 in the placebo group. This is an odds ratio of 2.9 (95% CI 1.2-7.1). The risk for peptic ulceration and anaemia was similar in both groups (Riggs *et al*, 1990). Gastrointestinal symptoms occurred significantly more often in 45 postmenopausal women treated with 34 mg fluoride plus 1500 calcium/day over four years than in 38 women receiving only calcium (16/46 versus 6/38) (Kleerekoper *et al*, 1991).

Table 5. Eleven therapeutical randomised studies in old-age osteopenia/ osteoporosis grouped according to fluoride dosis. Outcome with regard to vertebral and non-vertebral fractures and other side effects

Reference	Sample size (treatment/ placebo)	Age (years)	Duration (years)	Fluoride treatment: Fluoride compound	Fluoride treatment: Dose (mg/day)	Fluoride treatment: Dose (mg/kg/d)[3]	Other substances (mg/day)	Control group	Vertebral fractures: fluoride	Vertebral fractures: control / placebo	Non-vertebral fractures: fluoride	Non-vertebral fractures: placebo	Lower Limb Pain: verum	Lower Limb Pain: placebo	Gastrointestinal symptoms: verum	Gastrointestinal symptoms: placebo
Hansson and Roos, 1987	50 (25/25)	66	3	NaF	4.5		calcium 1000	calcium 1000	2/25	1/25						
	50 (25/25)	65	3	NaF	13.6		calcium 1000	placebo		1/25					4/25	
Christiansen *et al*, 1980	177 (29/121)	50	2	NaF	9		a) calcium 500	calcium 500	N.R.		N.R.		N.R.		N.R.	
	(27/121)	50	2	NaF	9		b) calcium 500 Vit. D 50 µg	calcium 500								
Grove & Halver, 1981	28 (14/14)	74	0.23	NaF	9		calcium 500 Vit. D 360 µg	-							2/12	2/10
Reginster[1] *et al*, 1998	164 (84/80)	64	4	NaMFP	20	(0.32)	calcium 1000	calcium 1000	2/84 2.4% (95% CI, 0.3-8.3)	8/80 10% (95% CI, 4.4-18.8%	15/84	13/80	N.R.			
									p=0.05		N.S.				N.S.	
Gambacciani *et al*, 1995	60 (30/30)	52	2	GluMFP	20		calcium 600	calcium 500	N.R.		N.R.		N.R.		7/21	6/21
Meunier *et al*, 1998									33%	25.4%	all 29/208 = 13.9%	17/146 = 11.6% p=0.53	all 17.8	4.8	59.2%	59.4%
	219 (73/146)	66	2	NaF	22.6	0.37	calcium 1000 Vit. D 20 µg	calcium 1000 Vit. D 20 µg			11/73		19.2%			
	214 (68/146)	66	2	NaMFP	19.8	0.33					7/68		13.2%			
	213 (67/146)	66	2	NaMFP	26.4	0.44			p=0.15		13/67		20.9%		N.S.	
													p=0.001			
Pak[1] *et al*, 1995	110 (54/56)	67	4[5]	NaF[4]	22.6	0.37	calcium 800	calcium 800	7/48	22/51	2/48	4/51	11.1%	14.3%	9.3%	7.1%
	after one year								p=0.001							
	99 (48/51)								=14.6%	=43.1%	p>0.2		p=0.78		p=0.74	
Sebert[2] *et al*, 1995	94 (35/41)	61	2	NaMFP	26.4	0.43	calcium 500	calcium 500	N.R.	N.R.	2/45	0/49	5/45	2/49	10/45	9/49
									p=0.84		p>0.2		p=0.3		p>0.2	

Reference	Sample size (treatment/ placebo)	Age (years)	Duration (years)	Fluoride treatment: Fluoride compound	Dose (mg/day)	Dose (mg/kg/d)[3]	Other substances (mg/day)	Control group	Vertebral fractures: fluoride	Vertebral fractures: control / placebo	Non-vertebral fractures: fluoride	Non-vertebral fractures: placebo	Lower Limb Pain: verum	Lower Limb Pain: placebo	Gastrointestinal symptoms: verum	Gastrointestinal symptoms: placebo
Kleerekoper *et al*, 1991	84 (46/38)	67	4	NaF	34	0.52	calcium 1500	calcium 1500	961/1000 patient years	723/1000 patient years	13/46 =28%	7/38[6] =18%	N.R.		16/46 =35%	6/38 =16%
									p=031		p=0.29				p=0.05	
Riggs *et al*, 1990	202 (101/101)	68	4	NaF	34	0.56	calcium 1500	calcium 1500	47.0/100 person years	52.5/100 person years	23/100 person years	7/100 person years	54	24	17	7
									RR 0.85 (95% CI, 0.6-1.2)		RR 3.2 (95% CI, 1.8-5.6)		RR 3.0 (95% CI, 1.9-4.8)		RR 2.9 (95% CI, 1.2-7.1)	
									N.S.							
Riggs[7] *et al*, 1994	151 (50/101)	66	2	NaF	31.5	0.52	calcium 1500	see Riggs *et al*, 1990	45/100 person years (0-6 years)		21/100 person years (0-6 years)		N.R.		N.R.	
									32/100 person years (4-6 years)		13/100 person years (4-6 years)					
Riggs[1] *et al*, 1982	165 (33/104)	63	4	NaF	57		calcium 800-1500 Vit. D 10 µg or 360 µg	10 µg Vit. D and calcium 1500-2000 and/or estrogens 0.625-2.5 and/or 360 µg Vit. D	304/1000 patient years	834/1000 patient years (10 µg Vit. D only)	N.R.		14/61	0/104	10/61	0/104
	(28/104)	63	4	NaF	57		calcium 1000-5000 estrogens 0.625-2.5 Vit. D 10 µg or 360 µg		53/1000 patients years							
									p<0.000001							

N.S. not significantly different
N.R. not reported
NaF sodium fluoride
NaMFP sodium monofluorophosphate
GluMFP L-glutamine monofluorophosphate

1) 10-33% of patients continued hormone replacement therapy
2) study population includes three male patients; 18 patients were not included in assessment of bone mineral density
3) dosis divided by reported mean body weight
4) slow release preparation
5) four cycles of 12 months treatment plus 2 months treatment pause
6) excluding "incomplete fractures" identified by ^{99}Tc-bone scans which occurred significantly (p=0.02) more often in the lower extremities of patients on fluoride treatment
7) 50 patients under fluoride treatment in the study of Riggs *et al*, 1990, continued treatment for another 2 years

4. DOSE-RESPONSE ASSESSMENT

4.1. Skeletal fluorosis, bone density, fractures

4.1.1. Bone density and bone strength

Bone density increases with increasing fluoride content of bone as a consequence of an increasing fluoride intake both in animals and in humans. This effect is observed predominantly in cancelleous bone. This increase in bone fluoride content is accompanied by an increase in bone strength up to a certain level, thereafter bone strength decreases. Turner *et al* (1992; 1995) showed in rats drinking for 16 weeks water with 16 mg fluoride/L (corresponding to an estimated intake of 0.11 mg/kg body weight/day), that the fluoride content in bone was ≤ 1200 mg/kg and bone strength increased by 38%, whereas rats drinking water with fluoride contents between 50-128 mg/L (corresponding to 2.5-7.2 mg/kg/day accumulated 10,000 mg/kg fluoride in bone and strength decreased by 20%.

Trabecular bone compressive strength in autopsy samples from the iliac crest was significantly ($p<0.05$) higher in women from Kuopio with fluoridation of drinking water (0.97 mg/L) than in women from an area with low fluoride content in the water (0.02-0.32 mg/L), however, no significant difference was found in men (Alhava *et al*, 1980). There are no reliable measures for bone strength in humans. The available data are of uncertain relevance with regard to the risk for bone fractures and insufficient for conclusions on effective fluoride doses.

4.1.2. Skeletal fluorosis

The asymptomatic stage of skeletal fluorosis is associated with fluoride contents in bone ash of 3500-5500 mg/kg. Clinical stages I and II plus III have been found to have fluoride contents of 6000-7000 and >7500 mg/kg bone ash, respectively (Hodge and Smith, 1977). There are no parallel data on the fluoride intake associated with these levels of fluoride in bone.

Fluoride content of the skeleton increases with increasing intake of fluoride via water. In areas with water fluoride contents of <0.3, 1.0 and 4 mg/L fluoride in bone ash was 140-790, 400-2300 and 6900 mg/kg, respectively (Alhava *et al*, 1980; Bergmann, 1994; Zipkin *et al*, 1958). From studies in China and India a correlation between the fluoride content in drinking water and skeletal fluorosis can be deduced. Prevalences of 4.4% at water fluoride levels of 1.4 mg/L and of 63% at water fluoride levels of 6 mg/L were observed in India. Crippling fluorosis was consistently found in villages with more than 3 mg fluoride/L. An estimated total fluoride intake of 20 mg/day was associated with a fluorosis prevalence of 34%, whereas no fluorosis was observed in areas with an estimated total fluoride intake of less than 10 mg/day. Skeletal fluorosis started to appear after 10 years of residence in a village with an estimated daily fluoride intake of 36-54 mg/day and concerned 100% of the population after 20 years. Precise intake estimates from regions with higher fluoride concentrations in drinking water are lacking. Fluoride intake from diet and water in adults was estimated to be 0.84-4.69 mg/day in Indian villages without endemic skeletal fluorosis and 3.4-27.1 mg/day in fluorosis-prone villages (IPCS, 2002).

Numerous epidemiological data support a linear relationship between fluoride intake and bone fluoride content and between bone fluoride content and both incidence and severity of skeletal fluorosis In the few cases of clinical skeletal fluorosis in which the fluoride intake could be estimated it ranged from 15 to 20 mg/day and the period of exposure was over 20 years. A more precise threshold dose for fluoride causing skeletal fluorosis can not be defined.

The Panel decided not to chose the data on skeletal fluorosis in relation to the fluoride content of the drinking water as the critical endpoint for setting an UL because too many assumptions on the effective fluoride dose were necessary.

The Panel decided also not to use the data on the relationship between fluoride intake via drinking water and radiographic skeletal changes decribed in Section 3.2.2.1.1 for setting a UL because of insufficient exposure estimates and the lack of more recent radiographic investigations.

4.1.3. Fractures

4.1.3.1. Observational Data

Although an association of an increased risk for hip fractures in the elderly with the fluoride content in drinking water has been reported, the opposite has been found as well (Jacqmin-Gadda *et al*, 1998; Kurttio *et al*, 1999; Li *et al*, 2001; Sowers *et al*, 1986) or no association (Hillier *et al*, 2000; Karagas *et al*, 1996; Kröger *et al*, 1994).

In one study from China a bimodal relationship between fluoride content in drinking water and fluoride intake per day and risk of overall fractures was apparent. Compared to an exposure of 3.4 mg fluoride/day there was a significantly increased risk for fractures at all sites (OR 1.47; p=0.01) and for hip fracture (OR 3.26; p=0.02) at an exposure of 14.1 mg fluoride/day. A fluoride exposure of about 6.5 mg/day was associated with a non-significant increase in the risk of hip fracture (OR 2.13; p=0.15) compared to an exposure of 3.4 mg fluoride/day. Compared to a fluoride exposure of 3.4 mg/day there was a significantly increased risk for fractures at all sites at an exposure of 0.7 mg/day (OR 1.5; p=0.01) (Li *et al*, 2001).

In the retrospective cohort study in Finland which involved 144,627 persons an increasing risk for hip fracture in women between 50 and 65 years of age with increasing fluoride concentration in drinking water was found. This relationship was significant for concentrations of 0.5-1.0 and of >1.5 mg/L compared to less than 0.1 mg/L (Kurttio *et al*, 1999).

The study by Li *et al* (2001) is considered as evidence that an increased risk of bone fractures occurs at a total intake of 14 mg fluoride per day and that there are data (although statistically not significant) suggestive of an increased risk of adverse bone effects at total intakes above about 6.5 mg fluoride/day. The study of Kurttio *et al* (1999) is considered as supportive (IPCS, 2002).

4.1.3.2. Therapeutic studies

From therapeutical studies with fluoride administration in postmenopausal women of 0.25-6 years duration and which employed fluoride doses between 0.13 and 1.1 mg/kg body weight per day either as sodium fluoride or monofluorophosphate it appears that side-effects in the form of lower limb pain occurred in a significantly higher frequency when fluoride doses of more than 0.4 mg/kg body weight were administered compared with the placebo group. Lower limb pain was indicative of incomplete fractures of the bone (Kleerekoper *et al*, 1991; Meunier *et al*, 1998; Riggs *et al*, 1982, 1990 and 1994).

In one study involving 101 subjects in the fluoride treatment group (0.56 mg fluoride/kg body weight/day) and 101 subjects in the control group, of which two thirds completed the four-year study period, there was a significant increase in the occurrence of non-vertebral fractures (72 versus 24), with an odds ratio of 3.2 (95 CI 1.8-5.6). Vertebral fracture rate increased by 11% for each 1 μM (19 μg/L) increase in serum fluoride over baseline and it decreased with increasing bone mineral density of the lumbar spine. However, if this increase in bone mineral density went beyond 1.2 g/cm^2 an increase in vertebral fracture rate was observed (Riggs *et al*, 1990; 1994). Fifty women from the fluoride group continued treatment for an additional two years, but only nine of these with 34 mg fluoride/day corresponding to 0.56 mg/kg/day (as sodium fluoride). The fluoride dosis in the other 41 women had been reduced because of side effects or by the patients themselves; four women took less than 18 mg fluoride/day. The lumbar spine, femoral neck, and femoral trochanter bone mineral density continued to increase and the bone mineral density of the radius continued to decrease. The vertebral fracture rate decreased compared to the years 0-4. The non-vertebral fracture rate decreased also but was still 3 times higher after six years than in the control group (Riggs *et al*, 1994).

The Panel considers the fluoride dose of 0.56 mg/kg body weight per day, rounded up to 0.6 mg/kg/day to include the usual dietary intake from food and water, as the dose associated with a significant increase in the occurrence of non-vertebral fractures.

4.2. Dental fluorosis

Enamel fluorosis is caused by fluoride ingestion during the preeruptive formation and maturation of enamel of teeth. Therefore, the sensitive period is before the age of eight years. There is a clear dose-response relationship with a prevalence of 48% of very mild and mild forms of dental fluorosis at fluoride intakes from water of 0.043 mg/kg/day (Fejerskov *et al*, 1996a). Very mild forms of dental fluorosis are of aesthetic concern only. From the data of Dean (1942), it appears that in areas with a fluoride content of water of 1 mg/L 10-12% of the residents had mild forms of fluorosis (very mild plus mild). The fluoride intake of children in these communities was found to be 0.02-0.1 mg/kg body weight/day. In areas with a fluoride concentration in water of ≤ 0.3 mg/L the fluorosis prevalence was 1%, whereas it was 50% in areas with a fluoride concentration in water of 2 mg/L, and in these areas a few cases (<5%) of moderate fluorosis were observed. The fluoride intake by children in these communities was 0.08-0.12 mg/kg/day. A fluoride dose of 0.1 mg/kg body weight/day was, therefore, described as a “threshold” dose for the occurrence of less than 5% of moderate forms of dental fluorosis in a population for the ages from birth to eight years (Dean, 1942; Fejerskov *et al*, 1996a).

The Panel concludes that an intake of 0.1 mg fluoride/kg body weight/day in children up to the age of eight years can be considered as the dose below which no significant occurrence of moderate forms of fluorosis in permanent teeth will occur.

4.3. Gastrointestinal effects

Gastrointestinal symptoms like nausea, vomiting, anorexia, diarrhoea occur with fluoride intakes that also result in skeletal effects, i.e., with doses above 0.5 mg/kg body weight/day (Kleerekoper *et al*, 1991; Riggs *et al*, 1990). However, these effects are more unpredictable and presumably dependent on other dietary factors like fluid intake and type of diet.

Severe clinical symptoms were observed in 22% of children on acute single dose ingestion of sodium fluoride amounts of about one mg fluoride/kg body weight (Augenstein *et al*, 1991).

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF THE TOLERABLE UPPER INTAKE LEVEL (UL)

The Panel has identified different critical endpoints for the derivation of the UL of oral fluoride intake for the age from one to eight years (moderate dental fluorosis) and for all ages above eight years (bone fracture). Different ULs are set for these groups.

1.1. Children up to the age of eight years

The data support a continuous relationship between fluoride intake during the period from birth to eight years of age and both incidence and severity of dental fluorosis. The occurrence of moderate enamel fluorosis was less than <5% in populations at fluoride intakes of 0.1 mg/kg body weight/day. Mild fluorosis is generally considered to be acceptable on a population basis, in view of the concomitant beneficial effect of fluoride in the prevention of caries. No uncertainty factor is deemed necessary to derive an UL from this intake, because it is derived from population studies in the susceptible group. For children up to the age of eight years this intake level of 0.1 mg/kg body weight/day is proposed as the UL. Calculated on a body weight basis the following age-related ULs for daily fluoride intake are set:

Age (years)	Tolerable Upper Intake Level (UL) for fluoride (mg/day)
1-3 years	1.5
4-8 years	2.5

1.2. Children older than eight years and adults

Therapeutic studies with fluoride in postmenopausal osteoporosis suggest an increasing risk for skeletal fractures at or above fluoride intakes of 0.6 mg/kg body weight per day. The Panel decided to apply an uncertainty factor of 5 to the intake of 0.6 mg fluoride/kg body weight/day, because, although the adverse effects were detected in a sensitive group of elderly postmenopausal women, the study duration was relatively short and the studies were not designed to systematically define a LOAEL. The epidemiological data with an observed significantly increased risk for fractures at all sites associated with a long-term total daily intake of fluoride of 14 mg/day are considered as supportive evidence. An intake of 0.12 mg fluoride/kg body weight/day converts on a body weight basis (60 kg) into an UL of 7 mg/day for adults.

On a body weight basis the following ULs are proposed:

Age (years)	Tolerable Upper Intake Level (UL) for fluoride (mg/day)
9-14 years	5
≥15 years	7

1.3. Pregnancy and lactation

There are no data which support the setting of a specific UL. The UL of 7 mg/day applies.

2. RISK CHARACTERISATION

There is a narrow margin between recommended intakes for the prevention of dental caries and the ULs.

2.1. Infants and children up to 8 years

The Panel did not establish an UL for infants. The Panel notes, however, that the maximum level recommended by the SCF for fluoride of 0.6-0.7 mg/L (0.1 mg/100 kcal; 600-700 kcal/L) in infant formula and follow-on formula will result in fluoride intakes of infants during the first half of the first year of life (body weight 5 kg) of about 0.1 mg/kg body weight per day. The maximum recommended fluoride content of formula will be exceeded if water containing more than 0.7 mg/L is used for preparation of the formula.

Breast-fed infants have very low fluoride intakes from human milk (2-40 μg/day) and are not at risk of developing enamel fluorosis even when given fluoride supplements of 0.25 mg/day.

Children will have fluoride intakes from food and water well below the UL provided the fluoride content of their drinking water is not higher than 1.0 mg/L.

An increase in the prevalence of mild dental fluorosis observed in some countries has been attributed to the inappropriate use of dental care products, particularly of fluoridated toothpaste.

2.2. Children older than eight years and adults

The probability of exceeding the UL of 5/7 mg fluoride/day on a normal diet is generally estimated to be low. However, consumption of water with a high fluoride content e.g. more than 2-3 mg/L predisposes to exceeding the UL.

3. RECOMMENDATIONS FOR FURTHER WORK

More reliable data on total daily fluoride intake and the identification of the main sources of fluoride, particularly in young children, are needed. The incidence and severity of dental fluorosis should be monitored as an indicator of fluoride exposure during childhood.

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Annex 1.

I. Development of deciduous teeth (Wei, 1974 cited in Bergmann, 1994)

Tooth	first formation hard substance (months of gestation)	mature enamel (months of life)	eruption (months of life)	root completed (years of life)
Mandibula				
Incisors				
central	4.5	2.5	6	1.5
lateral	4.5	3	7	1.5
Canine	5	9	16	3.25
first praemolar	5	5.5	12	2.25
second praemolar	6	10	20	3
Maxilla				
Incisors				
central	4	1.5	7.5	1.5
lateral	4.5	2.5	9	2
Canine	5	9	18	3.25
Praemolar first	5	6	14	2.5
Praemolar second	6	11	24	3

II. Development of permanent teeth (Wei, 1974 cited in Bergmann, 1994)

Tooth	formation of hard substance [age in months (m) or years (y)]	mature enamel (age in years)	eruption (age in years)	root completed (age in years)
Mandibula				
Incisors				
central	3-4 m	4-5 y	6-7 y	9 y
lateral	3-4 m	4-5 y	7-8 y	10 y
Canines	4-5 m	6-7 y	9-10 y	12-14 y
Praemolars				
first	1.75-2 y	5-6 y	10-12 y	12-13 y
second	2.25-2.5 y	6-7 y	11-12 y	13-14 y
Molars				
first	at birth	2.5-3 y	6-7 y	9-10 y
second	2.5-3 y	7-8 y	11-13 y	14-15 y
third	8-10 y	12-16 y	17-21 y	18-25
Maxilla				
Incisors				
central	3-4 m	4-5 y	7-8 y	10 y
lateral	10-12 m	4-5 y	8-9 y	11 y
Canines	4-5 m	6-7 y	11-12 y	13-15 y
Praemolars				
first	1.5-1.75 y	5-6 y	10-11 y	12-13 y
second	2-2.25 y	6-7 y	10-12 y	12-14 y
Molars				
first	at birth	2.5-3 y	7-8 y	9-10 y
second	2.5-3 y	7-8 y	12-13 y	14-16 y
third	7-9 y	12-16 y	17-21 y	18-25 y

Annex 2. Dental fluorosis

I. Grading of dental fluorosis ("Dean's fluorosis index") (Dean, 1934 and 1942)

Grade	Criteria
Normal (0)	The enamel presents the usual translucent semivitriform type of structure . The surface is smooth and glossy and usually of a pale creamy white colour. Included under this heading are all persons showing hypoplasia other than mottling of the enamel.
Questionable (0.5)	The enamel shows slight aberrations in the translucency of of normal enamel, ranging from a few white flecks to occasional white spots, 1 to 2 mm in diameter. It is recommended that this diagnosis is best made on a group basis comparing groups of children from different areas and with demonstrated use of a common water supply from birth.
Very mild (1)	Small opaque paper white areas are scattered irregularly or streaked over the tooth surface, principally on the labial and buccal surfaces and involving less than 25% of the surface of the affected teeth. Small pitted white areas are frequently found on the summit of cusps. No brown stains are present. Mottling of the enamel of deciduous teeth is invariably of the very mild type, while permanent teeth of the same individual may show severe mottling.
Mild (2)	The white opaque areas on the surfaces of the teeth involve at least half of the tooth surface. The surfaces of molars, bicuspids and cuspids subject to attrition show thin white layers worn off and the bluish shades of underlying normal enamel. Faint brown stains are sometimes apparent, generally on the upper incisors.
Moderate (3)	No change is observed in the form of the tooth, but generally all of the tooth surfaces are involved. Surfaces subject to attrition are definitely marked. Minute pitting is often present. Brown stain is frequently a disfiguring complication.
Severe (includes former grades moderately severe and severe) (4)	A greater depth of enamel is involved, with a smoky white appearance. Pitting is frequent, observed on all the tooth surfaces and is often confluent. The hypoplasia is so marked that the form of the teeth is at times affected. Stains are wide-spread and range from a chocolate brown to almost black in some cases. Teeth often present as corroded.

II. Tooth Surface Index of Fluorosis (TSIF) (Horowitz et al, 1984)

Score	Criteria
0	Enamel shows no evidence of fluorosis.
1	Enamel shows definite evidence of fluorosis, namely areas with parchment-white colour, that total less than one third of the visible enamel surface. This category includes fluorosis confined only to incisal edges of anterior teeth and cusp tips of posterior teeth ("snowcapping").
2	Parchment-white fluorosis totals at least one-third of the visible surface, but less than two-thirds.
3	Parchment-white fluorosis totals at least two-thirds of the visible surface.
4	Enamel shows staining in conjunction with any of the preceding levels of fluorosis. Staining is defined as an area of definite discoloration that may range from light to very dark brown.
5	Discrete pitting of the enamel exists, unaccompanied by evidence of staining of intact enamel. A pit is defined as a definite physical defect in the enamel surface with a rough floor that is surrounded by a wall of intact enamel. The pitted area is usually stained or differs in color from the surrounding enamel.
6	Both discrete pitting and staining of the intact enamel exist.
7	Confluent pitting of the enamel surface exists. Large areas of enamel may be missing and the anatomy of the tooth may be altered. Dark-brown stain is usually present.

III. Thylstrup-Fejerskov (TF)-Score (Thylstrup and Fejerskov, 1978; Fejerskov et al, 1996)

Score	Criteria
0.	Normal translucency of the glossy creamy-white enamel remains after wiping and drying of the surface.
1.	Thin white opaque lines are seen running across the tooth surface. Such lines are found on all parts of the surface. The lines correspond to the position of the perikymata. In some cases, a slight “snowcapping” of cusps/incisal edges may also be seen.
2.	The opaque white lines are more pronounced and frequently merge to form small cloudy areas scattered over the whole surface. “Snowcapping” of incisal edges and cusp tips is common.
3.	Merging of the white lines occurs, and cloudy areas of opacity occur spread over many parts of the surface. In between the cloudy areas, white lines can also be seen.
4.	The entire surface exhibits a marked opacity, or appears chalky white. Parts of the surface exposed to attrition or wear may appear to be less affected.
5.	The entire surface is opaque, and there are round pits (focal loss of outermost enamel) that are less than 2 mm in diameter.
6.	The small pits may frequently be seen merging in the opaque enamel to form bands that are less than 2 mm in vertical height. In this class are included also surfaces where the cuspal rim of facial enamel has been chipped off, and the vertical dimension of the resulting damage is less than 2 mm.
7.	There is a loss of the outermost enamel in irregular areas, and less than half the surface is so involved. The remaining intact enamel is opaque.
8.	The loss of the outermost enamel involves more than half the enamel. The remaining intact enamel is opaque.
9.	The loss of the major part of the outer enamel results in a change of the anatomic shape of the surface/tooth. A cervical rim of opaque enamel is often noted.

OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF POTASSIUM

(REQUEST N° EFSA-Q-2003-018)
(ADOPTED ON 22 FEBRUARY 2005)

SUMMARY

Potassium is an essential nutrient involved in fluid, acid and electrolyte balance and is required for normal cellular function. Dietary deficiency of potassium is very uncommon due to the widespread occurrence of potassium in foods. Available evidence suggests that potassium can modulate blood pressure and increasing dietary potassium intake is associated with lower blood pressure.

Gastrointestinal symptoms (discomfort, mucosal lesions and sometimes ulceration) have been seen in healthy subjects taking some forms of potassium supplements (e.g. slow-release, wax matrix formulations) with doses ranging from about 1 to 5 g potassium per day, or more, but incidence and severity seem to be more dependent on the formulation than on dose. In healthy adults administration of single doses of 5-7 g potassium or more (as chloride or bicarbonate solutions) have been reported to cause elevated plasma potassium, adverse changes in heart function and peripheral nerve symptoms in a limited number of case reports.

In subjects with impaired kidney function and reduced urinary potassium excretion, elevated plasma potassium with adverse effects on heart function have been reported with intakes of potassium in the form of supplements or sodium-reduced salts equivalent to 1 g potassium per day or more in addition to food.

The available data are insufficient to establish a safe upper intake level for potassium.

Based on estimates of current potassium intakes in European countries, the risk of adverse effects from potassium intake from food sources (up to 5-6 g/day in adults) is considered to be low for the generally healthy population. Long-term intakes of about 3 g potassium per day as potassium chloride supplements, in addition to intake from foods, have been shown not to cause adverse effects (elevated plasma potassium or gastrointestinal symptoms) in healthy adults. However, a few case studies have reported that supplemental potassium in doses of 5-7 g/day can cause adverse effects on heart function in apparently healthy adults. In addition, gastrointestinal symptoms have been seen in healthy subjects taking some forms of potassium supplements with doses ranging from about 1 to 5 g potassium per day.

Certain groups, particularly those with impaired kidney excretion of potassium, are sensitive to adverse effects of increasing potassium intake on heart function associated with increases in plasma potassium. These include subjects engaging in strenuous activities leading to dehydration, with diabetes mellitus, with impaired kidney function, on cardiovascular disease drug treatment or other metabolic disorders affecting potassium balance. Elderly people may be more vulnerable to adverse effects of potassium due to reduced kidney function or due to use of drugs affecting potassium balance.

KEY WORDS

Potassium, tolerable upper intake level, gastrointestinal effects, hyperkalaemia, food safety.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

1 - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Potassium is widely distributed in the earth's crust, seawater as a mono-valent cation. It occurs naturally in the form of several mineral salts but does not occur as metallic potassium. Potassium in foods is associated with salts of weak organic acids. Various potassium salts, e.g. KCl, are used in many applications, amongst others as ingredients in foods (e.g. additives), food supplements and drugs, household chemicals etc. In this opinion, the term potassium refers to ionic potassium, except where specific potassium compounds are stated. One mmol potassium is equivalent to 39.1 mg.

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

2.1. Food levels and dietary intake

Important potassium sources include potatoes, fruit and berries, vegetables, milk products (excl. cheese) and nuts. Potassium occurs in foods mainly associated with weak organic acids. Potassium is also found in mineral, spring, and table waters, but the content varies considerably. Some mineral waters available on the market can, when consumed in large quantities, contribute significantly to the daily intake. The average dietary intake of potassium according to European food consumption studies is in the range of 3000 to 4000 mg/day. (Table 1). The 95th to 97th percentile intake is in the range of 4000-5500 mg/day.

A number of food additives also contain potassium as the cation. The level of potassium added to foods as additives generally contribute only to a minor degree to the daily intake. Salt substitutes, in which part of the sodium chloride has been substituted with potassium salts (usually KCl), can contribute to the potassium intake.

Food supplements can contribute significant amounts of potassium (usually as KCl), but according to recent food consumption surveys average reported contributions were only up to 5 % of the total potassium intake (see Table 1).

Table 1. The daily intakes of potassium in some EU countries (mg/day)

	Population	N	Method	Supplements	Mean	97.5%
Denmark[a]	males 1-80 y females 1-80 y	1516 1582	7-day record	- -	3500 2900	5200 4300
Finland[b]	men women	912 1095	48-h recall	- -	4000 3200	- -
Germany[c]	men women men women	1268 1540 240 347	Computer-assisted dietary interview	- - + +	3930 3240 4140 3360	- - - -
Netherlands[d]	household	6250	2-day record	-	3448	5339
Sweden[e]	men women	589 626	7-day record	- -	3540 3060	5300 4400
UK[f]	men women men women	833 891 833 891	7-day record	- - + +	3367 2653 3371 2655	5504 4183 5504 4183

[a] Andersen *et al* (1995) - values are means and 95th percentiles.
[b] Männistö *et al* (2003).
[c] Mensink and Ströbel (1999).
[d] Hulshof *et al* (1998) - values are mean and 95th percentiles.
[e] Becker and Pearson (2002) - values are means and 95th percentiles.
[f] Henderson *et al* (2003).

2.2. Nutritional requirements and recommendations

Recommended daily intakes in Europe are in the order of 3.1-3.5 g/day (SCF, 1993). The US Food and Nutrition Board have set an intake of 4.7 g potassium per day from food as an adequate intake, mainly based on the beneficial effects on blood pressure (FNB, 2004).
The losses of potassium via the gastrointestinal tract, urinary excretion and sweat, comprises about 800 mg/day (20 mmol), but 1.6 g/day (40 mmol) is needed to avoid low plasma levels and loss of total body potassium in adults (SCF, 1993).

Potassium deficiency can develop as a consequence of increasing losses from the gastrointestinal tract and kidneys, e.g. during prolonged diarrhoea or vomiting, and in connection with use of laxatives or diuretics. Potassium deficiency due to low dietary intake only is very uncommon, due to the widespread occurrence of potassium in foods. Treatment with diuretics without potassium compensation can, however, lead to deficiency. Symptoms of potassium deficiency are associated with disturbed cell membrane function and include muscle weakness, disturbances in heart function, which can lead to arrhythmia and heart seizure. Mental disturbances, e.g. depression and confusion, can also develop.

The potassium intake may affect sodium balance and low potassium intakes (10-30 mmol/day) may induce sodium retention and an increase in blood pressure, both in normotensive and hypertensive subjects (Gallen *et al*, 1998; Morris *et al*, 1999; Coruzzi *et al*, 2001).

A number of studies of both normotensive and hypertensive subjects indicate that an increased potassium intake, mainly given as a supplement, can lower blood pressure and increase urinary sodium excretion (Whelton *et al*, 1997; Geleijnse *et al*, 2003; Sacks *et al*, 1998; Gu *et al*, 2001; Naismith and Braschi, 2003). However, not all studies showed a clear dose-response effect which could be due to factors such as differences in duration of studies, initial blood pressure, sodium intake, habitual diet, race and age. Other clinical trials and population surveys also indicate that a diet rich in potassium alone, or in combination with calcium and magnesium, may have a favourable effect on blood pressure (Appell *et al*, 1997; Sacks *et al*, 2001; Jula *et al*, 1990; Geleijnse *et al*, 1997; He and MacGregor, 2001).

2.3. Function, uptake and distribution

The total body potassium is estimated to be approximately 135 g in a 70 kg adult man. Extra-cellular potassium, which constitutes around 2% of the body pool, is important for regulating the membrane potential of the cells, and thereby for nerve and muscle function, blood pressure regulation etc.

Potassium also participates in the acid-base balance. The major part of the potassium in the body (98%) is found in the cells where it is the main intracellular cation. Thus intracellular concentrations are substantially greater than extracellular concentrations. A large proportion of the body pool of potassium is found in muscle and the skeleton, and it is also present in high concentrations in the blood, central nervous system, intestine, liver, lung and skin.

The absorption of potassium is effective and about 85-90% of the dietary potassium is normally absorbed from the gut (EGVM, 2003; FNB, 2004). The potassium balance is primarily regulated by renal excretion in urine. A small proportion can be lost in sweat. The major excretory route of potassium is via the kidneys. It is secreted by the renal tubules, in exchange for sodium of the glomerular filtrate (ion exchange mechanism). Excretion in sweat and faeces is negligible, the latter changing only slightly as dietary potassium intake varies over a wide range.

The concentration of potassium in plasma is tightly regulated within a narrow range of about 3.5 to 5 mmol/L. The body is able to accommodate a high intake of potassium, without any substantial change in plasma concentration by synchronized alterations in both renal and extra-renal handling, with potassium either being excreted in the urine or taken up into cells. Thus the plasma or extracellular concentration of potassium does not give a clear indication of the body content of potassium.

Both the renal and extra-renal mechanisms through which potassium homeostasis are achieved are complex in nature, and intimately linked to the cellular handling of other minerals, such as sodium, magnesium and calcium, as well as to water homeostasis. The main process through which the body content of potassium is regulated over extended periods of time is renal excretion. Most of the potassium which is filtered in the glomerulus is re-absorbed in the proximal tubule and loop of Henle. Regulated excretion is determined by the rate at which potassium is secreted in the distal tubule and collecting ducts (Wang, 2004). For the normal unadapted kidney, the maximum excretion rate following an oral dose of 8 g potassium chloride (4.2 g potassium) was up to 130 μmol potassium/minute (5 mg potassium/minute) (Berliner *et al* 1950). If sustained this would be equivalent to excreting 7.3 g K+/day (188 mmol/day). The usual diet provides 0.75 to 1.25 mmol potassium/kg body weight/day, or 29-49 mg/kg body weight/day. On a normal diet a glomerular filtration rate (GFR) below 10 mL/min is rate limiting for potassium secretion if the urine output is less than 600 mL/day. However, balance can be maintained with intakes up to 5 to 10 mmol potassium/kg body weight/day (195-390 mg/kg body weight /day), as renal excretion through a healthy kidney which is adapted to high intakes of potassium can effectively excrete potassium at 10 to 20 times the rate of a kidney which has not been adapted to a high intake.

There are effective mechanisms which enable the body to cope with a wide range of habitual intakes of potassium. These involve complex changes in the kidney, colon and muscle over the shorter and longer term. In response to a large increase in dietary potassium intake, insulin-mediated uptake into skeletal muscle (and probably liver) is increased (Wang, 2004). This transfer of potassium from the extra-cellular to the intracellular space minimizes any rise in plasma potassium concentration in the short term. The potassium which has been buffered by uptake into muscle is eventually released into the extra-cellular fluid during the post-prandial period, and excreted through the kidney. There is a short term renal response to increased potassium in the diet, with stimulation of potassium secretion in the collecting duct within hours of a potassium rich meal. The kidney responds to a sustained increase in potassium intake through a decrease in absorption of potassium in the proximal tubules and adaptive changes in the collecting duct leading to prolonged enhancement of excretion. The combination of insulin mediated buffering in muscle and enhanced renal secretion in the short term, and more marked renal adaptive changes in the long term combine to ensure that plasma levels are maintained within narrow limits when potassium intake is increased. The uptake of potassium into muscle appears reduced in insulin resistant states, such as obesity, and consumption of high fat diets. Presumably this capacity for muscle to hold potassium is finite and therefore on a sustained high intake of potassium, the ability to cope with the dietary intake will be determined by the maximal rate of renal excretion, plus any increase in loss through the distal colon. Colonic losses of potassium may achieve 10 to 20 mmol/day, when glomerular filtration rates fall below 30 mL/minute (from the normal 130 mL/minute).

Therefore, the adverse effects of prolonged higher intakes of potassium are determined by a) local effects on the gastrointestinal tract, and b) metabolic effects determined by the maximum capacity for renal excretion, and to a lesser extent colonic excretion.

3. HAZARD IDENTIFICATION

The available animal data are of limited relevance to human risk assessment and this section is limited to selected considerations of oral toxicity.

3.1. Animal data

3.1.1. Acute toxicity

Acute oral administration of potassium to animals causes changes in acid-base balance, hyperkalaemia, changes in respiratory rate and hypernatraemia. Acute oral administration of potassium chloride in animals has been reported to cause death by respiratory failure, with gastroenteritis and renal tubular necrosis (EGVM, 2003). In rats the oral LD_{50} of KCl is reported to be 2.4-3.0 g/kg body weight (Von Oettingen, 1956; Boyd and Shanas, 1961). The acute toxicity of potassium bromate and potassium iodate has been studied in rats and dogs (Kurata *et al*, 1992; Webster *et al*, 1966). Four of five rats given single intragastric doses of 600 mg potassium bromate died within 24 hours of dosing, while the minimal lethal dose in dogs given orally administered potassium iodate was estimated to 200-250 mg/kg body weight. The higher toxicity of these potassium salts compared to KCl can be attributed to the anions.

3.1.2. Subacute/subchronic toxicity

Effects produced with potassium nitrate (hypertrophy of the adrenal zona glomerulosa) and potassium iodate (haemosiderin deposition in the renal tubules) were attributed to the anions (i.e. the nitrate and iodate moieties) (EGVM, 2003).

3.1.3. Carcinogenicity

There are limited data on the carcinogenicity of potassium chloride (Lina and Kuijpers, 2004). Potassium bromate, potassium iodide and potassium hydrogen carbonate produced cancers in experimental studies, but the effects were attributed to the anions (i.e. the bromate, iodide and hydrogen carbonate moieties) and are thus not relevant to this risk assessment (EGVM, 2003).

3.1.4. Genotoxicity

There are no data on genotoxicity of potassium chloride.

3.1.5. Reproductive toxicity

There are no data on reproductive toxicity of potassium chloride.

3.2. Human data

Daily intakes of potassium from the habitual diet generally do not exceed 5-6 g/day and has not been associated with any negative effects in healthy individuals. Elderly people may be more vulnerable to potassium toxicity due to reduced physiological reserve in renal function. Ageing is associated with a progressive loss of kidney volume and GFR fall with each decade (Beck, 1998). This and changes in for example renin release leads to decreased capacity for potassium secretion and thus limits the ability to handle large potassium loads. Elderly are therefore more vulnerable to potassium overload due to increased intake from diet and/or supplements or due to drugs affecting potassium balance. Individuals with pre-existing renal disease, hyperkalaemia, adrenal insufficiency, acidosis or insulin deficiency are also vulnerable, as are those using certain drugs, such as potassium-sparing diuretics, β-adrenergic blockers, angiotensin-converting enzyme (ACE) inhibitors, digitalis, non-steroidal anti-inflammatory drugs. Infants may also be vulnerable to excessive potassium due to limited excretion capacity and immature function (EGVM, 2003).
In some situations for therapeutic purposes relatively large amounts of oral potassium chloride might be given as a matter of course with substantial benefit and no adverse consequence. Low body potassium is ubiquitous in people who are severely malnourished, and in treatment a high priority is given to the provision of potassium. The World Health Organization recommends that up to 4 mmol (156 mg) potassium/kg body weight/day is given as an oral supplement of potassium chloride once an adequate flow of urine has been established, in the acute treatment of infants and young children (WHO, 1999). One report suggests that over 7 mmol (274 mg)/kg body weight/day of potassium might be tolerated without adverse effects in some situations (Manary and Brewster, 1997).

Intake of potassium chloride has been associated with acute poisoning in humans. Case reports have described heart failure, cyanosis and cardiac arrest after ingestion of high doses of potassium chloride tablets (see section 3.2.1).

Gastrointestinal toxicity has also been described after chronic ingestion of potassium chloride in case studies and supplementation studies. This is characterised by abdominal pain, nausea and vomiting, diarrhoea, and ulceration of the oesophagus, stomach and duodenum and ileum.

3.2.1. Hyperkalaemia and cardiac effects

Short-term studies (2-3 weeks) on healthy adults have shown that serum potassium levels were within normal ranges at intakes up to around 15 g potassium per day, provided that fluid intake is sufficient and that intake is evenly distributed over the day (FNB, 2004; Rabelink *et al*, 1990). In a metabolic ward study by Rabelink *et al* (1990) six healthy young subjects (3 males and 3 females) were given a KCl solution to the meals (in total 3.9 g K per meal), which were provided every sixth hour during a 20-day period, i.e. one meal was given during the night, as well 200 mL water was given hourly. The total intake of potassium was 15.6 g/day. Plasma potassium levels rose initially and the mean level after 48 hours was 4.77 mmol/L. About 95% of the ingested potassium was excreted in the urine. The plasma levels then decreased and remained stable throughout the study period. A similar, but more pronounced pattern was seen for aldosterone and plasma renin activity. There was an indication of some initial volume loss, e.g. fall in body weight, which normalised during the study period. This study indicates that intakes up to about 15 g/day, distributed over the day and with adequate fluid intake, may be tolerated in healthy subjects without exceeding the normal range of serum potassium, at least under metabolic ward conditions.

In a long-term study supplementation of the sodium-restricted diet with 3.7 g potassium per day given as KCl tablets 3 times a day was not reported to lead to hyperkalaemia in hypertensive males, although serum potassium levels increased during the first six months compared to the placebo group (Grimm *et al*, 1990). In this study the dietary potassium intake was not given, but can be roughly calculated from baseline data on urinary excretion, which was given per 8 hours (overnight). The mean intake from diet is estimated to about 2.5-3 g/day and the 97.5 percentile to about 4.5-5 g/day (allowing for absorption and incomplete urinary data). The KCl tablets provided 3.7 g/day, but the mean intake was about 3.1 g/day, taking compliance into account. Thus, the estimated total high intake would be about 7-8 g/day.

Acute high doses of potassium might, however, exceed the capacity of the kidney to eliminate potassium and thereby lead to elevated serum potassium levels and disturbed clearance of for example urea. In a study by Keith *et al* (1941) seven normal subjects received single doses of 9.5-17.5 g potassium chloride or bicarbonate (4.9-6.8 g K) in solutions after having a standardized breakfast. In two of the subjects, who received a single dose of 12.5 or 17.5 g potassium chloride or bicarbonate, respectively (6.5-6.8 g K), symptoms as increased T-wave ECG and paresthesia of hands and feet in parallel with marked/or severe hyperkalaemia (8 mmol/L) were observed within 2-3 hours. However, symptoms did not appear in other subjects receiving the same amount of potassium. The data indicate that acute intakes of 80-100 mg/kg body weight (equivalent to 4.8-6 g for a 60 kg person) could cause acute adverse effects in some apparently normal subjects. In subjects with impaired kidney function the capacity to eliminate potassium is limited and a number of drugs also influence potassium elimination. In such cases lower doses of potassium may affect potassium homeostasis negatively.

Case reports of adverse effects associated with high doses of potassium containing supplements (KCl) and salt substitutes have described chest tightness, nausea and vomiting, diarrhoea, hyperkalaemia, shortness of breath and heart failure. The reported doses causing acute effects were 1-94 g/day in adults and 1.5-7 g/day in infants (see Annex 1). Fatal cases of acute or chronic potassium intake have been reported. For example, a fatality resulted from hyperkalaemia and resultant asystole after ingestion of 21 g of salt substitute representing an oral bolus of 11 g potassium (Restuccio, 1992). A 2 month-old boy died after being given three doses of 1.5 g potassium chloride in two days (2.3 g potassium in total), with breast milk over one and a half days (Wetli and Davis, 1978).

Severe cardiac complications (fatal and non-fatal) and hyperkalaemia have also been reported following sub-chronic and chronic ingestion of salt substitutes or supplements.

A 75 year-old women with previous myocardial infarction developed heart failure after 6 weeks of consuming salt substitutes and a low-sodium diet (Snyder *et al* 1975).

Schim van der Loeff *et al* (1988) report of a 29-year-old woman who suffered a cardiac arrest, due to profound hyperkalaemia, which was attributed to the use of a potassium-containing salt substitute. The patient was resuscitated, but post-hypoxic brain damage occurred.

A 31 year-old body builder developed ventricular tachycardia and collapse due to myocardial infarction, while consuming potassium supplements (5 g/day, duration unknown) in addition to anabolic steroids, amphetamines and potassium sparing diuretics (Appleby *et al,* 1994).

Parisi *et al* (2002) report a case of a 14-year old football player suffering from premature ventricular beats. He used to take regularly a hydrosaline supplementation, which gave him a daily intake of potassium of about 5 g. Hyperkalaemia was found. After refraining from potassium supplementation and sport for 3 months clinical examination showed no ventricular arrhythmias and plasma concentration was normal.

In subjects with impaired kidney function high potassium intakes from diet and potassium containing salt substitutes may lead to hyperkalaemia. A typical case is reported by Doorenbos *et al* (2003), in which a 74-year old woman with end stage renal disease developed severe hyperkalaemia after use of a potassium-containing salt substitute, of which at least two-thirds was potassium chloride. After ceasing to use the salt substitute, no further episodes of severe hyperkalaemia occurred.

These case reports emphasize the potential risk of excessive use of salt substitutes and supplements, especially when used by subjects who are predisposed to retain potassium.

3.2.2. Gastrointestinal effects

Administration of potassium as KCl supplements has been associated with negative effects on the gastrointestinal mucosa. The majority of studies refer to patients treated with potassium supplements. Reported side effects include mild mucosal lesions to ulceration, sometimes leading to death. The occurrence and severity of the effects depend on a number of factors of which formulation of the preparation, dose and gut transit time seem to be the most important.

Other symptoms such as nausea, stomach pain, vomiting and diarrhoea have been reported in supplementation studies but they were often seen in the control groups as well (Svetkey *et al*, 1987; Grimm *et al*, 1990; Gonzalez *et al*, 1998)

McMahon *et al* (1982) studied the effects of two types of potassium chloride supplements (microencapsulated or wax-coated) on gastrointestinal lesions. Forty-eight healthy volunteers were given a supplement of 96 mmol/day (3.7 g) or 24 mmol (0.9 g) potassium for a week. Twelve subjects were given glycopyrrolate and either of the KCl preparations. The remaining 24 subjects were randomised on either preparation without glycopyrrolate. Subjects were gastroscoped, the endoscopist being blind to the type of preparation taken. Wax-matrix formulations were associated with a higher incidence of upper gastrointestinal lesions (score 26-41) than microencapsulated (score 0-3). Lesion scores were of the same magnitude on 96 mmol/day and 24 mmol/day - score 30 and 26, respectively. The lesions were not accompanied by epigastric symptoms. Glycopyrrolate, given to delay gastric emptying, was associated with higher lesion score for gastric and duodenal side effects. Gastrointestinal erosions occurred with only mild symptoms being apparent. The total potassium intake was not stated.

In another study by the same group (McMahon *et al*, 1984), eight controlled 1- or 2-week experiments involving 225 healthy male subjects and one study of 18 patients with hypertension, nine of whom were long-term users of a wax-matrix potassium chloride preparation, were conducted to evaluate the upper gastrointestinal safety of oral KCl supplements. Subjects were given either wax-matrix KCl tablets, KCl liquid, microencapsulated KCl, a potassium-sparer, or placebo and were examined after treatment. Some subjects received an anticholinergic drug with treatment to induce delayed gastric motility. Results indicated that upper mucosal injury, particularly erosions (43%) and ulcerations (11%), were more frequent after wax-matrix tablets. These changes occurred less frequently after liquid KCl (0%), microencapsulated KCl (10.5% erosions, 1.2% ulcers), and the potassium-sparing drug (0%). More serious and more frequent lesions were associated with slowed motility. No occult bleeding was noted. Symptomatic complaints did not correlate with endoscopic findings. In the long-term study with patients with hypertension, endoscopic examination after 19 to 23 months on KCl showed that six of nine of the patients given a wax-matrix KCl supplement had significant lesions. One had developed ulceration after 7 days. However, the nature of the placebo given was not stated and the incidence of mucosal damage was higher in the placebo group than for subjects given some of the potassium preparations.

McLoughlin (1985) compared 7-day administration of three different forms of KCl preparations (wax matrix, microencapsulated and controlled release systems) administered three times daily in 45 healthy subjects. Oesophagus, stomach and duodenum were examined with endoscopy. The preparations were given in a random order and endoscopist was unaware of the order. Seven of the 15 subjects taking wax matrix KCl

showed erosions and two showed hyperaemia only. Of the 15 subjects taking microencapsulated KCl, one showed erosions and two showed hyperaemia only, while none of those taking the controlled release preparation showed erosions and four had hyperaemia. The dosage was not stated.

Small bowel ulceration at an incidence of 3 per 100,000 patient year of medication in 13 surgical clinics in Stockholm County treated with slow-release (wax matrix) KCl tablets during 1970-83 (Leijonmarck and Räf, 1985). The figure is higher than figures given by the authors for the USA, 1 per 100, 000.

A large number of studies have investigated the preventive effect of potassium supplementation on hypertension and heart disease (Whelton *et al*, 1997). The study groups included both normal, healthy subjects and subjects with hypertension and heart disease. The majority of these studies have shown beneficial effects of potassium supplementation (usually as KCl). Although adverse effects have not generally been reported, except gastrointestinal effects, it is often unclear whether adverse effects were investigated. In the study by Grimm *et al* (1990) supplementation with 96 mmol microcrystalline KCl (3.7 g K, with an effective dose of 3.1 g) or placebo for 2 years the reported incidence of side effects was comparable in the placebo and treatment groups. In a double-blind, placebo-controlled study by Svetkey *et al* (1987) 101 subjects with mild hypertension were allocated either 120 mmol microencapsulated KCl (4.7 g K in 5 capsules 3 times daily) or placebo for 8 weeks. Side effects of mostly mild character were reported in both groups and confined to a few subjects. Subjects in the treatment group reported somewhat more frequently abdominal pain (18% vs 9%) and belching or flatulence (20% *vs.* 10%). One subject in the treatment group and two in the placebo group discontinued the study due to side effects. The authors state that clinically evident irritation of the gastric mucosa did not occur, nor was occult gastrointestinal bleeding detected. Obel (1989) reported no notable untoward effects in 48 subjects with mild hypertension given 64 mmol/day of potassium (2.5 g K) supplements for 16 weeks.

A few studies have investigated gastrointestinal effects of other potassium salts than KCl. In a randomised, controlled trial Gonzalez *et al* (1998) compared effects on the gastric mucosa of potassium-magnesium citrate with potassium citrate and placebo in 36 healthy adults. Five tablets providing 70 mmol potassium (2.7 g K) or placebo per day were given for 7 days. In addition all subjects took 2 mg/day of glycopyrrolate to delay gastric emptying. On day 8, stools were examined for occult blood and an oesophago-gastroduodenoscopy was performed. Mucosal lesions were scored at five anatomic sites. No significant differences were observed in the endoscopic scores at any site, or in the total lesion scores among the three groups. Erosion or ulcers were found in about 20% of the subjects with no differences between the groups. However, subjects receiving both potassium supplements more frequently reported symptoms like epigastric pain, cramps and loose stools.

In a randomised, crossover study Overlack *et al* (1995) investigated the effect of potassium citrate or chloride supplementation on blood pressure in 25 patients with essential hypertension. Tablets providing 120 mmol/day (4.7 g K) or placebo were given for 8 weeks. Unwanted, unspecific gastrointestinal side effects were observed in 12 patients on KCl, 10 patients on potassium citrate and two patients on placebo. In two patients these were stated to be mild. More severe symptoms were reported in one patient receiving potassium citrate (headache, dizziness and fatigue) and in one patient receiving potassium chloride (*ulcus duodeni*), which needed treatment. Four patients ceased to take the potassium supplements for personal reasons. In another study (Overlack *et al* 1991) the authors compared the effect of a potassium citrate/bicarbonate supplement with placebo on blood pressure in 12 patients with essential hypertension. However, no information on side effects was given.

In summary, potassium supplementation in the form of tablets may cause gastrointestinal symptoms, from mild symptoms and damage of mucosa to ulcers. The effects seem to be more dependent on the formulation than on dose. Slow release, wax coated KCl tablets appear to induce more lesions than microencapsulated tablets (McMahon *et al*, 1982 and 1984). It is unclear if effects depend on type of potassium salt, since few studies have included other salts than potassium chloride.

4. DOSE-RESPONSE ASSESSMENT

4.1. Hyperkalaemia and cardiac effects

There are no reports of adverse effects associated with potassium naturally-occurring in food in healthy subjects. In adults high-level intakes (95 or 97.5 percentile) from diet are reported to 5-6 g/day. A few experimental studies indicate that healthy adults can tolerate potassium intakes up to about 15 g per day provided that the intake is evenly distributed over the day and that fluid intake is sufficient and the renal function is normal (Rabelink *et al*, 1990).

Long-term intake (more than 2 years) of KCl supplements providing an effective dose of 3.1 g potassium per day in 3 separate doses did not cause hyperkalaemia in middle-aged, hypertensive men (Grimm *et al*, 1990) on a sodium-restricted diet. The 97.5 percentile total potassium intake in this study is estimated to be about 7-8 g/day. However, single doses of 6.5-6.8 g potassium given as KCl solution to apparently healthy adults (80-100 mg/kg body weight) have been associated with acute hyperkalaemia, ECG characteristic of hyperkalaemia and paresthesia of hands and feet in a few subjects (Keith *et al*, 1941).

Case studies of adverse effects associated with high to very high doses (Annex 1) of potassium from salt substitutes or supplements (KCl) have described chest tightness, nausea and vomiting, diarrhoea, hyperkalaemia, shortness of breath and heart failure. The reported acute and chronic effects have usually been seen in subjects with impaired renal function, heart disease, on medication for various diseases including heart disease, e.g. diuretics and antihypertensive drugs, or in subjects taking anabolic steroids. A case of hyperkalaemia and compromised heart function in an apparently healthy young subject, which was associated with high intakes of a potassium salt containing beverage has been reported in the literature (Parisi *et al*, 2002). The reported dose was estimated to about 5 g potassium per day, in addition to the dietary intake, which was not stated. It is well known that plasma potassium levels increase during exercise (Lindinger, 1995). Therefore excessive potassium supplementation in connection with exercise may pose an increased risk for conductive heart effects, even in apparently healthy subjects.

4.2. Gastrointestinal effects

Controlled studies show that gastrointestinal symptoms (ranging in severity from discomfort to mucosal erosion and ulceration) can occur in healthy subjects taking some forms of potassium supplements, e.g. slow release, wax-matrix formulations, with doses ranging from 24 to 120 mmol/day (0.9 to 4.7 g K) or more, but incidence and severity seem to be more dependent on the formulation than on dose (McMahon *et al*, 1982 and 1984; McLaughlin, 1985; Overlack *et al*, 1995).

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The available data are insufficient to establish an upper level for potassium.

2. RISK CHARACTERIZATION

Potassium intakes from foods have not been associated with adverse effects in normal, healthy children and adults. The average intake in adults from the diet is 3-4 g and the intake generally does not exceed 5-6 g per day.

A long-term intake of potassium supplements as potassium chloride of about 3 g per day in addition to intakes from foods has been showed not to have adverse effects. Supplemental potassium in doses of 5-7 g/day in addition to dietary intake has in a few cases, however, been reported to cause conductive effects and compromised heart function in apparently healthy adults.

Gastrointestinal symptoms have been seen in healthy subjects taking some forms of potassium supplements, e.g. slow release, wax-matrix formulations, with doses ranging from 0.9 to 4.7 g/day or more, but incidence and severity seem to be more dependent on the formulation than on dose.

Elderly people may be more vulnerable to adverse effects of potassium due to reduced physiological reserve in renal function or due to drugs affecting potassium balance. Certain other groups are also sensitive to increases in potassium intakes. These include subjects engaging in strenuous activities leading to dehydration, with impaired renal function, on cardiovascular disease drug treatment or other metabolic disorders affecting potassium homeostasis. Case reports of various adverse effects such as hyperkalaemia, conductive effects and compromised heart function have been reported in such subjects after moderate to high acute or sub-chronic intakes of potassium in the form of supplements or potassium-containing salt substitutes.

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Annex 1. Potassium toxicity and adverse effects. Case reports.

Subject	Symptoms	Dose	Comment	Reference
Acute				
Woman, 62y	Gastric distention, inflamed stomach, necrotic mucosal lining sloughed off	94g K as 300 KCl slow-release tablets	Suicide attempt	Peeters&van der Weef 1998
Woman, 52y	Vomiting, sweaty, breathless, left ventricular failure, cyanosis, lung crepitations	0.63g KCl x 20 (c. 6.6 g K)	Bendrofluazide and phenylbutazone also taken	Illingworth&Proudfoot, 1980
Man, 26 y	Vomiting, fatal cardiac arrest	0.6g KCl x 40 (c. 12.5g K)	Distalgesic also taken	Illingworth&Proudfoot, 1980
Woman, 32 y	Presented with diarrhoea, subsequently found dead	47 KCl tablets		Wetli&Davis, 1978
Boy, 2 mo	Listlessness cyanosis, ceased breathing, fatal 28h later	3g KCl and 1.5 g in breast milk on 2 subsequent days (c. 1.56g and 0,78g K/day)	KCl given after infant being 'colic'	Wetli&Davis, 1978
Man, 56 y	Hyperkalaemia, ventricular fibrillation, fatal cariac arrest after aortic valve replacement.	Potassium supplement. 40 mmol after bicycle exercise test. Salt substitute 5.5g/day 2 wk before test	Existing heart disease. Digoxin, chlorthiazide. Low Na diet	Hultgren *et al* 1975
Man, 58 y	Hyperkalaemia, cariac arrest	Potassium supplement. 40 mmol after exercise test	Existing heart disease. Low Na diet (1.5g/day) 2 wks before. Moderate renal dysfunction	Hultgren *et al* 1975
Man, 53 y	Chest tightness, nausea, vomiting. Died of hyperkalaemia with asystole	283 mmol (c. 11g K) as Nu-salt (21g)	Imipramine, beer also taken	Restuccio, 1992
Infant, 8 mo	Stiffness, eye rolling back, breathing difficulties, severe hyperkalaemia	17.2g Morton's salt substitute, equiv. to 26 mmol K/kg BW (c. 0.66g K/kg)	Mild upper respiratory infection causing emesis and diarrhoea	Kallen *et al*, 1976
Man, 52 y	Hyperkalaemia	KCl solution, single oral dose, 32 mmol (1.3g)	Hypertension, hypoaldosteronism; chlorthalidone taken. Low Na and K diet 3 d before	Perez *et al* 1984
Man, 49 y	Hyperkalaemia	KCl solution, single oral dose, 47 mmol (1.8g)	Diabetes mellitus, periferal sensory neuropathy, hypoaldosteronism	Perez *et al* 1984

Subject	Symptoms	Dose	Comment	Reference
Sub-chronic and chronic				
Woman, 75 y	Shortness of breath, oedema, heart failure	Lite-salt substitute ad lib for 6 weeks	Previous myocardial infarction	Snyder *et al*, 1975
Patient	Near fatal hyperkalaemia	Soup seasoned with salt substitute		Hoyt 1986
Man, 31 y	Ventricular tachycardia. Collapse due to myocardial infarction	5g/day potassium supplements. Duration unknown	Body builder. Subject also taking anabolic steroids, amphetamines and potassium sparing diuretics	Appleby *et al*, 1994.
Woman, 68 y	Nausea and abdominal cramps, stenosis of the small bowel, probably caused by focal alteration.	2x 10mEq KCL tablets/day for several years (approx 0.78g/day)	Hypertension treatment, 50mg hydrochlorothiazide	Bronson&Gamelli, 1987
Man 63 y	Hyperkalaemia developed	No Salt' supplement, 35 mmol (1,4g K) per ½ teaspoon. Duration not stated	Existing cardiomyopathy	McCaughan 1984
Man, 74 y	Cardiac arrhytmia, oedema, hyperkalaemia	Salt substitutes used liberally several days prior diagnosis	Chronic reumatic valvular disease, digoxin, furosemide, spironolactone also taken	Yap *et al* 1976
2 men, 64 & 67 y	Hyperkalaemia, loss of conciousness, vomiting	Lo salt', c. 70-133 mmol/day (2.7-5.2g/day) > 1 wk	Hypertensive patients on ACE inhibitors	Ray *et al* 1999
Woman, 29 y	Hyperkalaemia. Cardiac arrest. Post-hypoxic brain damage	K-containing salt substitutes taken after period of diarrhoea as she suspected hypokalaemia	Frusemide also taken.	Schim van der Loeff *et al* 1988
Boy, 14 y	Hyperkalaemia, premature ventricular beats	Hydrosaline beverages c. 5 g K/day during 2 month	Football player	Parisi *et al* 2002

OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF CHLORIDE

(REQUEST N° EFSA-Q-2003-018)

(ADOPTED ON 21 APRIL 2005)

SUMMARY

Chloride is an essential nutrient involved in fluid and electrolyte balance and is required for normal cellular function. Dietary deficiency of chloride is very uncommon due to the widespread occurrence of chloride in foods.

Chloride is present in foods as a normal constituent at a low level. It is also added to foods, mainly as sodium chloride (commonly known as salt) or as mixtures of sodium chloride and potassium chloride (sometimes referred to as salt substitutes) during processing, cooking and immediately prior to consumption. The main reasons for the addition of salt during the processing of foods are for flavour, texture and preservation.

Mean daily chloride intakes of populations in Europe range from about 5-7 g (about 8-11g salt) and are well in excess of dietary needs (about 2 - 2.5 g chloride/day in adults). The main source of chloride in the diet is from processed foods (about 70-75% of the total intake), with about 10-15% from naturally occurring chloride in unprocessed foods and about 10-15% from discretionary chloride added during cooking and at the table.

The major adverse effect of increased intake of chloride, as sodium chloride, is elevated blood pressure. Higher blood pressure is an acknowledged risk factor for ischaemic heart disease, stroke and renal disease which are major causes of morbidity and mortality in Europe. For groups of individuals there is strong evidence of a dose dependent rise in blood pressure with increased consumption of chloride as sodium chloride. This is a continuous relationship which embraces the levels of chloride habitually consumed and it is not possible to determine a threshold level of habitual chloride consumption below which there is unlikely to be any adverse effect on blood pressure.

Gastrointestinal symptoms (discomfort, mucosal lesions and sometimes ulceration) have been seen in healthy subjects taking some forms of potassium chloride supplements (e.g. slow-release, wax matrix formulations) with doses ranging from about 1 to 4 g chloride per day, or more, but incidence and severity seem to be more dependent on the formulation than on dose.

Chloride is not carcinogenic but high intakes of sodium chloride can increase the susceptibility to the carcinogenic effects of carcinogens, such as nitrosamines, and gastric infection with H. pylori.

The panel concludes that the available data are not sufficient to establish an UL for chloride from dietary sources.

There is strong evidence that the current levels of chloride consumption (as sodium chloride) in European countries contribute to increased blood pressure in the population, which in turn has been directly related to the development of cardiovascular disease and renal disease. For this reason, a number of national and international bodies have set targets for a reduction in the chloride as sodium chloride consumed in the diet.

KEY WORDS

Chloride, salt, blood pressure, tolerable upper intake level, food safety.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

1. Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Chloride is found widely in nature and as a normal constituent of foods, generally as salts of sodium (NaCl) or potassium (KCl). Chloride is also added to food mainly as sodium chloride (commonly known as salt (1 mmol is equivalent to 35.5 mg chloride and approximates 58 mg sodium chloride) or mixtures of sodium chloride and potassium chloride (sometimes referred to as salt substitutes). Other chloride salts may be added, generally at lower amount, to food for nutritional or technological purpose, e.g. magnesium chloride. Ammonium chloride is permitted to be added to certain foods (e.g. liquorice) as an additive. In drinking water, the guide level of chloride is 25 mg/L (Council Directive 80/778/EC). Chloride is an essential dietary constituent and a dietary inadequacy leads to serious consequence. Chloride is present in biological systems as the main anion in the extracellular space, acting to maintain extracellular volume, and ionic balance. It crosses cell membranes and is involved in the regulation of osmotic pressure, water balance and acid-base balance.

It is sometimes difficult to differentiate the effects of the chloride moiety in chloride salts such as NaCl and KCl from that of the sodium or potassium moiety on physiology and metabolism. Therefore this Opinion should be read in conjunction with the Panel's Opinions on the tolerable upper level of sodium (NDA, 2005a) and potassium (NDA, 2005b).

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

2.1. Food levels and dietary intake

Chloride is found in plant and animal based foods in association with monovalent counter cations, mainly sodium and potassium. It is added as salt (NaCl or NaCl/KCl mixtures) to foods during processing, cooking and immediately prior to consumption. The main reasons for the addition of salt during the processing of foods are for taste, texture and preservation.
The chloride content of natural foods varies from around 0.1 to 3 mmol/100g, with fruit containing 0.1 mmol/100g, vegetables, 0.3 mmol/100g and meat fish or eggs 3.0 mmol/100g. The chloride content of processed foods may be much higher; bread 20 mmol/100g; cheese, 30 mmol/100g; salted butter, 40 mmol/100g; and lean raw bacon, 80 mmol/100g.

The assessment of the dietary consumption of chloride in individuals and populations is difficult because of the variable extent to which discretionary additions of salt contribute to the total. The use of dietary assessment methods to determine intake are likely to provide variable underestimates. The most accurate determinations of chloride consumption are derived from measurements of the excretion

in urine over 24 hours, although care has to be taken to ensure the completeness of the urine collection (Bingham and Cummings, 1985; Bingham *et al*, 1988).

The main source of chloride in the diet is from salt (NaCl) or (to a lesser extent) salt substitutes (NaCl/KCl mixtures) added during the processing and manufacture of foods (about 70-75% of the total intake) and added to food during cooking and at the table. Naturally occurring chloride in unprocessed foods contributes about 15% of total chloride intake. Discretionary sources of sodium chloride or sodium chloride/potassium chloride mixtures added during cooking and at table comprise about 10 to 15% of total chloride intake (Sanchez-Castillo *et al*, 1987). The main sources of chloride from foods in the diet are from cereals and cereal products, particularly bread, and meat and meat products (SACN, 2003). Other sources of chloride are from vegetables.

2.2. Nutritional requirements and recommendations

Chloride is an essential nutrient. The SCF did not establish a Population Reference Intake for chloride but concluded that the requirements should match those for sodium (on a molar basis), i.e. 25 - 150 mmol/day (SCF, 1993). The US Institute of Medicine established an Adequate Intake (AI) for chloride at a level equivalent on a molar basis to that of sodium, since almost all dietary chloride comes with the sodium added during processing or consumption of foods (FNB, 2004). The AI for chloride for younger adults is 2.3 g/day (65 mmol/day), and for older adults and the elderly 2.0 and 1.8 g per day respectively.

For most populations, the habitual levels of chloride consumption greatly exceed the physiological requirements, and there are few data which determine the minimal levels of chloride consumption required to maintain health in people who have adapted to low levels of chloride consumption over long periods of time.

2.3. Function, uptake, distribution and elimination

Dietary chloride is virtually completely absorbed along the length of the intestine. The total body chloride averages about 33 mmol/kg body weight (1.2 g/kg) in a normal adult male (Pike and Brown). Chloride is found in small amounts in a bound form related to connective tissue. Less than 15% of the body's content of chloride is located within cells. The chloride content of blood and the extracellular space is not related to dietary intake but is influenced by intake/plasma concentrations of other electrolytes. The ready transfer of chloride in exchange for bicarbonate between erythrocytes and plasma and in the gastrointestinal tract and renal collecting tubule is an important aspect of the control of blood pH.

The important role played by chloride in the control of electrolyte and acid base equilibria has been well characterised and chloride deficiency is most likely the consequence of an increase in losses, although dietary deficiencies have been described in infants consuming a commercial formula deficient in chloride (Rodriguez-Soriano *et al*, 1983). Chloride is essential for the formation of hydrochloric acid in the stomach, and hence is involved in the non-specific protection from food borne pathogens. It has generally been considered that chloride readily crosses cell membranes, although the permeability of some smooth muscle, such as vascular smooth muscle, is less than has been assumed. In vascular smooth muscle the active transport of chloride is energetically expensive and appears to be tightly regulated, playing a fundamental role in contraction, an observation of direct relevance to the development of high blood pressure (Chipperfield and Harper, 2000).

Experimental studies have shown that the chloride moiety makes a specific contribution to the effects of sodium chloride on blood pressure, by modulation of renal regulatory systems and plasma renin activity in the rat (Kirchner, 1978; Abboud *et al*, 1979), dog (Kotchen *et al*, 1980; Kotchen *et al*, 1983.), and in the human (Julian *et al*, 1982; Tomita *et al*, 1990). There are also specific effects on angiotensin II and aldosterone (Koletsky *et al*, 1981; Sato *et al*, 1991; Imig *et al*, 1993). Dietary loading with sodium chloride leads to positive chloride balance, expanded extracellular volume and increased renal vascular resistance (Passmore *et al*, 1985; Tomita *et al*, 1990). Chloride is rate limiting for the transport of sodium and chloride in the thin ascending loop of Henle, because of the differences in the affinities of sodium and chloride for the cotransporters. Thus the availability of chloride has a determinant effect on the release of renin (Kotchen *et al*, 1987).

Chloride is lost from the body in sweat (20-80 mmol/day) and other secretions, in stool (5-10 mmol/day) and in urine (1-500 mmol/day).

3. HAZARD IDENTIFICATION

There is evidence that prolonged consumption of excessive chloride as sodium chloride contributes to an elevated blood pressure, which is a risk factor for cardiovascular disease and renal disease (NDA, 2005a). Available evidence indicates that both chloride and sodium contribute to this effect.

In three selective breeds of rats which are especially prone to develop high blood pressure when exposed to dietary salt, chronic selective loading with either sodium or chloride has been found not to induce hypertension (Kotchen *et al*, 1983; Kurtz and Morris, 1983; Whitescarver *et al*, 1984; Kurtz and Morris, 1985; Whitescarver *et al*, 1986; Passmore and Jimenez, 1990; Reddy and Kotchen 1992; Imig *et al*, 1993; Kadota *et al*, 1993; Kunes *et al*, 2004).

Any effects of selective loading with dietary chloride without sodium have been attributed in part to direct effects on acidosis or indirect effects of acidosis on potassium or calcium (Kotchen *et al*, 1988). Boegehold and Kotchen (1989) conclude that the observations indicate that the concomitant provision of a high intake of both sodium and chloride in the diet is required for the expression of experimental salt-sensitive hypertension.

As early as 1929, it was reported that a diet high in sodium bicarbonate did not have the same effect on raising blood pressure as sodium chloride (Berghoff and Geraci, 1929). This has been confirmed by others (Morgan, 1982; Kurt and Morris, 1983; Luft *et al*, 1990). The effect of sodium chloride on blood pressure has not been seen with sodium phosphate (Shore *et al*, 1988), or sodium citrate (Kurtz *et al*, 1987; Tomita *et al*, 1990; Sato *et al*, 1991). Similarly, when the chloride ion is taken without sodium the effects on blood pressure are less evident (Grollman *et al*, 1945; Dole *et al*, 1950). Thus, the findings from human studies support the evidence from animal investigations that both sodium and chloride are required for the effects of salt on blood pressure to be manifest. The evidence would suggest that changes in blood volume underlie these effects (Tomita *et al*, 1990), which are closely related to alterations in the set point for renal salt and water homeostasis. Recent molecular studies implicate a specific role for the anion exchanger pendrin, and its expression in the kidney (Quentin *et al*, 2004).

Adverse changes in heart function and peripheral nerve symptoms associated with high to very high doses of potassium chloride from salt substitutes or supplements (KCl) (4.4-6.2 Cl/day, or more, in addition to diet) have been described, usually in subjects with impaired renal function, but occasionally in healthy adults (NDA, 2005b). These effects are mediated by hyperkalaemia and thus appear to be attributable to potassium intake rather than chloride.

Controlled studies show that gastrointestinal symptoms (ranging in severity from discomfort to mucosal erosion and ulceration) can occur in healthy subjects taking some forms of potassium chloride supplements, e.g. slow release, wax-matrix formulations, with doses ranging from 24 to 120 mmol/day (0.9-4.3 g/day) or more, but incidence and severity seem to be more dependent on the formulation than on dose (NDA, 2005b). It is not possible to distinguish between the possible contributions of chloride and potassium to these effects.

4. DOSE-RESPONSE ASSESSMENT

Higher blood pressure is an acknowledged risk factor for ischaemic heart disease, stroke and renal disease. For groups of individuals there is strong evidence of a dose response relationship between increased consumption of chloride as sodium chloride and higher levels of systolic, diastolic and mean blood pressure (Sacks *et al*, 2001). The effect of sodium on blood pressure is linked to that of chloride and he adverse effect on blood pressure associated with increasing salt intake appears to be attributable to both sodium and chloride (NDA, 2005a).

Gastrointestinal symptoms (ranging in severity from discomfort to mucosal erosion and ulceration) can occur in healthy subjects taking some forms of potassium chloride supplements, e.g. slow release, wax-matrix formulations, with doses ranging from 24 to 120 mmol/day (0.9 to 4.3 g chloride) or more (NDA, 2005b). The extent to which chloride, as distinct from potassium, contributes to these symptoms is not clear.

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The available data are not sufficient to establish an upper level for chloride from dietary sources.

2. RISK CHARACTERISATION

The habitual intake of chloride (mainly as sodium chloride) for populations across Europe is high and exceeds the amounts required for normal function. The current levels of chloride consumption as sodium chloride have been associated directly with a greater likelihood of increased blood pressure, which in turn has been directly related to the development of cardiovascular disease and renal disease.

For these reasons, national and international bodies have set targets for a reduction in the sodium chloride consumed in the diet (SACN, 2003; FNB, 2004; WHO, 2003 and 2004).

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF SODIUM

(REQUEST N° EFSA-Q-2003-018)
(ADOPTED ON 21 APRIL 2005)

SUMMARY

Sodium is an essential nutrient involved in fluid and electrolyte balance and is required for normal cellular function. Dietary deficiency of sodium is very uncommon due to the widespread occurrence of sodium in foods.

Sodium is present in foods as a normal constituent at a low level. It is also added to foods, mainly as sodium chloride (commonly known as salt) during processing, cooking and immediately prior to consumption, but also in other forms, for example as sodium nitrate, sodium phosphate or sodium glutamate. The main reasons for the addition of salt during the processing of foods are for flavour, texture and preservation.

Mean daily sodium intakes of populations in Europe range from about 3-5 g (about 8-11g salt) and are well in excess of dietary needs (about 1.5 g sodium/day in adults). The main source of sodium in the diet is from processed foods (about 70-75% of the total intake), with about 10-15% from naturally occurring sodium in unprocessed foods and about 10-15% from discretionary sodium added during cooking and at the table.

The major adverse effect of increased sodium intake is elevated blood pressure. Higher blood pressure is an acknowledged risk factor for ischaemic heart disease, stroke and renal disease which are major causes of morbidity and mortality in Europe. The effect of sodium on blood pressure is linked to that of chloride. For groups of individuals there is strong evidence of a dose dependent rise in blood pressure with increased consumption of sodium as sodium chloride. This is a continuous relationship which embraces the levels of sodium habitually consumed and it is not possible to determine a threshold level of habitual sodium consumption below which there is unlikely to be any adverse effect on blood pressure.

While blood pressure, on average, rises with increased sodium intake, there is well-recognised variation between individuals in the blood pressure response to changes in sodium chloride intake. Individuals with hypertension, diabetes, and chronic kidney disease, as well as older-age persons, tend to be more sensitive to the blood pressure raising effects of sodium intake. The blood pressure response to sodium can be modulated by a range of factors which include other components of the diet (e.g. potassium), relative body weight, and level of physical activity, as well as fixed factors which include age, gender and genetic factors.

Epidemiological studies indicate an association of increased risk of morbidity and mortality from cardiovascular diseases, including coronary heart disease and stroke, with increasing sodium intake. Evidence that high sodium intake may have a direct adverse effect on heart function, independent of any secondary effect due to changes in blood pressure, is not conclusive. Sodium is not carcinogenic but high intakes sodium chloride can increase the susceptibility to the carcinogenic effects of carcinogens, such as nitrosamines, and gastric infection with *H. pylori*.

The Panel concludes that the available data are not sufficient to establish an upper level (UL) for sodium from dietary sources.

There is strong evidence that the current levels of sodium consumption in European countries contribute to increased blood pressure in the population, which in turn has been directly related to the development of cardiovascular disease and renal disease. For this reason, a number of national and international bodies have set targets for a reduction in the sodium consumed in the diet.

KEY WORDS

Sodium, blood pressure, stroke, cardiovascular disease, tolerable upper intake level, food safety

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Sodium (Na) is a metal with an atomic mass of 23. It is found widely in nature and as a normal constituent of foods. It is added to foods, most frequently as sodium chloride (NaCl), common known as salt (1 mmol is equivalent to 23 mg sodium and approximates 58 mg sodium chloride), but also as other salts, e.g. nitrate, nitrite, phosphates, glutamate. In drinking water, the guide level of sodium is 20 mg/L (Council Directive 80/778/EEC). Sodium is an essential nutrient and a dietary inadequacy may lead to serious consequences. Sodium is present in biological systems as the main cation in the extracellular space, acting to maintain extracellular volume and plasma osmolality.

It is sometimes difficult to differentiate the effects of the sodium moiety from sodium salts such as NaCl on physiology and metabolism. Therefore this Opinion should be read in conjunction with the Panel's Opinion on the tolerable upper intake level of chloride (NDA, 2005).

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

2.1. Food levels and dietary intake

Sodium is found in plant and animal based food and also in drinking water. Sodium is added to foods, commonly as sodium chloride, during processing, cooking and immediately prior to consumption, but also in other forms, for example as sodium nitrate, sodium phosphate or sodium glutamate. The main reasons for the addition of salt during the processing of foods are for flavour, texture and preservation.

1. Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

The sodium content of natural foods varies from around 0.1 to 3 mmol/100g, with fruit containing 0.1 mmol/100g, vegetables 0.3 mmol/100g, and meat, fish or eggs 3.0 mmol/100g. The content of sodium as sodium chloride in processed foods may be much higher; bread 20 mmol/100g; cheese, 30 mmol/100g; salted butter, 40 mmol/100g; and lean raw bacon, 80 mmol/100g. It is difficult to obtain reliable information on the sodium chloride content of foods as consumed, because of variable practices in terms of processing, food preparation and personal preferences.

The assessment of the dietary consumption of sodium in individuals and populations is difficult because of the variable extent to which discretionary additions of salt contribute to the total. The use of dietary assessment methods to determine intake are likely to provide variable underestimates of the true intake, which has been assessed as around 20% for some populations. The most accurate determinations of sodium consumption are derived from measurements of the excretion of sodium in urine over 24 hours, although care has to be taken to ensure the completeness of the urine collection (Bingham and Cummings, 1985; Bingham *et al*, 1988).

The amount of sodium consumed, varies widely between populations, within populations, and within individuals with time. The Intersalt study was a study from 52 centres in 32 countries around the world, in which 24 hour specimens of urine were collected from 10,079 men and women aged 20-59 years of age (Intersalt Cooperative Research Group, 1988). There were 20 centres from 12 countries in Western Europe who participated, providing data from 3,942 men and women. In this group the median sodium excretion varied from 135 to 180 mmol/24 hours (equivalent to 3.1-4.1 g Na or 7.9-10.5 g NaCl) (Perry and Beevers, 1992). Estimates of the intake of sodium as sodium chloride were 8-9 g in Belgium, Denmark and The Netherlands and 9-11 g in Finland, Italy, Portugal, Spain and the UK. For Romania, the daily consumption of sodium as sodium chloride has been estimated to be 14 g/person (WHO, 2004). Although there are suggestions that manufacturers have attempted to reduce the sodium chloride content of some products, such as bread, the amount of sodium consumed in the UK did not change between 1987 (sodium excretion in urine for men 173 mmol/day, and for women 132 mmol/day) and 2001 (sodium excretion in urine for men 187 mmol/day, and for women 139 mmol/day), based on the measured excretion of sodium in urine in nationally representative samples (Gregory *et al*, 1990; Henderson *et al*, 2003). Data from the UK suggest that average daily intake of sodium in children aged 4-6 years and 7-10 years exceeds 5 g and 6 g, respectively (SACN, 2003). This compares with the average intake of sodium assessed for a group of children in the Dortmund Nutritional and Anthropometrical Longitudinally Designed Study of 751 children from 3 months to 18 years of age, where consumption was about 3g/day at age 4 to 6 years, and 4.5 g/day at 10 to 12 years, but these values exclude any discretionary sodium chloride added in the home (Alexy and Kersting, 1999).

The main source of sodium in the diet is from processed foods, and it is estimated that this non-discretionary consumption comprises about 70-75% of the total intake in most European countries. Naturally occurring sodium in unprocessed foods contributes about 10 to 15% of total sodium intake. Discretionary sources of sodium added during cooking and at the table comprise about 10 to 15% of total intake (Sanchez-Castillo *et al*, 1987).

Based on the National Diet and Food Survey in the UK, cereals and cereal products (particularly bread) were estimated to contribute 35% of total sodium consumption, or about 2.3 g/day, with 1.7 g/day coming from meat and meat products (particularly processed meats) to contribute 26% to the total. Vegetables contribute to 7%, milk and milk products, 8%, fats spreads, 3% and cheese, 4% made lesser contributions (NDNS, 2003), but individual composite foods or savoury snacks can be especially high in sodium.

2.2. Nutritional requirements and recommendations

Human populations survive on wide extremes of habitual sodium consumption from 10 to 450 mmol/day. The ability to survive at low levels of consumption is dependent upon adaptive mechanisms which reduced losses in sweat, stool and urine. For most populations, the habitual levels of sodium consumption greatly exceed the physiological requirements, and there are few data which determine the minimal levels of sodium consumption required to maintain health in people who have adapted to low levels of sodium consumption over long periods of time (Allsopp *et al*, 1998). As the metabolism of sodium is closely related to that of water and other nutrients, the need for sodium can only be adequately assessed when the provision of water and all other nutrients is adequate.

For sodium, the acceptable range of intakes for adults established by the Scientific Committee on Food was 25 to 150 mmol/day (SCF, 1993). This compares with the Adequate Intake identified in the USA as

65 mmol/day for adults up to 50 years of age, 55 mmol/day for those between 51 and 70 years of age, and 50 mmol/day for those aged over 70 years of age (FNB, 2004). The Reference Nutrient Intake was set at 70 mmol/day for the UK (Department of Health, 1991).

2.3. Function, uptake, distribution, and elimination

Dietary sodium is virtually completely absorbed along the length of the intestine and the active transport of sodium is closely linked to the wider ability of the small intestine to absorb other nutrients.

Sodium is the major extracellular cation in the body, and the total body content is tightly regulated. The normal adult contains around 5600 mmol sodium (129 g Na). About half of this, 2800 mmol, is dissolved in the extracellular fluid, with 300 mmol in the intracellular compartment. In healthy adults, the total exchangeable sodium is about 40-50 mmol/kg body weight. Bone contains about 2500 mmol of sodium, part of which is exchangeable with isotopically labelled sodium, and the remainder is deeper and less accessible as an intrinsic part of the crystal lattice of bone (Ganong, 1983).

Sodium is the main osmolyte in the extracellular fluid, maintained within a narrow range at a concentration of 135-145 mmol/L and therefore plays a fundamental role in maintaining extracellular fluid volume and together with potassium in maintaining total body water homeostasis. It contributes to the establishment of the membrane potential of most cells and plays a direct role in the action potential required for the transmission of nerve impulses and muscle contraction. The active absorption of sodium from the lumen of the gastrointestinal tract is important in the absorption of nutrients.

The regulation of total body content is closely related to the regulation of total body potassium, the main intracellular cation, and the regulation of total body water. The membrane bound sodium-potassium pump (the sodium-potassium-activated adenosine triphosphate Na+-K+ ATPase) plays a fundamental role in maintaining the partitioning of sodium and potassium between the extracellular and intracellular compartments respectively, and the energy required for this process represents a significant component of the metabolic rate.

Sodium is lost from the body in sweat (20-80 mmol/day) and other secretions, in stool (5-10 mmol/day) and in urine (1-500 mmol/day). Under normal conditions, gastrointestinal and cutaneous losses are minimal and the maintenance of the total body content of sodium is predominantly a characteristic of the regulation of renal excretion. Sodium is actively conserved in the body, with the kidneys playing a major role. Approximately 18 mmol of sodium are filtered per minute and over 99% is actively reabsorbed. Sodium depletion is seldom a consequence of a deficient intake, and more usually the result of excessive losses through excessive sweating or gastrointestinal losses. Sodium depletion may be manifest as low blood pressure and muscle cramps. Excessive sodium leads to an increase in plasma osmolality, resulting in the sensation of thirst, and an increase in the secretion of antidiuretic hormone which increases water retention in the kidney.

The excretion of sodium in urine is highly regulated, through the complex interaction of hormonal, nervous and other systems which enable tight homeostatic control. In this way urinary sodium excretion can be adjusted over a very wide range to achieve sodium balance, from virtually zero when sodium needs to be retained in the body, to 1,100 mmol/day when intake is high (Roos *et al*, 1985). The regulatory systems are highly efficient and include resetting of glomerular filtration rate, the reabsorption of sodium in the proximal and distal tubule under neurohumoral control, adapting in both the short and the longer term to variation in intake and status. Following a change in diet sodium balance may be restored in 5 to 7 days, as sodium excretion comes into equilibrium with intake. The period taken to restore balance is variable amongst individuals and takes longer in some people, especially at the extremes of age, in young infants and older people (Ganong, 1983; Pecker and Laragh, 1991). These mechanisms are responsive to changes in total body water and extracellular fluid volume, to the function and integrity of cells in general and specialised cells in different locations, to ensure the effective delivery of blood to all tissues, including the brain.

3. HAZARD IDENTIFICATION

3.1. Animal toxicity data

For sodium chloride, the oral LD_{50} in rats is 3000 mg/kg and in mice is 4000 mg/kg (EGVM, 2003; RTECS, 1998).

3.1.1. Carcinogenicity

Evidence from studies in laboratory animals indicates that high intakes of salt may increase the susceptibility to various carcinogens and the incidence of gastric cancer, but that salt is not of itself carcinogenic (Cohen and Roe, 1997). The possible biological pathways by which salt increases cancer of the stomach have been well articulated, although salt itself is not considered intrinsically carcinogenic, intake of higher concentrations of salt leads to damage to the protective mucin and the mucosal layer of the stomach, which leads to an inflammatory regenerative response with increased DNA synthesis and cell proliferation. These effects have been considered to be more closely related to local effects where high concentrations are achieved, which physically damage cells, rather than any direct effect in damaging DNA (Cohen and Rose, 1997). By contrast specific highly salted foods have been shown to enhance chemically induced carcinogenesis in the glandular stomach of rats (Takahashi *et al*, 1983 and 1994). Quite apart from the high salt content, these foods also contain many other compounds which might increase the risk of cancer (IARC, 1993). The consumption of salt is thought to create an increased susceptibility, which typically enhances carcinogenesis induced by other specific carcinogens, such as nitrosamines (Watanabe *et al*, 1992; Takahshi *et al*, 1994; Furihata *et al*, 1996; Iishi *et al*, 1999; Cohen and Roe, 1997; IARC, 1990).

Infection with *Helicobacter pylori* has been considered to be an important aetiological factor in the development of gastric cancer (IARC, 1994). Mongolian gerbils were treated with 20 ppm of N-methyl-N-nitrosourea (MNU) in their drinking water alternate weeks for 5 weeks. At 11 weeks the animals were inoculated with *H. pylori*, and after 12 weeks the animals were fed a 10% high salt diet. A high salt diet enhanced the effects of *H. pylori* infection on gastric carcinogenesis, and these two factors acted synergistically to promote the development of stomach cancer. *H. pylori* promoted stomach cancer more than the high salt diet (Tatematsu, 2003). Big Blue transgenic mice were inoculated with *H. pylori* and developed severe gastritis, and after 12 months they exhibited mutagenic effects. There was no added effect of a high salt diet (Touati *et al*, 2003).

3.1.2. Genotoxicity

Studies carried out *in vitro*, have shown that when cells are exposed to high concentrations of sodium chloride, there are physical effects which can lead to direct damage to chromosomes or to DNA which are non-specific in nature.

3.1.3. Reproductive toxicity

In rats fed experimental diets from one week prior to conception and throughout gestation, the proportion of male pups was lower as the sodium chloride content of the diets increased from 0.8 to 4%. No differences were observed in litter sizes or general health of the offspring (Bird and Contreras, 1986).

Dams were maintained on diets which contained 0.12, 1.0, and 3.0% sodium chloride throughout pregnancy, and the offspring were given the same diets until day 30 of life, followed by a diet containing 1.0% sodium chloride (Contreras and Kosten, 1983). Preference tests for solutions of sodium chloride, potassium chloride or glucose were carried out at 90 days of age. Preferences were non-specific. Although those raised on a high salt diet showed an elevated preference for sodium chloride solutions, males also showed a preference for glucose solutions. The weights of adrenal glands of the males in the highest salt group were significantly lower than controls.

Borderline hypertensive rats fed a diet containing 8% sodium chloride from conception to weaning: offspring demonstrated higher blood pressure as adults (Hunt and Tucker, 1993).

Pregnant Brattleboro rats fed 8.5% sodium chloride in the diet showed a higher concentration of sodium chloride in amniotic fluid, and supplementation of pups with sodium post weaning led to an elevation of arterial blood pressure (Hazon *et al*, 1988).

3.1.4. Cardiovascular health and blood pressure

There is a large body of data on rodent models which indicate that depending on genetic strain there may be different degrees of sensitivity to dietary sodium. The immediate relevance of this work for long term human health is not clear (Swales, 1994).

Perhaps the most informative intervention study of relevance to human physiology is that conducted by Denton *et al* (1995) on the effect of increased levels of sodium chloride on the blood pressure of chimpanzees. A colony of 26 chimpanzees, aged 5 to 18 years, was maintained on a vegetable and fruit

diet, very low in sodium and high in potassium. Sodium chloride in the range habitually consumed by human populations was added to the diet of one half of the animals over a period of 20 months. The stepwise addition of sodium chloride caused a highly significant, stepwise rise in systolic and diastolic blood pressure. No threshold effect was observed. The increase in blood pressure reversed completely following the cessation of the added sodium chloride to the diet. The effect was variable amongst different animals, with some having a larger and others a smaller or no rise in blood pressure.

3.2. Human toxicity

3.2.1. Acute effects

The acute ingestion of 0.5 to 1 g sodium chloride per kg body weight can be toxic to fatal to most people. There are local effects within the gastrointestinal tract leading to ulceration. There may be direct or secondary systemic metabolic perturbations in fluid and acid base balance. There is neurological dysfunction leading to seizures and damage to the central nervous system, muscle dysfunction and renal damage.

3.2.2. Chronic effects

3.2.2.1. Carcinogenicity

The evidence for an effect of NaCl on the incidence of cancer is less clear in humans than in experimental animals. The data have been recently reviewed by the World Cancer Research Fund (WCRF, 1997) and the Institute of Medicine (FNB, 2004). Salt consumption has been associated with cancer of the naso-pharynx and cancer of the stomach. An increased risk of cancer of the larynx, mouth and pharynx has been associated with consumption of salt-preserved meat and fish (Zheng *et al*, 1992; Anderson *et al*, 1978). Eight case control studies of people from China living in different parts of the world found statistically significant increases in risk for nasopharyngeal cancer, with odds ratio varying from 2.1 to 37.7, and suggesting that childhood exposure was particularly important (WCRF, 1997). Experimental studies in rats and hamsters corroborate the findings, and consideration of aetiological factors indicates that volatile carcinogenic nitrosamines might be particularly important. In areas where mortality from nasopharyngeal carcinoma was particularly high, there were highly significant correlations with total volatile nitrosamines in salted fish samples. There is some evidence to suggest a possible interaction with Epstein-Barr virus, which appears to be activated by the chemicals found in salted fish (Ho *et al*, 1976; Shao *et al*, 1988).

The main body of evidence relates to cancer of the stomach. Throughout the world, a strong and consistent relationship has been found between the intake of salt and salted foods and the incidence of cancer and pre-cancerous lesions of the stomach. Biological pathways by which salt increases cancer of the stomach are best explained by salt in the diet acting to damage the protective mucosal layer of the stomach, thereby enhancing carcinogenesis. Thus, although salt is not considered to be intrinsically carcinogenic, a high intake of salt leads to damage of the protective mucosal layer of the stomach, and results in an inflammatory regenerative response, increased DNA synthesis and cell proliferation. Prolonged damage results in chronic atrophic gastritis. Over time these changes provide favourable conditions for mutations to occur, and typically enhance carcinogenesis induced by specific carcinogens (Sugimura, 2000). There is a significant relationship between infection of the stomach with *H. pylori* and cancer of the stomach, and it is suggested that this is a consequence of the bacterial infection acting as a co-factor with salt on a damaged gastric epithelium (Joossens *et al*, 1996; Tsugane, 2005).

3.2.2.1.1. Ecological study

Based on the data collected in the Intersalt study, a comparison has been made to explore the possibility that high urinary excretion of sodium and/or high nitrate might be considered as risk factors for stomach cancer in 39 populations from 24 countries, based on sodium excretion in 24 hour urine specimens for 5756 people. The data were age and sex standardised between 20-49 years and the Pearson correlation for stomach cancer was 0.70 in men and 0.74 in women. The analysis indicated that the importance of nitrate as a risk factor for stomach cancer mortality increased markedly with higher sodium levels. The relationship of stomach cancer mortality with sodium was stronger than with nitrate and the authors conclude that salt intake is likely the rate limiting factor for stomach cancer mortality at the population level (Joossens *et al*, 1996). There was no increase noted in the incidence of cancer mortality for men with sodium intakes less than 117 mmol (2.7 g sodium) per day, and for women with sodium intakes less than 91 mmol (2.1 g) per day.

3.2.2.1.2. Cross-sectional studies

A significant positive association has been observed between salt/sodium intake and risk of gastric cancer in most, but not all, of the cross sectional studies (WCRF, 1997; FNB, 2004). More recent evidence suggests that the risk might be greatest in individuals with specific genotypes (Chen *et al*, 2004). In an area of Taiwan where there is a high mortality for stomach cancer, a survey to determine risk factors was carried out in 312 subjects (174 men and 138 women) in 1995. The presence of intestinal metaplasia was determined using gastro-endoscopic examination. The consumption of salted foods was associated with an increased risk of intestinal metaplasia, with an odd-ratio (OR) 2-3. The risk appeared to be greatest among subjects with specific genotypes: GSTMI null, GSTTI non-null and CYP2EI cl/cl.

3.2.2.1.3. Case-control studies

WCRF (1997) reported that of sixteen case control studies, eight estimated overall dietary salt or sodium intake, and of these four have shown strong statistical increase in risk, with four showing no substantial association. The relative risk for gastric cancer, associated with higher intakes of salt, has varied from 1.4 to 6.7 (FNB, 2004). In a hospital-based case control study carried out in Korea, 69 patients newly diagnosed as early gastric cancer were compared with 199 healthy subjects. There was *H. pylori* seropositivity in 88% cases and 75% controls (OR=5.3). Adaptive salt concentration was significantly and positively associated with early gastric cancer risk ($p<0.01$). Subjects seropositive for *H. pylori*, and a high salt preference had 10-fold higher risk of early gastric cancer than those without *H. pylori* infection and low salt preference (p for interaction 0.047) (Lee *et al*, 2003).

3.2.2.1.4. Prospective studies

In two prospective studies, salt intake was significantly and directly associated with gastric cancer in dose response fashion in men but not in women.

A cohort study was carried out in The Netherlands of 120,852 men and women aged 55-69 years at baseline in 1986. The exposure to salt was determined using a diet questionnaire. After 6.3 years of follow up, 282 incident cases of cancer were available for analysis, with 3123 sub-cohort members. In multivariate analyses adjusted for age, sex, smoking, education, stomach disorders, history of stomach cancer in the family, rate ratios for increasing quintiles of energy adjusted intake of dietary salt were not significant. An inverse trend was found between stomach cancer and salt added at the hot meal (p for trend 0.04). Positive associations were found for bacon (relative risk [RR] 1.3), and other sliced cold meats (RR 1.29). Separate analyses among subjects with self-reported stomach disorders revealed higher RR of stomach cancer for dietary salt and types of cured meat. It was concluded that the intake of dietary salt and several types of cured meat were weakly positively associated with stomach cancer risk (van den Brandt *et al*, 2003).

A population-based prospective study was carried out in Japan, where the majority of men had been infected with *H. pylori*. A total of 18,684 men and 20,381 women aged 40-59 years completed a food frequency questionnaire and were followed from 1990 to 2001. A total of 486 cases, 358 men and 128 women were documented with gastric cancer confirmed by histology. The quintile category of salt intake was associated with gastric cancer in a dose dependent fashion for men (p for trend <0.001; median g/day, 2.9, 4.8, 6.1, 7.5, 9.9), while there was no clear trend identified for women (p for trend 0.48; median g/day 2.6, 4.2, 5.3, 6.4, 8.2). In men there was no obvious break point below which the risk was reduced (Tsugane *et al*, 2004).

3.2.2.2. Bone health

In surveys of free living individuals and in physiological studies where dietary loads of sodium chloride have been administered, there is consistent evidence that increased sodium ingestion induces a substantial increase in the urinary excretion of calcium (SACN, 2003; FNB, 2004; New and Bonjour, 2003). On this basis, it has been hypothesised that higher levels of sodium ingestion might compromise bone health. The available evidence suggests that sodium-induced increase in urinary calcium excretion leads to an increased calcium absorption by the gastrointestinal tract, mediated via parathyroid hormone (Cashman and Flynn, 2003). However, this adaptive mechanism may be insufficient to prevent an increase in bone resorption in some individuals, e.g. postmenopausal women with a particular vitamin D receptor genotype (Harrington *et al*, 2004). Whether this increase in bone resorption leads to net bone loss is unclear and thus the evidence for a direct effect of sodium ingestion on bone health is not conclusive.

3.2.2.3. Cardiovascular function

Systematic reviews of the evidence which relates consumption of sodium in the diet with cardiovascular health have been reported recently (SACN, 2003; FNB, 2004).

3.2.2.3.1. Heart

An increase in the mass of the left ventricle of the heart is a powerful predictor of cardiovascular morbidity and mortality, including myocardial infarction, stroke, congestive heart failure and sudden death (Bikkina *et al*, 1994; Casale *et al*, 1986; Koren *et al*, 1991; Levy *et al*, 1990). In the Framingham study, elevated left ventricular mass as measured by echocardiography was associated with an increased incidence of cardiovascular disease after adjustment for traditional risk factors (Levy *et al*, 1990). For people with left ventricular hypertrophy, the 5-year mortality was 33% men and 21% for women (Koren *et al*, 1991).

An increase in the mass of the left ventricle might be the consequence of high blood pressure, but there is evidence that a high sodium intake might have direct effect on heart leading to an increase in left ventricular mass. In most studies, the association between urinary sodium and left ventricular mass persists after adjustments for other determinants of left ventricular mass, such as blood pressure (du Cailar *et al*, 2002; Liebson *et al*, 1993).

In a cross sectional study of 42 patients with essential hypertension Schmieder *et al* (1988), using stepwise multiple linear regression analysis, found that sodium excretion was the strongest predictor for left ventricular posterior wall thickness independently of other variables (sodium excretion correlated with ventricular posterior wall thickness, $r=0.64$, $p< 0.001$, and septal wall thickness, $r=0.78$). Sodium excretion varied from 37 to 356 mmol/day. No break point was identified in the relationship. Alderman *et al* (1995) carried out a cross-sectional analysis of 1900 men and 1037 women and concluded that there was a non-significant relationship between urinary sodium and left ventricular hypertrophy assessed by electrocardiography. Du Cailar *et al* (2002) in a cross-sectional study of 839 men and women, using multivariate analysis, found a strong relationship between sodium excretion and left ventricular mass in men and women. Liebson *et al* (1993 and 1995) examined the effect of different therapeutic interventions on left ventricular mass in a randomized double blind trial of 902 people with mild essential hypertension. There were significant changes in left ventricular mass determined by echocardiography from 3 months and maintained to the end of the study period at 48 months. The authors concluded that sodium reduction was effective in reducing left ventricular mass, which was greater than could be accounted for by a change in blood pressure, leading to the conclusion that sodium reduction might be more effective in improving left ventricular mass than in reducing blood pressure, although an effect of weight loss could not be excluded.

These studies raise the possibility that sodium has a direct adverse effect on left ventricular structure and function, independent of any secondary effect due to changes in blood pressure. Recent evidence suggests that the primary mediator of the effects of salty diets on the left ventricle might be marinobufagenin. Marinobufagenin is produced endogenously in response to high levels of sodium chloride consumption. It is an ouabain-like compound which has a specific effect on the alpha-1 isoform of the sodium-potassium pump in the coronary microvasculature, which is suggested might lead to altered ventricular structure and function (Manunta and Ferrandi, 2004; McCarty, 2004).

3.2.2.3.2. Blood vessels

There is experimental evidence that a high sodium diet results in dilatation and reduced distensibility of arteries. Consumption of a higher sodium diet, leading to an increase in plasma sodium, may be sufficient to stimulate vascular reactivity (Simon and Kocks, 2003). Thus increased sodium consumption may exert a direct effect on the rigidity of vessels and also on their responsiveness to vasodilatory stimuli (Kocks *et al*, 2004). In untreated, older hypertensives, 4 weeks of sodium restriction increased compliance of the carotid artery significantly by 27% (Gates *et al*, 2004).

3.2.2.3.3. Blood pressure

Usual blood pressure is strongly and directly related to overall mortality and to mortality from stroke, ischaemic heart disease, and other vascular mortality, without any evidence of threshold, down to blood pressures of at least 115mm Hg (systolic) and 75 mm Hg (diastolic) (Prospective Studies Collaboration, 2002). Thus, the risk is increased even within what is considered to be a normal range for blood pressure (less than 140/90 mmHg in adults, WHO, 1996). Analysis of the burden of ill-health and disease which can be attributed to different risk factors indicates that a considerable proportion of

cardiovascular disease is related to non-optimal blood pressure, translating to deaths and years of life lost to death and disability (Lawes *et al*, 2004).

Blood pressure tracks throughout life, and therefore a higher blood pressure at an earlier age is more likely to be associated with a higher blood pressure in later life. It has been suggested that the salt content of the diet during early life might have an important determinant effect on the likelihood of higher blood pressure in adulthood. The longer term effects may be related to the acquisition of a preferential taste for salt, but there is also evidence that it reflects a change in the ability of the individual to handle salt. Salt sensitivity is the term used to identify those individuals in whom the acute ingestion of salt leads to identifiable metabolic changes, either an alteration in blood pressure, or a constraint on the renal ability to respond to a salt load (SACN, 2003; FNB, 2004). There is some evidence of a genetic predisposition to salt sensitivity, as the prevalence is higher in some racial groups, for example African-Americans. The increased susceptibility to the adverse effect of sodium on blood pressure with ageing is thought to reflect a declining renal capacity to handle salt with the decline in the number of nephrons and their function with time.

Although much of the effect on blood pressure has been attributed to the sodium moiety, there is a body of evidence which indicates that the chloride ion also has a role to play in a predisposition to salt-sensitive hypertension (Boegehold and Kotchen, 1989). As early as 1929, it was reported that a diet high in sodium bicarbonate did not have the same effect on raising blood pressure as sodium chloride (Berghoff and Geraci, 1929). This has been confirmed by others (Morgan, 1982; Kurt and Morris, 1983; Luft *et al*, 1990). The effect of sodium chloride on blood pressure has not been seen with sodium phosphate (Shore *et al*, 1988), or sodium citrate (Kurtz *et al*, 1987; Tomita *et al*, 1990; Sato *et al*, 1991). Similarly, when the chloride ion is taken without sodium the effects on blood pressure are less evident (Grollman *et al*, 1945; Dole *et al*, 1950). Thus, the findings from human studies support the evidence from animal investigations that both sodium and chloride are required for the effects of salt on blood pressure to be manifest. The evidence would suggest that changes in blood volume underlie these effects (Tomita *et al*, 1990), which are closely related to alterations in the set point for renal salt and water homeostasis. Recent molecular studies implicate a specific role for the anion exchanger pendrin, and its expression in the kidney (Quentin *et al*, 2004).

The impact of dietary sodium (salt) on blood pressure may be affected by consumption of potassium or calcium. The urine sodium-potassium ratio is a stronger correlate of blood pressure than sodium or potassium alone (Intersalt study, 1988; Kwah and Barrett-Conner, 1988; Mc Carron *et al*, 1984). Oral potassium supplementation lowered blood pressure and the magnitude of this effect was found to be more pronounced in subjects consuming a diet high in salt (Cappuccio and Mc Gregor, 1991).

3.2.2.3.3.1. Infants and children

There is evidence that blood pressure during early life might be related to the concurrent consumption of sodium, and that the level of sodium consumption during early life might have longer term effects on blood pressure.

Pomeranz *et al* (2002) compared the effect on blood pressure in term infants of consuming human milk (sodium content 7 mmol/L), or consuming a formula with a low content (sodium 9.5 mmol/L), or a higher sodium content (16.6 mmol/L), during the first 8 weeks of life. Compared with infants consuming human milk, systolic blood pressure was significantly increased in infants fed a formula with the higher sodium content at 8 weeks of age. Even following reversion to a lower sodium intake, this group of infants had higher blood pressure than the breast fed infants at 24 weeks of age.

Hofman *et al* (1980) carried out an observational study of the relationship between blood pressure and the sodium content of drinking water in 348 children aged 7.7 to 11.7 years of age in The Netherlands and demonstrated a relationship between sodium consumption and blood pressure in the short term. They went on to recruit 476 healthy newborns in a single centre during 1980 who were randomized to receive either a low (4.9 mmol/day) or a high sodium diet (13.9 mmol/day), with measurements of blood pressure being taken every 4 weeks. After 25 weeks of intervention systolic blood pressure was 2.1 mm Hg lower in the low sodium group than in the high sodium group, after adjustment for weight and length at birth and systolic blood pressure in the first week of life (Hofman *et al*, 1983). Longer term follow-up was carried out after 15 years in 35% of the original cohort, and the adjusted systolic blood pressure was 3.3 mm Hg lower in the children who had been assigned to the low sodium group during infancy (Geleijnse *et al*, 1996). A cohort of 233 children aged from 5 to 17 years from The Netherlands were followed for 7 years with annual measurements of sodium excretion based on timed overnight specimens. Mean

systolic blood pressure increased with age, but this change was lower when the sodium intake relative to potassium intake was lower and when the intake of potassium was higher (Geleijnse *et al*, 1990).

An intervention study was carried out to determine the effect of reducing sodium intake in 80 children aged 6 to 9 years who had high blood pressure. Sodium consumption was determined from diet diaries and overnight urine collections. One year after randomisation sodium intake was significantly lower in the intervention group, 87 compared with 130 mmol/24 hours, but no significant differences in blood pressure were recorded (Gillum *et al*, 1981).

Cooper *et al* (1980) measured urinary sodium in seven consecutive 24 hour urine collections from 73 children aged between 11 and 14 years. They demonstrated a significant correlation between mean individual sodium excretion and blood pressure, which persisted when they controlled for height, weight, pulse, age, sex and race, but was eliminated when they controlled for creatinine excretion. They were not able to reproduce these findings in later studies carried out according to the same protocol (Cooper *et al*, 1983).

Complete collections of urine and ambulatory blood pressure over 24 hours were determined in 85 obese and 88 non-obese children aged 3 to 19 years. Weight and sodium excretion were directly associated with systolic blood pressure, but the relationship was modified in obese children compared with controls (Lurbe *et al*, 2000).

3.2.2.3.3.2. Adults

Observational and ecological studies show that across populations there is a strong direct relationship between average intakes of salt and prevalence of hypertension (Dahl, 1960; Gleiberman, 1973; Elliott, 1991). The results are less consistent for studies within populations, which often lack sufficient statistical power, or variation in habitual levels of salt consumption (FNB, 2004).

Systematic reviews

A number of systematic reviews have been carried out to characterise the literature on the relationship between dietary sodium consumption and blood pressure. Adherence to a diet low in sodium over long periods of time is difficult to achieve, because salt is so widely distributed throughout different foods (Kumanyika *et al*, 2005). In reviewing the effect of dietary salt on blood pressure, the Institute of Medicine (FNB, 2004) assessed studies of shorter and longer duration separately, to take some account of these difficulties in compliance, and also the possibility of time-related differences in biological responses. Although on average a reduction in the sodium intake appears to lower blood pressure, the individual response may be highly variable, either because of methodological difficulties related to measurement, or to genuine biological variability (Miller *et al*, 1987).

A number of reviews have included studies with a duration of observation of less than 6 months (Law *et al*, 1991 a and b; Midgley *et al*, 1996; Cutler *et al*, 1997; Graudal *et al*, 1998; Alam and Johnson, 1999). For example, the objective for Midgley *et al* (1996) was to determine whether restriction of dietary sodium lowers blood pressure in hypertensive and normotensive individuals. Fifty-six trials met inclusion criteria. Publication bias was evident. Studies were included which used urinary sodium as a proxy measure of dietary sodium intake. The age of the normotensive participants was 26 years, and for the hypertensive subjects 47 years. The median urinary sodium in the hypertensive group was 158 mmol/day, and in the normotensive group 164 mmol/day and the average change in sodium consumption in the hypertensive group was 95 mmol/day, and in the normotensive group 125 mmol/day. Following adjustment for measurement error, the decrease in blood pressure for a 100 mmol/day reduction in daily sodium excretion was 3.7 mm Hg systolic and 0.9 mm Hg diastolic in the hypertensive, and 1.0 mm Hg systolic and 0.1 mm Hg diastolic in the normotensive trials, respectively. There was wide variability in response between studies. The decreases were greater in individuals who were older and in those who had hypertension. Trials of shorter duration tended to show greater decreases in blood pressure. This raises the question of the extent to which variability in response may be related to the degree of compliance. There were no changes in urinary potassium or in body weight where these had been measured.

The studies included in more recent systematic reviews have sought to include studies in which the period of observation extends for longer than 6 months (Ebrahim and Smith, 1998; Hooper *et al*, 2002). In general the longer studies provide an insight into the ability of individuals to adhere to advice to consume a diet low in salt, as much as the effect of a lower salt diet on outcomes. Hooper *et al* (2002) were able to identify three trials in people with hypertension (n=2326); five in people with untreated

hypertension (n=387); three in people with treated hypertension (n=801). They had been followed up for periods from 6 months to 7 years and the endpoints were mortality and cardiovascular events. There were reductions in both systolic and diastolic blood pressure at both intermediate and late follow up, and reductions in urinary sodium excretion at both intermediate and late follow up. The low salt diets seemed to allow people with hypertension to stop taking medication. In eleven long-term randomised controlled trials of dietary salt reduction there were few data on mortality from cardiovascular events or quality of life but they did show significant falls in systolic blood pressure and urinary sodium excretion 13 to 60 months after initial advice. In their review, He and MacGregor (2004) included 734 subjects from 17 trials of individuals with high blood pressure and 2220 individuals with normal blood pressure from 11 trials. For individuals with normal blood pressure there was a reduction in urinary sodium of 74 mmol/24 hours (4.4 g salt/day) and a fall in systolic blood pressure of 2.03 mmHg, and a fall in diastolic blood pressure of 0.99 mmHg. For those with elevated blood pressure there was a reduction in urinary sodium of 78 mmol/24 hours (4.6 g salt/day) and a fall in systolic blood pressure of 4.97 mmHg, and in diastolic blood pressure of 2.74 mmHg.

Miller *et al* (1983) studied 16 healthy husband-wife pairs (men aged 40 years, women aged 37 years) to determine the effect on blood pressure of a reduction in dietary sodium from 152.7 (SE 10.1) mmol/day to 69.5 (SE 4.5) mmol/day for periods up to 12 weeks. In the men blood pressure decreased from 102/71 mm Hg to 99/68 mm Hg, and in women from 114/77 mm Hg to 109/73 mm Hg. There was no change in potassium excretion or body weight. There was variability in the blood pressure response amongst individuals and in 24 of 31 systolic blood pressure decreased, whereas 7 of 31 showed no decrease. In one person there was an increase that achieved statistical significance. Individuals with higher initial blood pressure were more likely to respond with a decrease in blood pressure. The decrease in blood pressure was significantly correlated with the magnitude of the sodium restriction ($r=0.36$, $p<0.03$)

Whelton *et al* (1998) carried out a trial of non-pharmacological interventions in the elderly to determine whether weight loss or reduced sodium intake was effective in treatment of older persons with hypertension. The study comprised 975 older people aged 60 to 80 years with systolic pressure <145/85 mm Hg, receiving treatment with single hypertensive medication. There were 585 obese and 390 non-obese subjects. The goal for sodium reduction was to maintain an intake below 80 mmol/day (1800 mg/day), based upon measurements made by 24 hour urine collection. The objective was to reduce the medication being given to reduce blood pressure medication and follow up was up to 30 months. The results showed that a 30% reduction in the need for antihypertensive therapy was achieved by reducing the average sodium intake by about 40 mmol/day, and by decreasing body weight on average by 3.5 kg. Best blood pressure control was achieved with combined interventions of both sodium reduction and weight reduction.

The strongest evidence that a change in dietary sodium leads to a change in blood pressure, with or without other dietary changes, in normotensive and hypertensive individuals, who are white or black, comes from the Dietary Approach to Stop Hypertension-sodium trial (DASH-sodium) (Sacks *et al*, 2001). The original DASH trial explored the effect of a diet rich in vegetables and fruit compared with a standard American diet. In the DASH-sodium trial the effect of a diet rich in vegetables and fruit and containing low-fat dairy products was compared with a standard American diet, and for each dietary pattern the level of salt was modified at three levels, high (about 150 mmol/day), intermediate (about 100 mmol/day) and low (about 50 mmol/day). 412 persons were randomly assigned to the control or DASH, foods at the three levels of sodium intake for 30 consecutive days. Actual consumption was assessed from 24 hour urinary excretion of sodium. The urinary sodium in the three groups was 144 mmol/day for the high groups, 106 mmol/day for the intermediate, and 65 mmol/day for the low, indicative of good compliance. At all levels of salt intake those on the DASH diet had significantly lower blood pressure than those on the control diet. When the sodium intake was reduced from the high to the intermediate level of consumption there was a reduction in systolic blood pressure by 2.1 mmHg in control and 1.3 mm Hg during DASH. When the sodium intake was reduced from the intermediate level to the low level of consumption there was a reduction in systolic blood pressure by 4.6 mm Hg in control and 1.7 mm Hg during DASH. As compared with the control diet high in sodium, the DASH diet low in sodium led to a mean systolic blood pressure which was 7.1 mm Hg lower in those without high blood pressure and 11.5 mm Hg in those with high blood pressure.

The level of dietary sodium had approximately twice as great an effect on blood pressure on the control diet compared with the DASH diet, for every level of intake. As the level of sodium intake was lowered progressively the response in terms of a lower blood pressure was greater. Consumption of the DASH diet compared with control diet resulted in a significantly lower systolic blood pressure at every level of

sodium consumption, and the effect on blood pressure was greater at high sodium levels than at lower sodium levels. The effect was seen in those with and those without hypertension, but was greater in those with hypertension.

The relationship between dietary sodium consumption and blood pressure has also been investigated in short-term salt loading studies. Murray *et al* (1978) observed consistent increases in blood pressure when normotensive human adult subjects were subjected to a sodium intake greater than 800 mmol/day, but Roos *et al* (1985) were unable to detect consistent changes in blood pressure with intakes as high as 1200 mmol/day.

Luft *et al* (1979) studied 14 normotensive healthy male volunteers (age 32 years, range 18-40 7 white and 7 black), who were given different levels of salt supplementation, 10 mmol/24 hours for 7 days, and 300, 600, 800, 1200, 1500 mmol/24 hours for 3 days each. In 6 subjects the study was repeated on a sodium intake of 1500 mmol/24 hours, while urinary potassium losses were replaced on a 24 hour basis. Urine was collected throughout for each 24 hour period, and echocardiography was used to measure cardiac size and function. The subjects experienced no ill effects, other than some diarrhoea at the highest levels of salt consumption which was corrected when water consumption increased. Total sodium excretion approached total sodium intake by 72 hours. There was considerable variability in the response within individual subjects. Most developed a significant increase in both systolic and diastolic blood pressure with the higher sodium intake, although there was no increase in a few. In white subjects the increase in blood pressure was significant at an intake of 1200 mmol/day, relative to the baseline of 10 mmol/day, whereas black subjects developed significant increased blood pressure by 800 mmol/day. Overall blood pressure increased significantly for sodium intakes between 10 and 1500 mmol/24 hours from 113 to 131 mm Hg systolic, and for sodium intakes between 10 and 1200-1500 mmol/24 hours from 69 to 85 mm Hg diastolic. There was a significant linear regression relationship between urinary sodium excretion and increase in blood pressure ($y=111.5 + 0.0083$ x: $r=0.48$, $p <0.001$).

There was no consistent change in plasma sodium or potassium concentration, but there was an increase in body weight of up to 5 kg. Cumulative potassium balance became negative on day 10 or 11 in subjects taking more than 600 mmol Na/day with a net deficit of -163 mmol or more. The effects of high sodium intake were attenuated when the study was repeated with subjects taking supplements of potassium to correct for urinary losses of potassium. Blood pressure returned to baseline values within 3 days of the completion of the study. It was concluded that blood pressure can be raised acutely in normotensive men by increasing sodium consumption to very high levels.

Myers *et al* (1982) reported a study in 136 normal individuals, aged 10 to over 60 years of age who were randomly assigned to an intake of sodium which was either 70 mmol/day or 200 mmol/day for periods of 14 days. Adherence was checked from urinary measurements and weight and blood pressure were recorded at the end of each study period. There was a significant increase in blood pressure for the group as a whole. The proportion which might have been considered to be hypertensive increased from 14 on the lower salt intake to 23% on the higher salt intake, which was more marked in the older people (13 to 50%) than in the younger people (0 to 4%).

3.2.2.4. Cardiovascular risk

He *et al* (1999) reported a prospective cohort study based upon the NHANES of 1971-75, in those aged 25 to 75 years, of whom 2688 were overweight and 6797 were normal weight. Sodium consumption estimates were based on a single 24 hour recall for dietary sodium. Over a period of 19 years of follow up there were 680 stroke events (210 fatal), 1727 coronary events (614 fatal), 895 cardiovascular disease deaths and 2486 deaths from all causes. Among overweight people, 100 mmol higher sodium intake was associated with 32% increase in stroke incidence, 89% increase in stroke mortality, 44% increase in coronary heart disease mortality, 61% increase in cardiovascular disease mortality and 39% increase in mortality from all causes. Dietary sodium was not significantly associated with cardiovascular disease risk in non-overweight persons. It was concluded that the effects on mortality and morbidity were greater than could have been expected from a simple effect on blood pressure. This raised the possibility of an independent direct effect of sodium intake on cardiovascular disease. Obesity activates the sympathetic nervous, the renin-angiotensin systems and causes insulin resistance and hyperinsulinaemia, and alters intra-renal vascular resistance. Therefore, these changes separately or together may have been related to enhance renal tubular sodium reabsorption and sodium retention.

In a prospective study of 1173 Finnish men and 1263 women aged 25-64 years, an increase in urinary sodium of 100 mmol/day was associated with hazard ratio for coronary heart disease, 1.51;

cardiovascular disease, 1.26; all cause mortality, 1.26 (Tuomilehto *et al*, 2001). The frequency of acute coronary events rose significantly with increasing sodium excretion and body mass index. High sodium intake predicted mortality and risk of coronary heart disease independent of other coronary risk factors, including blood pressure. There was direct evidence of harmful effects of high salt intake in the adult population. The median intake for men was 205 mmol/day, and for women, 154 mmol/day. Quartiles of excretion for men were <159, -205, -262, >262 mmol/24 hours; and for women <119, -154, -194, >194. Sodium was a more powerful predictor of mortality in men who were overweight.

In a prospective study in Japan, the relationship between deaths from stroke and sodium intake was assessed using a semi-quantitative food frequency questionnaire in 13,355 men and 15,724 women (Nagata *et al*, 2004). There were 269 deaths from stroke, and after controlling for covariates, there was a significant positive relationship between stroke deaths and sodium intake: hazards ratio (HR) 3.85 between the highest and lowest tertile for sodium consumption for men. The relationship was less strong for women (HR 1.7), and of borderline significance. Thus, although there is some evidence of a direct relationship between consumption of salt and death from stroke which is independent of blood pressure and most marked in people with a higher body mass index (He *et al*, 1999; Nagata *et al*, 2004), the finding is not consistent for all studies (Kagan *et al*, 1985; Alderman *et al*, 1998; Tuomilehto *et al*, 2001).

4. DOSE-RESPONSE ASSESSMENT

There are data which indicate that the human acute consumption of large doses of sodium chloride, such as 0.5 to 1.0 g per kg body weight can have fatal consequences, but these doses are far greater than those generally consumed on a habitual basis. The ingestion of concentrated solutions of sodium chloride can lead to local gastrointestinal irritation and mucosal damage, both in rodents and humans. This has been considered to increase the susceptibility to the carcinogenic effects of other carcinogens, such as nitrosamines, infection with *H. pylori*, or inadequate protection against free radical induced damage both in rodents and humans. Based on data collected in the Intersalt population study, no increased stomach cancer mortality was reported with the sodium intakes less than 2.7 g/day for men and 2.1 g/day for women. High concentration of salt may indirectly damage, through physical effects, DNA or chromosomes of bacterial and mammalian cells *in vitro*.

Higher blood pressure is an acknowledged risk factor for ischaemic heart disease, stroke and renal disease. For groups of individuals there is strong evidence of a dose response relationship between increased consumption of sodium as sodium chloride and higher levels of systolic, diastolic and mean blood pressure (Sacks *et al*, 2001). The effect of sodium on blood pressure is linked to that of chloride. This is a continuous relationship which embraces the levels of salt habitually consumed, and continued benefit is seen at levels of salt consumption which are much lower than have been recommended as targets for population consumption (SACN, 2003; WHO/FAO, 2003). The character of the dose response can be modulated by a range of factors which include other components of the diet, relative weight, and level of physical activity, as well as fixed factors which include age, gender and genotype. It is not possible to determine a threshold level of habitual sodium consumption below which there is unlikely to be any adverse effect on blood pressure.

The evidence for adverse cardiovascular effects of sodium, which is supported by number of prospective studies, indicate an association of increased risk of morbidity and mortality from cardiovascular diseases, including coronary heart disease and stroke, with increasing sodium intake. While it has been suggested that the magnitude of the observed effects was greater than could have been expected from a simple effect on blood pressure, there is no direct evidence for this, and evidence that high sodium intake may have a direct adverse effect on left ventricular structure and function, independent of any secondary effect due to changes in blood pressure, is not conclusive.

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The available data are not sufficient to establish an upper level for sodium from dietary sources.

2. RISK CHARACTERISATION

The habitual intake of sodium for populations across Europe is high and exceeds the amounts required for normal function. The current levels of sodium consumption as sodium chloride have been associated directly with a greater likelihood of increased blood pressure, which in turn has been directly

related to the development of cardiovascular disease and renal disease. For these reasons, national and international bodies have set targets for a reduction in the sodium consumed in the diet (SACN, 2003; FNB, 2004; WHO, 2003 and 2004).

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF PHOSPHORUS

(REQUEST N° EFSA-Q-2003-018)

(ADOPTED ON 1 JULY 2005 BY WRITTEN PROCEDURE)

SUMMARY

Phosphorus as phosphate is an essential nutrient involved in many physiological processes, such as the cell's energy cycle, regulation of the whole body acid-base balance, as a component of the cell structure (as phospholipids), in cell regulation and signalling, and in the mineralisation of bones and teeth (as part of the hydroxyapatite).

Estimates of habitual dietary intakes in European countries are on average around 1000-1500 mg/day, ranging up to about 2600 mg/day. The contribution of food supplements to phosphorus intake is low.

Adverse effects of excessive phosphorus intake, such as hyperphosphatemia, leading to secondary hyperparathyroidism, skeletal deformations, bone loss, and/or ectopic calcification have been reported in animal studies. However, such effects were not observed in studies in humans, except in patients with end stage renal disease. Although in acute or short term loading studies an increase in serum parathyroid hormone (PTH) levels has been found, no significant changes could be demonstrated in longer term studies with dosages up to 3000 mg/day (for 6 weeks). In these studies no evidence was found for effects on markers of bone remodelling and the Panel does not consider these to be adverse effects. Similarly, the Panel found no convincing evidence to support suggestions that high phosphorus diets would aggravate the effects of a state of secondary hyperparathyroidism induced by inadequate calcium intakes, or an inadequate vitamin D status.

Gastrointestinal symptoms, such as osmotic diarrhoea, nausea and vomiting, have been seen in some healthy subjects taking phosphorus (phosphate) supplements with dosages >750 mg/day. The Panel considered that these are not a suitable basis to establish an upper level for phosphorus from all sources.

The Panel concludes that the available data are not sufficient to establish an upper level for phosphorus.

The available data indicate that normal healthy individuals can tolerate phosphorus (phosphate) intakes up to at least 3000 mg/day without adverse systemic effects. In some individuals, however, mild gastrointestinal symptoms have been reported if exposed to supplemental intakes >750 mg phosphorus per day. There is no evidence of adverse effects associated with the current dietary intakes of phosphorus in EU countries.

KEY WORDS

Phosphorus, tolerable upper intake level, food safety.

BACKGROUND

In 2002, the European Parliament and the Council adopted the Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

1. *Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.*

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.

ASSESSMENT

1. INTRODUCTION

Phosphorus is an element of group 5 of the periodic table and has an atomic weight of 30.97. It is one of the most abundant elements on earth, most commonly found as the phosphate ion (PO_4^{3-}), with phosphorus in its pentavalent form. Phosphorus (as phosphate) is an essential dietary constituent, involved in numerous physiological processes, such as the cell's energy cycle (high-energy pyrophosphate bonds in adenosine triphosphate [ATP]), regulation of the whole body acid-base balance, as component of the cell structure (as phospholipids) and of nucleotides and nucleic acids in DNA and RNA, in cell regulation and signalling by phosphorylation of catalytic proteins and as second messenger (cAMP). Another important function is in the mineralization of bones and teeth (as part of the hydroxyapatite).

Reviews and safety evaluations for phosphorus are available from the Joint FAO/WHO Expert Committee on Food additives (JECFA, 1982), the Food and Nutrition Board of the Institute of Medicine (FNB, 1997), and the UK Expert Group on Vitamins and Minerals (EGVM, 2003). JECFA has used the nephrocalcinosis, induced by excessive phosphate intake in rats, as the critical effect to set a maximum tolerable daily intake (MTDI) of 70 mg/kg for phosphoric acid and phosphate salts. The FNB set an upper level for phosphorus of 4.0 g/day for adults, based upon a NOAEL which represents the extrapolation of the phosphorus intake to serum phosphorus concentration curve in adults up to the intake of phosphorus which would result in serum phosphorus levels of infants, which are considered to be safe for tissues with respect to metastatic mineralization. The UK Expert Group on Vitamins and Minerals used the gastrointestinal effects due to high supplemental phosphate intake, to establish a NOAEL of 750 mg/day for supplemental phosphorus.

2. NUTRITIONAL BACKGROUND, FUNCTION, METABOLISM AND INTAKE

2.1. Food levels and dietary intake

Phosphorus is widely found in foods as phosphates; especially foods rich in protein are usually high in phosphorus, such as dairy products (100-900 mg/100 g), meats (200 mg/100 g), fish (200 mg/100 g) and grain products (100-300 mg/100 g). The average intake from foods in adults is usually between 1000-2000 mg/day. Dietary phosphorus intakes in various European countries are given in Table 1.

Table 1. The daily intakes of phosphorus in some EU countries (mg/day)

	Population	N	Method	Supplements	Mean	97.5 percentile
Italy[a]	Household	2374	7-day record	+	1304	2076
Germany[b]	Individual (M) Individual (F)	862 1144	7-day record + food frequency record	- -	1488 1188	2517 1988
Netherlands[c]	Household	5958	2-day record	-	1480	2601
Sweden[d]	Individual (M) Individual (F)	1214	7-day record	- -	1570 1290	2517 1988
UK[e]	Individual (M) Individual (F)	656 803	7-day record	- -	1493 1112	2381 1763

[a] Turrini (INRAN)
[b] Heseker *et al* (1994) - values are median and 95 percentiles.
[c] Hulshof and Kruizinga (1999)
[d] Becker and Pearson (2002) - values are mean and 95 percentiles.
[e] Henderson *et al* (2003)

Phosphoric acid and phosphate salts are also added as food additives in processed foods and in soft drinks as acidity regulators and stabilizers.

In the US the contribution from phosphorus-containing food additives is estimated at 320 mg/day, i.e. 20-30% of the adult phosphorus intake (Calvo and Park, 1996). No specific data on the contribution of phosphorus-containing food additives to the total intake of phosphorus in the EU have been identified, but this is likely increasing due to the higher consumption of processed foods and carbonated soft drinks. Cola soft drinks contain between 120-200 mg/L phosphorus. Fruit flavoured soft drinks are mostly acidulated with citrate rather than phosphoric acid, and contain little or no phosphate.

Phosphorus is also present in drinking water, with a maximum allowed content of 2.2 mg/L (Council Directive 80/778/EEC).

Dietary supplements may also contain phosphorus (as phosphates), but dose levels are generally low. A survey by the Netherlands consumer organisation indicated low to moderate phosphorus levels in the commonly sold supplements, i.e. <400 mg/tablet (Gezondgids Consumentenbond, 2002). The phosphorus content in multivitamin supplements, sold in the UK, varies between 15 and 1100 mg per supplement dose (EGVM, 2003). The contribution of dietary supplements to the average population intakes in the UK was found to be zero or negligible for all age groups (EGVM, 2003). High-protein bars and other products marketed for “enhanced” athletic performance and muscle mass building may also contain high levels of phosphorus.

2.2. Nutritional requirements and recommendations

The phosphorus requirement has often been linked to the calcium requirement, allowing a Ca: P weight ratio of about 1. The SCF based their requirements on a molar basis (1:1) with the calcium requirements and established a Population Reference Intake (PRI) of 550 mg/day in adults (17+) (SCF, 1993). For young children (6 months up to 3 years) the PRI is 300 mg/day, increasing up to 775 mg/day in young males (11-17 years), and 625 mg/day in young females. For lactating women the PRI was set at 950 mg/day, allowing an incremental 400 mg/day for breast milk production (SCF, 1993). In other countries, such as The Netherlands, lower ratios have been used, i.e. between 0.5 and 1.0. More recently the D-A-CH (2000) established a recommended intake of 700 mg/day for adults (19+), and up to 1250 mg/day for adolescents (10-19 years). The FNB make similar recommendations based upon maintenance of the serum inorganic phosphate level (Pi) within the normal range (FNB, 1997). For the younger age groups, a factorial approach was used, taking into account phosphorus accretion in bone and (lean) tissues.

As the body can maintain a phosphate (and calcium) balance over a wide range of intakes and Ca:P ratios, this ratio is nowadays considered of limited value for evaluation of dietary adequacy. Only in infants and children under conditions of rapid growth such a ratio has some relevance to enable optimal growth.

In human milk the Ca:P ratio is 1.5:1 (w:w), and this is considered the optimal ratio for the infant. This ratio equals more or less the ratio in human bone mineral (Nordin, 1976). The infant formula and follow-on formula Directive in the EU states that the Ca:P ratio should be between 1.2 and 2.0.

2.3. Function, uptake and distribution

Net absorption from a mixed diet has been reported to vary between 55-70% in adults (Lemann 1996; Nordin, 1986) and between 65-90% in infants and children (Ziegler and Fomon, 1983). There is no evidence that, contrary to calcium, absorption efficiency varies with dietary intake. Phosphate absorption is greatest in jejunum and takes place by a saturable, active transport mechanism, facilitated by 1,25-dihydroxyvitamin D, as well as by passive diffusion (Chen *et al*, 1974).

Some forms of dietary phosphorus are less bioavailable, especially phosphorus present in phytic acid in the outer coatings of cereal grains. The actual bioavailability depends on the way these grain products are processed and the amount of residual phytate. Some dietary components, as well as colonic bacteria, contain phytase activity rendering phytate phosphorus more available.

The phosphorus content of the adult human body is about 700 g (as elemental phosphorus). About 85% of total phosphorus is present in the skeleton, the remainder in the soft tissues and in the extracellular fluids (*circa* 1%) (Lloyd and Johnson, 1988). Total body phosphorus content increases from 0.5% on a body weight basis in infancy up to 0.65-1.1% in adults (Fomon and Nelson, 1993). Total phosphorus concentration in whole blood is approximately 13 mmol/L (40 mg/dL). Approximately 70% is present as organic phosphates, such as in the phospholipids of red blood cells and in the plasma lipoproteins. The other 30% is present as inorganic phosphate, of which 15% is protein bound. About 50% of the inorganic phosphate is in the soluble divalent cation form (HPO_4^{2-}), the remaining as the monovalent anion (H_2PO_{4-}, 10%) and the trivalent cation (PO_4^{3-}, <0.01%), or as HPO_4^{2-}complexed with sodium, calcium and magnesium salts. These anion forms are interconvertible and effective buffers of blood pH and involved in regulation of the whole body acid-base balance.

The inorganic phosphate (Pi) fraction in the extracellular fluid is of critical importance and under endocrine control of parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D, secondary to the regulation of serum calcium concentrations. Serum Pi is less tightly controlled than serum calcium and varies throughout the day following a circadian rhythm with peaks in the early morning and the afternoon (Portale *et al*, 1989; Calvo *et al*, 1991). Serum Pi in normal adults varies between 0.97-1.45 mmol/L (3.0-4.5 mg/dL), and shows a slight increase with increasing phosphorus intakes (Heaney, 1996). Hyperphosphatemia, associated with clear clinical symptoms, has only been reported in patients with end-stage renal disease, i.e. when glomerular filtration rate (GFR) has decreased below 20% of the adult value (FNB, 1997). In males, but not females, serum Pi values decrease with increasing age. Serum Pi values in young infants are about two times higher than in adults (1.29-2.26 mmol/L), due to a lower GFR, and a consequently lower phosphate excretion capacity in the kidneys in the first months of life (Manz, 1992; Endres, 1996).

PTH secretion can be induced by low calcium and high phosphorus intake, and results in a decrease in serum Pi by increasing urinary phosphate excretion (Calvo *et al*, 1988, 1990 and 1996). This PTH effect is mediated through the production of the active vitamin D metabolite 1,25-dihydroxyvitamin D by an effect on the 1-α-hydroxylase activity in the kidney. Excretion of endogenous phosphorus is mainly through the kidneys. Phosphate is filtered at the glomerulus and 80-90% is reabsorbed in the proximal renal tubules. Until recently, tubular reabsorption was thought to be mainly controlled by PTH and 1,25-dihydroxyvitamin D, but more recent evidence suggests an important regulatory role for a novel group of circulating phosphaturic hormones, called phosphatonins, such as the fibroblast growth factor 23 (FGF-23) (Quarles, 2003). Pi transport in the proximal tubule is driven by a Na+-dependent Pi transporter protein, residing in the brush-border membrane. Most proximal tubular Pi reabsorption occurs via the type 2a (NPT2) co-transporter. Dietary phosphorus intake also has a direct effect on the renal Pi excretion rate. Feeding a high phosphorus diet results in down-regulation of the NTP2 co-transporter (Keusch *et al*, 1998), while a low phosphorus intake will increase the amount of NTP2 cotransporter, and thus increase Pi reabsorption (Tenenhouse, 1997).

Genetic defects in the proximal tubular NTP2 co-transporters can impair renal Pi reabsorption and result in hyperphosphaturia, such as in X-linked hypophosphatemia and hereditary hypophosphatemic rickets with hypercalciuria (Tenenhouse, 1997; Murer *et al*, 1999).

2.4. Deficiency

The development of a dietary-induced phosphorus deficiency is very unlikely due to the ubiquitous presence in foods. Hypophosphatemia can occur in patients and infants receiving poorly managed parenteral nutrition, and in patients suffering from liver disease, sepsis, antacid therapy with aluminium containing drugs, and in diabetic ketoacidosis. Symptoms include anorexia, anaemia, muscle weakness, bone pain, rickets, and ataxia (Lotz *et al*, 1968). Inadequate intake of calcium and phosphorus has been associated with pathogenesis of bone disease in newborn infants (Bishop, 1989).

3. HAZARD IDENTIFICATION

The inorganic phosphate fraction in the extracellular fluid is under endocrine control of the parathyroid-vitamin D axis. Excess phosphorus intake might result in hyperphosphatemia and a consequent increase in serum PTH level. Secondary hyperparathyroidism leads to increased bone resorption which might adversely affect bone mineral density and skeletal integrity, and result in ectopic calcification. Such phosphorus induced effects have been observed in animal studies, but not in humans, except in patients with end-stage renal disease. As long as the kidney function, i.e. renal capacity, is adequate the excess phosphate is excreted. In some supplementation studies using high phosphorus dosages, osmotic diarrhoea and mild gastrointestinal symptoms have been reported.

3.1. Adverse effects in animals

3.1.1. Acute toxicity

Histological and histochemical changes have been described in the kidneys of rats fed for 24 to 72 hours a diet containing 10% disodium acid phosphate (providing approximately 5 g/kg body weight/day equivalent to about 1200 mg elemental phosphorus/kg body weight/day elemental phosphorus) (Craig, 1957).

Ritskes-Hoitinga *et al* (1989) found marked kidney calcification and a rise in albumin concentration in urine in female rats fed for 28 days a diet containing 0.6% sodium hydrogen phosphate (approximately 300 mg phosphorus/kg body weight/day). It was concluded that dietary phosphorus-induced nephrocalcinosis is associated with impaired kidney function in rats.

3.1.2. Sub-chronic toxicity

Pathological effects in the parathyroid, kidneys and bones have been observed in mature male rats fed a diet containing an excessively high level of sodium orthophosphate (8% in the diet which is approximately 4 g/kg body weight/day, providing about 1 g/kg body weight/day elemental phosphorus or 38 mmol phosphorus/kg body weight/day) for 7 months or until the animals succumbed (Saxton and Ellis, 1941). Microscopic examinations of the tissues at the time of death revealed hypertrophy and hyperplasia of parathyroid cells. Calcium deposits were present in the tubules of the kidneys and other organs. The long bones of the animals appeared thickened and more fragile than those of control animals.

In a study with three groups of 12 rats an adequate absorption and utilisation of calcium, phosphorus and iron was found after feeding a control (P: 210 mg/kg body weight/day; Ca: 280 mg/kg body weight/day), a normal orthophosphate (P: 215 mg/kg body weight/day; Ca: 235 mg/kg body weight/day), and a high orthophosphate diet (P: 650 mg/kg body weight/day; Ca: 250 mg/kg body weight/day). The experiment was conducted in three stages, with experimental observations made when animals had consumed the test diets for 50, 60 and 150 days (Dymsza *et al,* 1959). No adverse physiological effects were observed clinically, at autopsy, or on histological examination. The authors concluded that at both high and normal levels of dietary phosphorus the calcium, phosphorus and iron absorption and utilisation were adequate.

Haut *et al* (1980) investigated phosphate-induced renal injury in uninephrectomised, partially nephrectomised and intact rats. Phosphorus was administered in the diet at levels of 0.5, 1.0 and 2% (approximately 250, 500 and 1000 mg/kg body weight/day, respectively) for 18 weeks. None of the animals on a normal phosphorus intake (250 mg/kg body weight/day) showed any abnormalities. Four of 6 intact animals on the 1 % phosphorus diet (500 mg/kg body weight/day) had normal kidney calcium concentrations (one animal showed histological alterations in the kidneys). In contrast, all but one of the partial and uninephrectomised animals on a 1 % phosphorus diet (500 mg/kg body weight/day) had increased kidney calcium concentrations; 5 of the six animals in the group exhibited histological changes in the kidney. It was concluded that as renal functional mass is reduced, the nephrotoxicity of phosphorus is greatly enhanced.

Pettifor *et al* (1984) fed young baboons with semisynthetic, vitamin D-containing diets differing in calcium and/or phosphorus content over a 16 month study period. Diets low in calcium alone (40 mg/100 g) or low in both calcium and phosphorus (90 mg/100 g) led to the development of radiologic rickets and histologic features of osteomalacia at both 8 and 16 months. The diet which was low in calcium but which had a normal phosphorus content (310 mg/100 g) was associated with histologic features of hyperparathyroidism at 16 months; such features did not develop in animals fed the low calcium, low phosphorus diet. Biochemically the low calcium, normal phosphorus diet was associated with a transient fall in serum calcium around 8 months and a more persistent elevation in serum phosphorus and alkaline phosphatase values during the latter half of the study. These biochemical changes were not seen in the baboons on the low calcium, low phosphorus diet.

Based upon an evaluation of the available data from short-term and long-term studies with phosphoric acid and inorganic phosphate salts in rats and other species, JECFA concluded that excessive dietary phosphorus might result in homeostatic adjustments and subsequent bone loss and calcification of soft tissues (JECFA, 1982). The lowest level of phosphorus that produced nephrocalcinosis in the rat (i.e. 1% P in the diet) was used as the basis to set a maximum tolerable daily intake (MTDI) of 70 mg/kg for phosphoric acid and phosphate salts. This value was endorsed by the SCF (1991).

3.1.3. Genotoxicity

No genotoxic effect of inorganic salts of phosphorus was identified (JECFA, 1982).

3.1.4. Reproductive toxicity

Long-term effects of dietary phosphoric acid in three generations of rats have been investigated (Bonting and Jansen, 1956). The animals received diets containing 1.4% and 0.75% phosphoric acid (equivalent to approximately 200 and 375 mg phosphorus/kg body weight/day) for 90 weeks. No harmful effects on growth or reproduction were observed, and also no significant differences were noted in haematological parameters in comparison with control animals. There was no acidosis, nor any change in calcium metabolism. The quality of these older studies would be considered limited by current standards.

JECFA reviewed the available data from studies in mice and rats and concluded that dosing with phosphoric acid and inorganic phosphate salts does not induce maternal toxicity or teratogenic effects. Maximum dose levels tested for the various inorganic phosphate salts varied between 130 and 410 mg phosphorus/kg bodyweight (JECFA, 1982).

3.2. Adverse effects in humans

3.2.1. Acute effects

Osmotic diarrhoea and other mild gastrointestinal effects, including dyspepsia, nausea and vomiting have been observed as side effects in some individuals participating in supplementation studies using higher supplemental dosages (between 750-2250 mg/day; total oral intakes up to 3008 mg phosphorus/day) (Bernstein and Newton, 1966; Bell *et al*, 1977; Broadus *et al*, 1983; Grimm *et al*, 2001, Brixen *et al*, 1992; Whybro *et al*, 1998). Details of these studies are summarized in Table 2.

3.2.2. Adjustment in calcium-regulating hormones and effect on calcium balance and skeletal mass

High phosphorus intake results in the post-absorptive state in an increase in the serum Pi fraction and a subsequent temporary decrease in the serum ionized calcium level. These temporary changes in serum calcium are likely due to the phosphorus induced effects on the serum PTH and 1,25 dihydroxyvitamin D (1,25-$(OH)_2$D) levels, as part of the normal homeostatic control of the serum calcium concentration.

Most of the studies, summarized in Table 2, are of relatively small size (number of subjects included) and of short duration (single dose up to treatment of maximum 6 weeks), except some studies in patient groups, such as hypercalciuria patients (maximum 5 years) (Bernstein and Newton, 1966), multiple myeloma patients and osteoporotic women (maximum 15 months) (Goldsmith *et al*, 1968 and 1976), and hyperparathyroidism patients (12 months) (Broadus *et al*, 1983).

Table 2. Short description of relevant studies on phosphorus supplementation

Study/Authors	Subjects	Dose/Duration (as phosphorus)	Findings
Bernstein and Newton, 1966	Patients with idiopathic hypercalciuria (16-69 yr); n=10	Treatment with 700-2000 mg/day between 4 months and 5 years	Urine Ca ↓; reduced urinary stone formation → Mild to moderate diarrhoea
Reiss *et al*, 1970	Healthy subjects (23-46 yr); n=5	Single dose of 1000 mg/day	60-125% increase in PTH within 60 min. PTH increase abolished after Ca-infusion
Goldsmith *et al*, 1968	Multiple myeloma patients (n=14)	Intravenous and/or oral treatment with 1000-2000 mg/day; up to 15 months	Reduction in bone pain and in urinary calcium excretion. No indication of extra-skeletal calcification; one case of dyspepsia reported
Goldsmith *et al*, 1976	Postmenopausal women with osteoporosis (63-75 yr); n=7	1000 mg/day for 3-15 months Total P-intake: 1696-2740 mg/day; Ca-intake: 616-1459 mg/day	Urine Ca ↓; serum P ↓; no effect on PTH; evidence for increased bone resorbing surface; skeletal mass not measured
Van den Berg *et al*, 1980	Patients with idiopathic hypercalciuria; n=11	2000 mg/day for 2 weeks	Small increase in S-PTH (within normal range) and small decrease in 1,25-$(OH)_2$D; no effect on serum Ca and P-levels → No side effects reported
Broadus *et al*, 1983	Hyperparathyroid patients (mean age 53 yr), n=10	1500 mg/day for 12 months	s-PTH ↑; s-1,25-$(OH)_2$D ↓; urinary Ca ↓ → Several patients reported transient loosening of bowel movement
Portale *et al*, 1986	Healthy men; n=6	3000 mg/day for 10 days, after depletion period on 500 mg/day (+ aluminium hydroxide)	s-1,25-$(OH)_2$D ↓ (-30%); after initial rise in s-Pi, no significant change
Heaney and Recker, 1987	Premenopausal women (43.1 ± 4.4 yr); n=8	Basal diet (1166 mg/day) + supplements (1114 mg /day): total intake 2280 mg/day) for 4 months	no evidence for bone remodelling as measured by radiocalcium kinetics and histomorphometry
Calvo and Heath, 1988	Healthy men (n=8) and women (n=8); 18-25 yr	8 days on test diet with 420 mg Ca and 1660 mg P, after 8 days on control diet (820 mg Ca; 930 mg P)	s-PTH ↑; s-Pi ↑; s-1,25$(OH)_2$D ↑; urine c-AMP ↑; urine Hydroxyproline ↑
Calvo *et al*, 1990	Healthy women (mean age 22 yr); n=15.	Basal diet with 800 mg Ca and 900 mg P; after 28 days 10 women switched to diet with 400 mg/day Ca and 1700 mg/day P for 28 days	s-PTH ↑; no change in s-1,25-$(OH)_2$D; no change in s-osteocalcin and in bone resorption markers
Brixen *et al*, 1992	Postmenopausal women (50-75 yr); n=79	750, 1500 and 2250 mg/day (19-21 subjects per dose) for 7 days; 4 months follow-up. P-containing tablets given on top of regular diet (not specified)	s-PTH ↑ at P-intakes >1500 mg/day; no changes in serum Ca and P; urine P/creatinin-ratio; S-osteocalcin ↑ at 1500 mg, but not 2250 mg dose. No evidence of bone remodelling → Nausea, vomiting and diarrhoea in 2/19 patients at 750 mg dose, 3/19 at 1500 mg dose; 7/20 at 2250 mg dose
Wybro *et al*, 1998	Healthy men (19-38 yr); study 1: n=10 study 2: n=12	Study 1: 1000 mg/day for 1 week; standard diet: 800 mg/day of Ca and P each. Study 2: escalating dose study with 0, 1000, 1500, 2000 mg/day for 1 week; Standard diet: 1000 mg/day Ca and P each	Urine P ↑; urine Ca ↓; no changes in s-Pi, osteocalcin, and urinary N-telopeptide excretion. S-PTH increased only in study 1 → Diarrhoea in 1 subject receiving 2000 mg/day (study 2)
Grimm *et al*, 2001	Healthy women (20-30 yr); n=10.	3008 mg/day P and 1995 mg/day Ca for 6 weeks (incl. 1700 mg P from basal diet	No significant changes in s-PTH, osteocalcin, s-creatinin, and bone resorption markers → Mild intestinal distress (diarrhoea)

Abbreviations: S: serum; ↓: decrease; ↑: increase.; Ca: calcium; P: phosphorus; PTH: parathyroid hormone; 1,25-(OH)2D: 1,25 dihydroxyvitamin D; c-AMP: cyclic adenosine monophosphate.

In nearly all studies phosphorus supplementation resulted in an increased phosphate excretion and decreased calcium excretion. Some, but not all, studies show an increase in serum PTH after acute or long-term exposure to phosphorus loading. The variability in PTH response might, at least in part, be explained by the circadian rhythm in PTH secretion, and, as a consequence, of differences in time of blood sampling between studies. Acute oral phosphorus loading with dosages of 1.0-1.5 g phosphorus

did not result in an increase in serum PTH in young adults (Calvo and Heath, 1988). However, the same authors showed that feeding a "high phosphorus, low calcium" diet (1700 mg and 400 mg phosphorus/day, respectively) for 28 days resulted in a persistent increase in PTH in young healthy women, concurrent with a decrease in the serum ionized calcium level, while no such changes were seen in a control group fed a basal diet containing 900 mg P and 800 mg Ca (Calvo, 1994).

In the study by Brixen *et al* (1992) the PTH increase was not significantly different between the two highest dose groups (i.e. +1500 and +2250 mg phosphorus/day, respectively), but 3 times higher as compared to the control and low dose supplementation group (+750 mg phosphorus/day). In the studies by Whybro *et al* (1998) and Grimm *et al* (2001), PTH levels remained essentially unchanged after supplementation with 1500-2250 mg phosphorus daily for 1 and 6 weeks, respectively.

Under normal conditions an increase in PTH would induce renal synthesis of the active vitamin D metabolite 1,25-$(OH)_2$D. Studies from Portale *et al* (1986 and 1987), however, showed that high phosphorus dose levels decreased serum 1,25-$(OH)_2$D levels due to suppressed renal synthesis. This effect was already reported in the study by van den Berg *et al* (1980) and Broadus *et al* (1983), but not found in the studies from Calvo *et al* (1990) and Brixen *et al* (1992). In the latter study serum 1,25-$(OH)_2$D tended to be lower at the highest dose level (2250 mg/day), but this change was not significant.

It has been suggested that postmenopausal women might be more sensitive to the modulating effects of phosphorus on serum 1,25-$(OH)_2$D levels, i.e. less stimulation of 1,25-$(OH)_2$D production on a low Ca: high P diet, as compared to younger, but actual data are inconclusive (Calvo and Park, 1996).

Variable effects after phosphorus supplementation have been reported for markers of bone resorption. In the study by Goldsmith *et al* (1976) a decrease in bone-forming surface and bone-resorbing surfaces was found in a group of postmenopausal women with osteoporosis, given a daily dose of 1 gram phosphorus (as inorganic phosphate) on top of their normal diet. In an earlier study from the same authors (Goldsmith *et al*, 1968) in multiple myeloma patients on radiation or drug therapy (cyclophosphamide or melphalan), and suffering from bone pain and urinary calcium losses, oral and/or intravenous treatment with phosphate supplements (1000-2000 mg/day phosphorus) reduced the hypercalciuria, even in absence of hypercalcemia. X-ray examination did not indicate extra-skeletal calcification. In one patient even recalcification of the cervical spine was noted after 9 months on the 2 g per day oral phosphorus dose.

In a short-term study by Calvo *et al* (1988) in a group of young men and women (8 days on a test diet containing 420 mg/day Ca and 1660 mg/day P) an increase in urinary hydroxyproline and c-AMP excretion was found, also suggestive of increased bone turnover. However, in a follow-up study by the same authors (Calvo and Heath, 1990) using similar dose levels, but for a longer period (28 days), urinary hydroxyproline excretion did not significantly change. Plasma osteocalcin, a sensitive and specific marker of osteoblastic activity, an indicator for bone formation, remained unchanged.

In a controlled metabolic balance study in premenopausal women (n=8) doubling of the basal phosphorus intake from 1166 mg/day up to 2310 mg/day, by giving an additional mixture of sodium and potassium phosphate supplement for at least 4 months, while maintaining the calcium and protein intake constant, no evidence for bone remodelling was found as measured by radiocalcium kinetics and histomorphometry (Heaney and Recker, 1987).

Also in the more recent studies using more specific, "state of the art" markers of bone resorption, such as the urinary N-telopeptide excretion, it was concluded that high dose phosphorus supplementation did not significantly affect bone turnover (Whybro *et al*, 1998; Grimm *et al*, 2001).

In a cross-sectional epidemiological study among 510 healthy Danish women, aged 45-58 years, a positive association was found between the dietary Ca:P ratio and bone mineral density, apparently related by the inverse relationship observed between the Ca:P ratio and serum 1,25-$(OH)_2$D levels (Brot *et al*, 1999). These associations were found within the normal physiologic range of the 1,25-$(OH)_2$D levels.

However, no clinical studies have linked high phosphorus intake, with or without adequate calcium intake, to lower bone mass, or higher rates of bone loss in humans.

3.2.2.1. Newborns, young infants and children

Hypocalcaemia has occasionally been observed in neonates fed infant formula based on cows' milk, and related to the higher phosphorus load of these formulae (Venkatamaran *et al*, 1985; Specker *et al*, 1991). Infant formula based on unmodified cows' milk protein used to have a relatively high phosphorus level as compared to human milk, but a Ca:P ratio of about 1.0 to 2.0 is requested in modern formulae (Dorea 1999; Fomon, 1993). Neonates and young infants have a lower renal excretion capacity and, as a consequence, higher serum Pi values than older infants and adults at comparable (relative) intakes. This favours skeletal mineralization, but too high levels might adversely effect bone accretion, and in severe cases lead to rickets, and hypocalcaemic tetany. Specker *et al* (1991) demonstrated a linear relationship between serum PTH and mean phosphorus intake (range 100-250 mg/day) in neonates from 1-6 days of age, but not at 7-14 days of age.

Consumption of soft drinks with added phosphoric acid has also been associated with hypocalcaemia in children (Mazarlegos-Ramos *et al*, 1995). In a group of 57 children, ages between 1.5 -10 years, with a low serum calcium level (<2.2 mmol/L), soft drink consumption was much higher (>1.5 L per day) as compared to an age-matched control group with normal serum calcium values. This might be related however to low calcium intakes, rather than soft drink consumption as such, but no data on calcium intake were provided.

3.2.2.2. Women

Comparative studies in 20-40 year old women with carbonated beverages (567 mL) containing phosphoric acid or citric acid as the acidulant, did not indicate an effect on urinary calcium excretion (Heaney and Rafferty, 2001).

3.2.3. Ectopic calcification

Ectopic calcification as a result of high dietary phosphorus intake, as has been observed in mice and rats with normal kidney functions before exposure, has not been reported in humans with an adequate renal function. This might occur however in patients with end-stage renal disease associated with a variety of syndromes and (malignant) conditions. However, in these conditions, the hyperphosphatemia is not a direct, but a secondary effect.

3.2.4. Interaction with mineral and trace element absorption

Bour *et al* (1984) reported that high intakes of polyphosphates could interfere with absorption of iron, copper and zinc. However, this was not confirmed in a study by Snedeker *et al* (1982) who found no significant effect on iron, copper and zinc balance in 9 adult males after feeding a high phosphorus diet (2383 mg daily), in combination with a moderate (780 mg) or high (2442 mg) calcium diet for 39 days.

There is also no evidence that phosphorus interferes with calcium absorption. Studies from Spencer *et al* (1965) and Heaney (2000) have shown that over a wide range of Ca:P ratios in the regular diet, i.e. between 0.18 and 1.88, this ratio does not determine calcium absorption efficiency.

4. DOSE-RESPONSE ASSESSMENT

4.1. Osmotic diarrhoea and other mild gastrointestinal effects

Mild gastrointestinal complaints were reported in some individuals in some, but not all supplementation studies, at supplemental phosphorus intakes ≥750 mg/day (see Table 2). The Panel did not consider this effect as a suitable critical endpoint for setting an upper level.

4.2. Effect on calcium regulating hormones, calcium balance and skeletal mass

An increase in phosphorus intake can induce an increase in serum PTH, as part of the normal homeostatic control to maintain serum calcium levels. This effect depends on the actual increase in serum Pi, and the subsequent (small) decrease in the serum ionized calcium level. PTH adjusts renal clearance of Pi and through this mechanism the phosphate balance is maintained. It is not clear at what dietary intake level of phosphorus PTH secretion is stimulated. Only in the first days of life a (linear) relationship exists between serum PTH and phosphorus intake in the normal nutritional range. After the renal excretion capacity has fully developed and remains intact, the excess absorbed phosphate is excreted and serum Pi levels are kept within the normal range, i.e. no hyperphosphatemia develops.

Acute oral phosphorus loading with dosages of 1.0-1.5 g phosphorus did not result in an increase in serum PTH in young adults (Calvo and Heath, 1988). Also in an earlier study in osteoporotic postmenopausal women a supplemental phosphorus dosage of 1000 mg (total intake up to 2740 mg/day) for 12 months did not affect fasting serum PTH (Goldsmith *et al*, 1976). Also in more recent chronic supplementation studies in young healthy men (Whybro, 1998), and women (Grimm, 2001), with total intakes up to 3000 mg phosphorus/day, no significant effect on changes in serum PTH could be demonstrated. However in a study with a similar group of postmenopausal women supplemental dosages >1500 mg phosphorus/day resulted in an increase in PTH levels (+35%) (Brixen, 1992). A normal phosphorus intake (1700 mg/day) in combination with a low calcium diet (400 mg/day) also resulted in a persistent increase in PTH, but is likely due to the low calcium content, rather than the relatively higher phosphorus content (Calvo *et al*, 1990).

The skeletal effects of carbonated beverage consumption, if any, as reported in a relatively small study in children (Mazarlegos-Ramos *et al*, 1995) might be due to milk displacement, i.e. a low calcium intake rather than an effect of phosphoric acid. Such a trend in the consumption of milk and soft drinks, i.e. a decrease in milk consumption with a concurrent increase in soft drink consumption, resulting in a lower calcium, but higher phosphorus intake, has been reported for the US (Anderson *et al*, 2001).

A comparative study in adult women showed no effect of phosphoric acid compared to citric acid as the acidulant in carbonated beverages on urinary calcium excretion (Heaney and Rafferty, 2001).

Ectopic calcification (e.g. nephrocalcinosis) and skeletal deformations and bone loss, as observed in animal studies with high phosphorus loads, have not been reported to occur in humans as long as the renal excretion capacity is not seriously compromised, such as in end-stage renal disease.

It should also be noted that standard diets for laboratory animals generally have a relatively high phosphorus and low calcium content (JECFA, 1982). It cannot be excluded therefore that the observed effects in some of the animal studies were associated with the relatively low calcium intakes, rather than the high phosphorus intake as such. Besides the sensitivity of the PTH-vitamin D axis to variations in calcium and phosphorus intake might be different between animals and humans.

The suppression of the renal 1,25-$(OH)_2$D synthesis by an increase in the serum Pi level (Portale, 1986) contributes to the already decreased intestinal phosphate absorption, and increased renal phosphate excretion. This effect may be mediated by phosphatonins, under conditions of high phosphorus intake, due to down-regulation of the NTP2 co-transporters (Blumsohn, 2004).

Decreased absorption and increased renal excretion therefore protect the human body against the development of chronic hyperphosphatemia under conditions of high phosphorus intake. The observed decrease in responsiveness of osteoclasts to PTH with increasing serum Pi levels likely explain why a (temporary) increase in serum PTH, if any, induced by a high phosphorus load does not result in an increased rate of bone resorption (remodelling), as compared to a low calcium intake which induces both an increased PTH and 1,25-$(OH)_2$D synthesis. The role of the phosphatonins in these processes remains to be established (Blumsohn, 2004).

The phosphorus-induced nephrocalcinosis as observed in rats (JECFA, 1982), and the upper boundary of the normal distribution curve for serum Pi in infants (FNB, 1997) have been used as critical endpoints to set a maximum tolerable daily intake level. Both approaches have their limitations however. The phosphorus-induced nephrocalcinosis in rats seems less relevant for humans. Also derivation of a hypothetical NOAEL based upon serum Pi distribution curves is doubtful as no actual adverse effects on bone mineralization have been observed. The Panel decides therefore not to use these endpoints to derive an upper level.

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The Panel considered that the adverse gastrointestinal effects that have been observed in some individuals exposed to high supplemental dosages (>750 mg/day) are not a suitable basis to establish an upper level (UL) for phosphorus from all sources.

An UL cannot be established based on the effect of a high phosphorus intake on the activity of calcium regulating hormones, which the Panel considers not to be adverse in themselves, and which have no demonstrable effects on bone mineral density and skeletal mass.

The Panel therefore concludes that the available data are not sufficient to establish an UL for phosphorus.

2. RISK CHARACTERISATION

The available data indicate that normal healthy individuals can tolerate phosphorus intakes up to at least 3000 mg phosphorus per day without adverse systemic effects. In some individuals, however, mild gastrointestinal symptoms, such as osmotic diarrhoea, nausea and vomiting, have been reported if exposed to supplemental intakes >750 mg phosphorus per day.

Estimates of current intakes of phosphorus in European countries indicate total mean dietary and supplemental intakes around 1000-1500 mg phosphorus per day, with high (97.5 percentile) intakes up to around 2600 mg phosphorus per day. There is no evidence of adverse effects associated with the current intakes of phosphorus.

Observational data suggest that high phosphorus intakes might aggravate the effects of a state of secondary hyperparathyroidism in individuals with inadequate calcium intakes, or an inadequate vitamin D status, e.g. postmenopausal women. The Panel considers that these data are not sufficient to establish the occurrence of such effects.

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OPINION OF THE SCIENTIFIC PANEL ON DIETETIC PRODUCTS, NUTRITION AND ALLERGIES ON A REQUEST FROM THE COMMISSION RELATED TO THE TOLERABLE UPPER INTAKE LEVEL OF TIN

(REQUEST N° EFSA-Q-2003-018)

(ADOPTED ON 6 JULY 2005)

SUMMARY

The European Food Safety Authority is asked to derive an upper level for the intake of tin from food that is unlikely to pose a risk of adverse health effects.

Tin has not been shown to be nutritionally essential for humans. Tin occurs naturally in foods as stannous and stannic salts, and stannous chloride ($SnCl_2$) is a permitted food additive (E512). Data on tin intake in EU countries are limited. In the UK mean intake in adults from food is estimated at 1.8 mg/day, ranging up to about 6 mg/day, and appears to be decreasing, while in France the mean daily intake was estimated to be 2.7 mg tin/day. The main dietary sources of tin are tinned fruit and vegetables.

The absorption of inorganic compounds of tin from the gastrointestinal tract in humans and animals is very low with as much as 98% being excreted directly in the faeces. Because of their limited absorption, inorganic tin compounds have low systemic toxicity in man and animals.

In man and animals, gastrointestinal effects are the main acute manifestation of toxicity associated with ingestion of tin. These are caused by the irritant action of soluble inorganic tin compounds on the mucosa of the gastrointestinal tract. In humans, acute effects resulting from consumption of tin-contaminated foods and drinks have resulted in gastrointestinal symptoms, including abdominal distension and pain, vomiting, diarrhoea, and headache. The balance of evidence suggests that the concentration of tin in contaminated foods is critical to the development of acute gastrointestinal effects, and that tin concentrations of 250 mg/kg in canned foods and 150 mg/kg in canned beverages are more likely to be associated with this.

In rats, growth depression, loss of appetite and reduced feed conversion efficiency are observed at doses of stannous chloride greater than 150 mg tin/kg diet. This appears to be due to reduced absorption and status of trace elements, particularly zinc, but also iron and copper, as a result of formation of insoluble complexes (probably with phosphates) in the gastrointestinal tract. There is evidence of reduced status of iron, zinc and copper when rats are fed diets containing 50 mg tin/kg diet or greater. It is likely that other effects which occur at this or higher levels e.g. reduced calcium content of bone at 50 mg tin/kg diet or pancreatic atrophy at a dose level of 2000 mg tin/kg diet, are not systemic effects of absorbed tin but rather manifestations of deficiency of one or more trace elements.

Short term studies in human adults indicate that high intakes of tin (about 30-50 mg tin/day or per meal) may reduce the absorption of zinc, but not other minerals such as iron, copper, manganese or magnesium. However, the possible long-term effects, if any, of such intake levels on status of zinc or other minerals have not been investigated.

The Panel considered that the available data from human or animal studies are insufficient to derive a tolerable upper intake level for tin. The current daily intake of tin in the EU (e.g. ranging up to about 6 mg/day in the UK) appears to be well below the lowest intakes reported to cause adverse effects on zinc absorption. Regulatory limits of 200 and 100 mg/kg for the concentration of tin in canned foods and beverages, respectively, have been established to protect against the occurrence of acute gastrointestinal effects of tin.

KEY WORDS

Inorganic tin, stannous chloride, stannous oxide, stannic chloride, toxicity.

BACKGROUND

In 2002, the European Parliament and the Council adopted Directive 2002/46/EC[1] related to food supplements containing vitamins and minerals.

In addition, and as announced in its White Paper on Food Safety, the Commission aims to put forward a proposal for harmonising legislation concerning the addition of vitamins and minerals to foods.

With a view to provide scientific support to the European Commission's legislative work in this field, the Scientific Committee on Food (SCF) issued, from October 2000 to April 2003, a series of opinions on tolerable upper intake levels of individual vitamins and minerals and safety factors in relation to their use in fortified foods and food supplements (available on the Internet at: http://europa.eu.int/comm/food/fs/sc/scf/out80_en.html).

The SCF opinions covered 22 out of the 29 nutrients, which were considered to be within their mandate for this task. The SCF did not have sufficient time to adopt opinions for the following vitamins and minerals: vitamin C, chloride, fluoride, iron, phosphorus, potassium and sodium. In addition, during the decision making process for the adoption of Directive 2000/46/EC on food supplements the Parliament requested that boron, nickel, silicon, vanadium and tin should be allowed to be used in food supplements. Therefore, the European Food Safety Authority is asked to provide scientific opinions on the remaining 12 vitamins and minerals in accordance with the present terms of reference.

TERMS OF REFERENCE

With respect to the outstanding 12 vitamins and minerals, the European Food Safety Authority is asked 1) to review the upper levels of daily intakes that are unlikely to pose a risk of adverse health effects; 2) to provide the basis for the establishment of safety factors, where necessary, which would ensure the safety of fortified foods and food supplements containing the aforementioned nutrients.
ASSESSMENT

1. INTRODUCTION

1.1. Chemistry

Tin is a metal of group 14 of the periodic table with an atomic weight of 118.71. It has 10 naturally occurring isotopes of atomic masses between 112 and 124 with abundances ranging from 0.34-32.97%. The oxides of tin are amphoteric, commonly forming stannous and stannic salts (oxidation state +2 and +4, respectively). Several reviews and evaluations have been prepared on inorganic tin (WHO, 1980; Thomas, 1984; Smith and Kumar Das, 1996; EGVM, 2002; ATSDR, 1992 and 2002; JECFA, 1982 and 2001).

1.2. Natural occurrence

Natural occurrence of tin in the metallic state is rare. The occurrence of tin in various organisms from different localities has been reviewed by Schroeder *et al* (1964) and they noted that the concentrations found are highly dependent on the existence of local mineral sources of tin. Although not commonly present in fresh waters it occurs at a concentration of about 3 µg/L in sea water. Tin was present in all vegetable samples obtained in Vermont, an area with soil containing considerable amounts of tin, from 0.07-9.07 µg/g wet weight. It is found in tissues of local farm and wild animals (Schroeder *et al*, 1964; WHO, 1980; EGVM, 2002; ATSDR, 1992 and 2002).

1.3. Occurrence in food, food supplements and medicines

The widespread distribution in soils of some areas results in the presence of tin within certain foodstuffs. Within the European Union stannous chloride is a permitted food additive (E512) for bottled and canned

1 - Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements. OJ L 183. 12.7.2002, p. 51.

white asparagus only (25 mg Sn/kg). The highest concentrations of tin in foods are found in tinned fruit and vegetables. Tin is present in some multi-vitamin and mineral food supplements (levels up to 10 μg tin/tablet) (EGVM, 2002).

At the 55th meeting of the Joint FAO/WHO Expert Committee on Food Additives in June 2000 the Provisional Tolerable Weekly Intake (PTWI) of 14 mg Sn/kg body weight was reconsidered and maintained (JECFA, 2001). In an opinion of the SCF on acute risks posed by inorganic tin in canned food, the Committee concurred with the JECFA conclusion that levels of 150 mg/kg in canned beverages or 250 mg/kg in other canned foods or higher may cause gastric irritation in some individuals (SCF, 2001).

Maximum levels for inorganic tin in canned foods (200 mg/kg) and canned beverages (100 mg/kg) have been established in EU legislation (EC, 2004).

2. NUTRITIONAL BACKGROUND

2.1. Deficiency

Tin has not been shown to be essential for humans or animals, and there are no data on deficiency effects resulting from an inadequate intake of inorganic tin (EGVM, 2002).

2.2. Absorption, distribution, metabolism and elimination

2.2.1. Absorption

The absorption of inorganic compounds of tin from the gastrointestinal tract in humans and animals is reported to be low with as much as 98% being excreted directly in the faeces. The nature of the inorganic tin compound and its oxidation state appears to determine the extent of absorption (Calloway and McMullen, 1966; Hamilton *et al*, 1972b; Tipton *et al*, 1966 and 1969; Fritsch *et al*, 1977a; WHO, 1980; ATSDR, 1992 and 2002).

2.2.1.1. Animals

It has been reported that gastrointestinal absorption of tin by the rat is extremely low. In one study, groups of 8 male Wistar rats (approximately 250 g) were fasted for 17 hours after which a dose of radiolabelled $^{113}SnCl_2$ (50 mg/kg body weight; 0.5 μCi/mg tin) was administered by gavage in either: (1) water; or with (2) aqueous sucrose at 5 g/kg body weight; (3) aqueous ascorbic acid at 0.5 g/kg body weight; (4) aqueous potassium nitrate 0.1 g/kg body weight; (5) an aqueous solution of all three compounds at the same dose; or in (6) 20% alcohol solution, equivalent to 2 g ethanol/kg body weight; or (7) a solution of albumin at 2.5 g/kg body weight; or (8) 1:1 (v/v) sunflower oil-1% Tween 20 emulsion at 10 mL/kg body weight. Rats were placed in metabolic cages, fasted for another 6 hours and then received a basal diet *ad libitum*. Urine and faeces were collected from 0-24 and 24-48 hours. Animals were then sacrificed and excreta and selected organs and tissues analysed for radioactivity. Group mean values of the proportion of the administered dose excreted in the faeces within 48 hours or remaining in the gastrointestinal tract ranged from 98.7-99.8%. The mean percentage of the ^{113}Sn dose detected in the urine was less than 1.1% and in the organs and tissues examined was less than 0.005% (Fritsch *et al*, 1977a; WHO, 1980; ATSDR, 1992 and 2002).

The effect of the anion and oxidation state on the gastrointestinal absorption of inorganic tin salts, labelled with ^{113}Sn, was studied in the rat. Following a 24-hour fast, groups of 10 female rats (Charles River, 200-225 g) were given a single 20 mg Sn/kg body weight oral dose of Sn^{2+} citrate, fluoride or pyrophosphate or Sn^{4+} citrate or fluoride. Changing the anion from citrate to fluoride did not alter the absorption of either oxidation state and approximately 2.8% and 0.6% of the Sn^{2+} and Sn^{4+}, respectively, were absorbed. With pyrophosphate as the anion, absorption of Sn^{2+} was significantly lower than with the citrate or fluoride, an observation which the author ascribed to the greater tendency of pyrophosphate to form insoluble complexes with tin as compared to the citrate and fluoride anions (Hiles, 1974). In a 28-day study in which groups of 6 weanling female rats were fed with the Sn^{2+} and Sn^{4+}fluoride salts (20 mg Sn/kg body weight, on 6 days/week) the steady state urinary excretion was *circa* 0.35% and 0.12% of the total dose of tin from the Sn^{2+} and Sn^{4+} salts, respectively, confirming the greater absorption of the Sn^{2+} ion (Hiles, 1974).

In a comparative study of the absorption of tin, tracer dose of $^{113}SnCl_2$ (2.6-4.4 mg Sn) were administered intravenously, intraperitoneally and by gavage to female RF mice, male Sprague-Dawley rats, male African white-tailed rats, male rhesus monkeys and male beagle dogs. In all species more than 95% of the oral

gavage dose was excreted via the faeces within 3 days, whereas a greater percentage (15.9-62.8%) of the parenteral doses was excreted via the urine during the same time (Furchner and Drake, 1976).

Orange juice containing 540 mg Sn/kg derived from corrosion of the can or a solution of tin citrate (1200 mg Sn) was administered to Wistar rats (gender not given), and faeces and urine were collected over 48 hours or 18 hours respectively. No tin was detected in the urine collections whereas the faecal excretion of tin was 99% or 94-98% respectively (Benoy *et al*, 1971).

2.2.1.2. Humans

The absorption of inorganic compounds of tin from the gastrointestinal tract in humans is very low with as much as 98% being excreted directly in the faeces at intakes around 10 mg/day or higher. Schryver (1909) reported the urinary excretion of tin in normal health adults weighing 65 kg, who ingested daily doses of sodium tin tartrate for 3 weeks. The doses were taken four times a day with meals and the total daily dose in the first week, of approximately 64.5 mg tin, was increased to 129 mg in the second week and to 193.5 mg in the third week (equivalent to approximately 1, 2 and 3 mg Sn/kg body weight per day in successive weeks). After the first week the tin excreted in 5-day periods in each week was related to the amount ingested, with 7.9 and 8.6% of the total excreted in the urine during the second and third weeks, respectively. There was no control period and no measures of dietary tin content were made (Schryver, 1909). In a study by Calloway and McMullen (1966) faecal excretion of tin was high and approximated dietary intakes when the diet provided 9-190 mg tin per day. In adults given 50 mg tin per day the apparent absorption was around 3%, while it was about 50% when the intake was 0.1 mg/day (Johnson and Greger, 1985).

2.2.2. Distribution

2.2.2.1. Animals

When expressed as a percentage of a dose administered orally to rats, tissue distributions for Sn^{2+} and Sn^{4+}, respectively were skeleton, 1.02% and 0.24%, liver, 0.08% and 0.02%; and kidneys 0.09% and 0.02% (Hiles, 1974). When radioactive stannous chloride was administered by stomach tube to anaesthetised rats the bulk of the dose was excreted in faeces, and there was highly variable distribution of the absorbed fraction in the internal organs as measured for periods of up to 21 days (Kutzner and Brood, 1971).

Tin concentrations were measured in the liver, kidneys and femur of groups of 6 male weanling Wistar rats administered 0, 0.3, 1.0 and 3.0 mg Sn^{2+}/kg body weight orally every 12 hours for a period of 90 days. There was a clear dose-related increase in femur concentration with statistical significance achieved at the 1.0 mg Sn^{2+}/kg body weight dose. In the highest dose group the femur concentrations were 10-fold higher than the control values of 2.05 ± 0.41 μg/g wet tissue and these were associated with significant reductions of the diaphysis and epiphysis concentrations of calcium. The concentrations of tin in the livers of control rats were 0.24 ± 0.01 μg/g wet tissue and the levels were significantly increased by 58% at the highest dose. There were no significant increases in the kidney concentrations of 0.22 ± 0.41 μg/g wet tissue (Yamaguchi *et al*, 1980).

Male Wistar rats were given $SnCl_2.2H_2O$ in their drinking water for 1-18 weeks at concentrations of 100 mg/L (0.44mM), 250 mg/L (1.11 mM) or 500 mg/L (2.22 mM). Tin accumulated in the brain at the highest concentration (2.22 mM) throughout the experiment, but elevated tin concentrations in brain were found only after 15 and 18 weeks at 1.11 mM and tin did not increase in the brains of rats given 0.44 mM. Blood tin increased after one week at the highest dose (2.22 mM) without further accumulation, whereas blood tin levels did not differ from controls at the 2 lower doses. Tin exposure caused a dose-dependent increase in the cerebral and muscle acetylcholinesterase activity at the two highest doses (Savolainen and Valkonen, 1986).

2.2.2.2. Humans

The mean concentration (± S.E.) of tin in 102 samples of human blood obtained through the UK National Blood Transfusion Service was 0.009 ± 0.002 μg/g wet weight. The concentrations ± S.E. (n) in various organs obtained at autopsy were: whole brain, 0.06 ± 0.01 (10); whole kidney (0.2 ± 0.04 (8); liver, 0.4 ± 0.08 (11); lung, 0.8 ± 0.2 (11), lymph node, 1.5 ± 0.6 (6); muscle, 0.07 ± 0.01 (6); testis, 0.3 ± 0.1 (5); ovary, 0.32 ± 0.19 (6), all μg/g wet weight; bone (hard water area), 4.1 ± 0.6 (22); bone (soft water area), 3.7 ± 0.6 (22), μg/g ash (Hamilton *et al*, 1972a).

2.2.3. Metabolism

The methylation of inorganic tin compounds by a mechanism involving the oxidation of a stannous compound to the Sn (III) radical and the reaction of this with the cobalt-carbon bond of vitamin B_{12} to give a methylated tin derivative have been described. However, it is probable that this can only occur in anaerobic conditions (Ridley *et al*, 1977 a and b; Wood *et al*, 1978; ATSDR, 1992 and 2002).

2.2.4. Elimination

2.2.4.1. Animals

The disappearance of radioactivity following intraperitoneal injection of a tracer of $^{113}SnCl_2$ into 5 Swiss mice was followed by whole body counting. The biological half life of tin was estimated as 29 days (Brown *et al*, 1977). Intravenous injection of single bolus doses of 2 mg/kg body weight of either Sn^{2+} or Sn^{4+} (citrate and fluoride) resulted in the excretion of 30% of the dose in the urine, with 11% and 0% of Sn^{2+} and Sn^{4+} eliminated in the bile (Hiles, 1974).

2.2.4.2. Humans

A baby fed on evaporated milk from an unlacquered tin can for the first 5 weeks of life was estimated to have ingested 11.23 mg Sn/24 hours. Excretion in the faeces was estimated as 10.64 mg Sn/24 hours and in the urine as 0.23 mg Sn/24 hours. The faecal excretion of tin decreased by 98% within 36 hours after changing to milk from a lacquered can (Hamilton *et al*, 1972b). A 30-day balance study on a husband and wife aged 35 and 34 respectively, involved the collection of duplicate samples of their food and drink and total collection of faeces and urine. Mean daily faecal and urinary excretion of tin (measured by emission spectroscopy on dry-ashed samples) were, 2.13 and 0.11 mg, respectively for the wife, and 1.55 and 0.08 mg, respectively for the husband. The wife was in negative balance and the husband in positive balance (Tipton *et al*, 1966). The same group studied two males, 23 and 25 years old, using similar procedures for a period of 347 days. Both subjects were in positive balance and their mean daily faecal and urinary excretions of tin (mean ± S.E.) were 3.6 ± 0.7 and 0.085 ± 0.011 mg, respectively for the first subject, and 3.6 ± 0.5 and 0.058 ± 0.006 mg for the second subject. It was calculated that less than 10% of the amount of tin ingested was excreted within 24 days (Tipton *et al*, 1969). A study of the tin content of army rations which had been stored at either 1 or 37° C for a period of 20 months indicated that these would provide mean tin intakes of 26.3 and 162.8 mg per day respectively as compared with a freshly prepared control diet (9.5 mg Sn per day). During ingestion of the control diet the faecal excretion of tin by 9 young adult male volunteers was slightly greater than the estimated intake and, during consumption of the high tin diet, faecal excretion was slightly lower than the intake; only trace amounts of tin were detected in the urine and these were unaffected by the diet (Calloway and McMullen, 1966). One study has reported results which were somewhat different from the other toxicokinetic studies. In adult males fed daily diets containing either 0.1 or 50 mg of tin in a 40-day study with a 20-day cross over period apparent absorption was 50 and 3% of the ingested tin, respectively (Johnson and Greger, 1982).

2.3. Interactions

2.3.1. Animals

Groups of 7 young male Sprague-Dawley rats were assigned to study groups in which they were: a) fed for 7 days with either a diet supplemented with 1954 mg Sn/kg or were pair-fed with a control diet; b) fed for 27 days with various concentrations of tin and zinc in a block design; and c) fed as in b with different concentrations of tin and zinc for 6 or 7 days. The absorption of zinc by rats on diets containing 200 or 500 mg/kg tin was decreased. At the higher dietary concentration, the retention of zinc in tibia, kidney, liver and plasma was significantly decreased and the plasma, liver and kidney levels of copper were reduced (Johnson and Greger, 1984 and 1985).

Groups of 10 weanling male long-Evans rats were administered diets containing 0, 100, 330 or 1100 mg Sn/kg diet, and a control group were pair-fed with 0 mg Sn/kg to match the 1100 mg/kg diet group, for a period of 28 days. The copper, zinc, iron contents of various tissues were then assessed. There were significant dose-related reductions of body weight gain in all treatment groups. The highest dietary concentration of tin significantly reduced the copper and zinc concentrations of most tissues studied. There were dose-related reductions in the liver concentrations of copper and in the kidney concentrations of zinc. Also, when the rats were fed a diet deficient in copper, the 100 mg Sn/kg diet caused a significant reduction of blood haemoglobin and serum ceruloplasmin concentrations (Rader *et al*, 1990; Rader, 1991). Similar results were reported in a study on the effects

of 100 mg Sn/kg diet in rats fed additional dietary glucose or fructose while consuming Cu-deficient or Cu-adequate diets (Reicks and Rader, 1990).

Groups of 12 weanling Wistar rats were fed semi-synthetic diets containing 1 (control) or 100 mg Sn/kg diet (the tin was added as $SnCl_2$) for 28 days. The test diet had no effects on body weight gain or feed intake but resulted in significantly reduced concentrations of copper in plasma, liver and kidneys. The biliary excretion rate of copper was reduced to approximately 40% of that of the control group. The authors concluded that tin affected copper status by inhibiting the copper absorption (Yu and Beynen, 1995).

A 28-day study with male Wistar rats fed diets containing 0, 1, 10, 50, 100 and 200 Sn mg/kg diet, incorporated as stannous chloride, provided evidence for dose-related changes to the iron, copper and zinc status of the animals, with changes claimed to occur at dietary concentrations lower than 50 mg/kg (Beynen *et al*, 1992; Pekelharing *et al*, 1994; EGVM, 2002; ATSDR, 1992 and 2002).

Tissues of rats and mice fed tin in drinking water for life were analyzed for the essential metals, chromium, copper, manganese and zinc. Contrary to what has been shown for competitive effects of tin on copper in some other studies, the concentrations of copper were significantly higher in the livers of rats fed tin than in controls (Schroeder and Nason, 1976).

2.3.2. Humans

The consumption of canned foods providing 163 (116-203) mg of tin per day by nine adult males was associated with an apparent greater retention of iron in the body; however, the iron content of the diet was higher than that of a low-tin-content diet (Calloway and McMullen, 1966).

During a 40-day study, with 20-day cross over periods, eight adult males (69-82 kg body weight) were fed either a mixed diet providing 0.1 mg tin per day (controls) or a similar test diet providing 50 mg tin per day. When the subjects were fed the test diet the subject's excretion of zinc was increased in the faeces and decreased in the urine, and the apparent absorption of zinc was reduced by 16%: there was no significant effect on the excretion of copper, iron, manganese and magnesium (Johnson *et al*, 1982). In a similar, and possibly the same study, there was no effect of incorporation of tin in the diet on calcium excretion or serum levels of calcium (Johnson and Greger, 1982). Also, on the high-tin diet there was a statistically significant increase in the faecal excretion of selenium, while the urinary excretion and overall apparent retention of selenium were decreased but non-significantly (Greger *et al*, 1982).

A single test meal containing 36 mg Sn as stannous chloride dihydrate administered to 10 healthy volunteers aged 18-46 years reduced the absorption of radiolabelled $^{65}ZnCl_2$ (molar ratio of Sn:Zn = 5) from the test meal by *circa* 29%, as measured by whole body counting 2-4 hours and 7-10 days after the meal (Valberg and Chamberlain, 1984). However, Solomons *et al* (1983) found no effect on zinc absorption, assessed by plasma zinc concentrations during 4 hours post-dose, in humans given increasing amounts of stannous chloride (from 25, 50 and 100 mg Sn, respectively) together with 12.5 mg zinc (as zinc sulphate) in 100 mL soft drink in single-meal studies.

2.4. Requirement and recommended daily intake

Tin has not been shown to be essential for humans. Although some authors (Gelfert and Stauffebiel, 1998) suggested that tin could be essential, there is no experimental evidence that tin is an essential element for animals or man.

2.5. Dietary intake

Information on dietary intakes of tin is limited, as it is not in the nutrient databanks for dietary surveys.

A total diet study in The Netherlands was used to investigate the content of minerals in market basket samples representing the diet of Dutch 18 year old males and purchased at 3-monthly interval over a period of 2½ years (1984-1986). The mean daily intake of tin was estimated to be 0.65 mg as compared with 1.7 mg in a study carried out eight years earlier. Canned fruits contributed 82% of the dietary intake of tin (Van Dokkum *et al*, 1989), which probably is due to migration from the can into the food.

A study in the UK of the concentration of tin in canned foods provided evidence that canned fruits (tomato and tomato products, pineapple, orange, grapefruit and pear) are the most likely to contain

elevated concentrations of tin, with the tomato, tomato products and pineapples categories each having some samples containing more than 250 mg Sn/kg. The mean upper and lower bound estimated total dietary intakes of tin for the years 1976 to 1982 ranged from 4.35-2.41 (upper bound) and 4.42-2.30 (lower bound) mg Sn per day. There was a decreasing tendency until 1982, and the authors quote references suggesting that intake via inhalation and drinking water are likely to be 2 to 3 orders of magnitude less than dietary intake (Sherlock and Smart, 1984). In a duplicate diet study of 29 adult females in north-eastern England in 1982, it was noted that the concentration of tin in the duplicate diets was higher than in the earlier total diet samples (Evans and Sherlock, 1987).

Analyses of samples from 1997 Total Diet Studies (TDS) showed that the population average intake of tin was 1.8 mg/day, and the upper level (97.5 percentile) tin intake was estimated at 6.3 mg/day using the TDS concentrations combined with consumption data from the 1986/87 Dietary and Nutritional Survey of British Adults. The population average intake of tin had decreased since the previous TDS estimate of 2.4 mg/day in 1994. Table 1 shows the concentrations of tin in each of the 1997 TDS food groups and the intake from each group. This shows that the highest concentrations of tin were in the canned vegetables group followed by the fruit products group. Canned food products are the main contributors to the intake of tin in the UK (EGVM, 2002).

In a study in France, the tin contents in fresh food or in food stored in lacquered or unlacquered cans were determined in order to estimate the average daily tin intake in a French citizen. Tin levels were 76.6 ± 36.5 mg/kg in foods preserved in unlacquered cans, 3.2 ± 2.3 mg/kg in foods stored in lacquered cans, and 0.03 ± 0.03 mg/kg in fresh foods. Tin intake is essentially dependent on food stored in tin cans (98%), which represents 5.6% of the total daily consumption of foods by a French citizen. The estimated tin intake was 2.7 mg/day which is equivalent to 0.04 mg/kg body weight (Biégo *et al*, 1999).

3. HAZARD IDENTIFICATION

3.1. Animal toxicity data

3.1.1. Acute toxicity

Single oral administration of inorganic tin caused a number of acute symptoms, such as severe salivation and emesis, with vomiting in cats and dogs (Benoy *et al*, 1971). LD_{50} values for inorganic tin compounds are shown in Table 2.

3.1.2. Short-term toxicity

Feeding rats with $SnCl_2$ in tap water at intakes equivalent to 1.4 or 14 mg $SnCl_2$/kg body weight/day or in an aqueous suspension of yeast at intakes equivalent to 14 mg $SnCl_2$/kg body weight/day for 21 days resulted in a reduction of about 30% in the activity of serum lactate dehydrogenase for all treatments but no effect was observed on serum glutathione peroxidase, carbonic anhydrase, alkaline phophatases or leucine aminopeptidase (Pfaff *et al*, 1980; EGVM, 2002; ATSDR, 1992 and 2002).

Table 1. Concentrations of tin in 1997 Total Diet Study samples and estimated average intake (EGVM, 2002).

Food Group (TDS)	Mean Sn concentrations (mg/kg fresh weight)	Intake of Sn (mg/day)
Bread	0.025	0.003
Misc. cereals	0.771	0.078
Carcass meat	0.007	0.00015
Offal	0.014	0.00001
Meat products	0.18	0.008
Poultry	0.006	0.00011
Fish	0.032	0.00045
Oils & fats	0.011	0.00030
Eggs	0.003	0.00004
Sugars and preserves	0.046	0.003
Green vegetables	0.003	0.0001
Potatoes	0.004	0.00049
Other vegetables	0.05	0.004
Canned vegetables	41[1]	1.353
Fresh fruit	0.019	0.001
Fruit products	7.21[1]	0.317
Beverages	0.002	0.002
Milk	0.003	0.001
Dairy produce	0.297	0.018
Nuts	0.029	0.00006
Total Intake (mg/day)		1.8 mg/day

[1] High levels resulting from migration from cans

Table 2. LD50 values for inorganic tin compounds

Compound	Species, gender	Dose route	LD50 (mg/kg body weight)	Duration	Reference
$SnCl_2$	Rat, M	p.o	700	over 21 days	Pfaff *et al*, 1980
$SnCl_2$	Rat, M	p.o	>1500	16 days	NTP, 1982
$SnCl_2$	Rat, F	p.o	>1500	16 days	
$SnCl_2$	Mouse, M, F	p.o	< 600	16 days	
$SnCl_4$/Na citrate complex	Mouse, M	p.o	2700	Unknown	Omori *et al*, 1973
SnF_2	Rat, M	p.o	188.2	Fasted, 24h	Lim *et al*, 1978
SnF_2	Mouse, M	p.o	128.4	Fasted, 24h	
$NaSnF_5$	Mouse, M	p.o.	592.9	24h	Conine *et al*, 1975
$NaSnF_5$	Rat, F	p.o	218.7	Fasted, 24h	
$NaSnF_5$	Rat, M	p.o	573.1	24h	
$NaSnF_5$	Rat, M	p.o	223.1	Fasted, 24h	

p.o.: oral gavage

Consumption of diets containing 1900-30,000 mg $SnCl_2$/kg diet for a period of 14 days by rats resulted in a dose-related decrease in weight gain in rats and, at the highest dietary concentrations, roughened coats and distended abdomens. In mice similarly treated, there was reduced weight gain on diets containing 15,000 and 30,000 mg $SnCl_2$/kg (NTP, 1982).

Consumption of diets by rats and mice containing (in mg/kg) $SnCl_2$ at levels of 0, 500, 1000, 1900, 3800, 7500 (in both species) and 15,000 and 30,000 (in mice only) mg $SnCl_2$/kg diet for 13 weeks resulted in reduced weight gain in rats at the 7500 mg/kg dose level and distension of the caecum and reddening of the mucosal surface of the stomach at the 3800 and 7500 mg/kg dose levels. No

histological changes were observed in any tissue. In the mice, there was a 30% decrease in body weight gain in the animals receiving 30,000 mg $SnCl_2$/kg diet and gross distension of the caecum was observed in all groups receiving dietary concentrations of 3800 mg/kg diet or greater. No histological changes were observed in any tissue (NTP, 1982).

Increasing dietary concentrations of $SnCl_2.H_2O$ from 0.1% in the first week to 0.8% in weeks 8 to 13 in rats reduced body weight gain, haemoglobin concentration and haematocrit and induced pancreatic atrophy, histological changes to the gastrointestinal tract, liver, kidney and thyroid. The authors considered the pancreatic changes to be the most specific manifestation of the toxicity of tin (Dreef-van deer Mullen *et al*, 1974).

Administration of diets containing 0, 4000 or 8000 mg Sn/kg diet as $SnCl_2$ to rats for 6 months caused pancreatic atrophy and histological changes in the kidney, adrenal medulla and adrenal cortex, signs of irritation in the gastrointestinal tract in both treatment groups (Fritsch *et al*, 1978).

Feeding a diet containing 5000 mg Sn/kg food as $SnCl_2$, labelled with radioactive $^{113}SnCl_2$, equivalent to about 700 mg Sn/kg body weight, to young male rats for one month resulted in reduction of body weight and food consumption, anaemia characterised by a significant drop in haemoglobin and haematocrit values, congestion of the kidneys and the cortex of the adrenals, and congestion and desquamation of the mucosa in the upper gastrointestinal tract from the stomach to ileum. About 99% of the administered labelled tin was excreted in the faeces with less than 1% in the urine, while radioactivity in the gastrointestinal tract, organs and carcass was negligible (Fritsch *et al*, 1977b).

Feeding weanling rats diets containing 0, 300, 1000, 3000 and 10,000 mg/kg diet as SnO_2, $SnCl_2$, $Sn_3(orthophosphate)_2$, $SnSO_4$, SnS_2, Sn-oleate, Sn-oxalate or Sn-tartrate for 4 weeks resulted in no deleterious effects of SnO_2, SnS_2, and Sn-oleate (all insoluble tin compounds) but there was severe growth retardation, decreased food efficiency, slight anaemia and slight histological changes in liver with 3000 mg Sn/kg diet with $SnCl_2$, $Sn_3(orthophosphate)_2$, $SnSO_4$, Sn-oxalate or Sn-tartrate (all water soluble tin compounds). Dietary supplements of iron partly protected against tin-induced anaemia but did not protect against the other adverse effects. The authors suggested that the observed adverse effects of these tin compounds might be explained by the inhibition of iron absorption (de Groot *et al*, 1973).

In a 90-day study weanling rats were fed diets containing either SnO or $SnCl_2$ at dietary concentrations of 0, 300, 1000, 3000, 10,000 mg/kg. There were no toxic effects of SnO at any dose. Animals receiving $SnCl_2$ at 10,000 mg /kg diet showed loss of appetite, retarded growth and abdominal distension within 7 days; autopsy after 9 weeks showed distension of the intestines, severe pancreatic atrophy, testicular degeneration, and histological damage to liver, and brain, Animals fed $SnCl_2$ at 3,000 mg/kg diet showed some abdominal distension and loss of appetite and retarded growth during the first 2 weeks. After the second week, appetite returned to normal as did growth. Significantly lower haemoglobin levels were determined between the fourth and ninth week but this returned to control values for female, but not male, animals by the end of the study. Minor histological changes were observed in liver. There were no treatment-effects in rats fed $SnCl_2$ at 300 or 1000 mg/kg diet (equivalent to 450-650 mg Sn/kg diet or 22-33 mg Sn/kg body weight) (De Groot *et al*, 1973).

An additional 90-day study investigated the influence of iron concentration in the diet on the toxicity on inorganic tin. Rats were fed diets containing either 35 or 250 mg iron/kg diet which also contained 0, 50, 150, 500, or 2000 mg Sn/kg diet as $SnCl_2$. Growth depression, reduced appetite and reduced feed conversion efficiency were observed at the 500 and 2000 mg Sn/kg diets. Distinct signs of anaemia occurred in the 2000 mg Sn/kg diet group, but only a transitory decrease in haemoglobin was seen in rats receiving the 500 mg Sn/kg diet. Pancreatic atrophy and histological changes in the liver, kidneys, spleen, testicles and heart were seen in some animals in the highest tin group. In all instances where effects of dietary tin were determined, the degree of severity was usually more pronounced in animals receiving the lower iron diets (De Groot *et al*, 1973b as cited by JECFA, 1982).

De Groot *et al* (1973) considered that the adverse effects seen in these studies were due to inhibition of iron absorption. However, this only partly explains effects on haemoglobin and it does not at all explain the other effects. For example, the paper describes distinctly improved haemoglobin levels (although they remained low) at 10,000 mg/kg stannous chloride by further enrichment of the test diet with iron but the reduced growth rate was not improved. There may be alternative explanations which were not considered by the authors, e.g. interference with intestinal absorption of other trace elements (as described in Section 2.3) which were only reported some years after this study. Loss of appetite is the earliest sign of zinc depletion in rats. The authors did not consider interaction of Sn with any trace element except iron and made no tissue measurements for zinc or copper.

3.1.3. Long-term toxicity and carcinogenicity studies

Available evidence indicates that orally ingested tin salts are not carcinogenic.

In lifetime studies in rats and mice in which $SnCl_2$ was given in drinking water at dose levels of 0.35 mg Sn/kg body weight per day (mice) and 0.34-0.38 mg Sn/kg body weight per day (rats) there was no evidence of any effect of tin on the survival of the animals, or on the incidence or classification of tumours (Kanisawa and Schroeder, 1967 and 1969; Schroeder and Balassa, 1967; Schroeder *et al*, 1968).

Treatment of mice for one year from birth (with the mothers being given the appropriate diet or drinking water solution) through weaning and into adulthood with stannous oleate (5000 mg/kg diet) or sodium chlorostannate (5000 or 1000 mg Sn/L in the drinking water) had no effect on the incidences of hepatomas, malignant lymphoma and lung adenoma (Walters and Roe, 1965). Feeding rats diets containing either sodium chlorostannate (5000 mg Sn/diet) or stannous 2-ethylhexoate (4500 mg Sn/kg diet) until 8 weeks of age, and then 2250 mg Sn/kg diet until 80 weeks did not result in any increase in tumours in either group which could be attributed to the treatment (Roe *et al*, 1965).

Based on long-term feeding studies (105 weeks) with $SnCl_2$ in the diet at concentrations of 0, 1000 and 2000 mg $SnCl_2$/kg diet to rats and mice commencing at an age of 6 week, it was concluded that $SnCl_2$ was not carcinogenic in either species (NTP, 1982; ATSDR, 1992 and 2002; EGVM, 2002).

In a study reviewed by JECFA but not otherwise available (Sinkeldam *et al* 1979b) in rats at a high dose (800 mg Sn/kg diet) there were no compound-related effects on tumour site or incidence were observed (JECFA, 1982).

3.1.4. Reproductive and developmental effects

Available evidence indicates that orally ingested tin salts are not teratogenic.

Pregnant rats were administered $SnCl_2$ by gavage on gestation days 7-12 inclusive at doses of 0, 20, 100, 500 mg/kg body weight; teratogenic effects in the form of protruding tongue of foetus were reported but at unspecified doses (Wu *et al*, 1990). Stannous and stannic chloride had estimated LD_{50} of 10 and 20 mg/egg respectively when injected into yolk of 4-day old White Leghorn chicken embryos. No abnormalities of embryonic development were detected (Ridgway and Karnofsky, 1952).

A multi-generation reproduction study in rats with an incorporated developmental toxicity study with dose levels of 0, 200, 400 and 800 mg stannous chloride in the diet revealed transient adverse effects only at specific stages. These were a decrease in body weight gain during lactation, decreased haemoglobin in pups prior to weaning, and microscopic changes in the liver and spleen of pups of the F3b generation at weaning. The iron content in the diet for these pregnant rats was, respectively, 70 and 140 mg/kg feed, greater than the minimal adequate level of iron for adult non-pregnant rats (35 mg/kg feed). At the higher iron content in the feed the effects were less in the suckling pups. This led the investigators to the conclusion that the 70 mg iron/kg feed is a sub-optimal content for pregnant dams. No adverse effects were observed in the dams. Visceral and skeletal examination did not reveal any tin-related teratogenic effects (Sinkeldam *et al*, 1979a). As the effects in the pups seen in this study were transient and disappeared after the animals were weaned, the NOAEL is 800 mg stannous chloride/kg feed which is equivalent to 40 mg/kg body weight.

3.1.5. Genotoxicity

A non-standard *in vitro* mutagenicity test was carried out with the aim of studying the role of DNA repair genes in the repair of $SnCl_2$-induced damage in *Escherichia coli* (Cabral *et al*, 1998). The results showed that the product of the *x tha gene*, exonuclease III, was required for the repair of DNA lesions induced by $SnCl_2$, most of which were gene mutations of base substitution type. Cytogenetic studies gave positive responses with $SnCl_2$ for chromosomal aberrations and sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells with or without metabolic activation (Gulati *et al*, 1989; ATSDR, 1992 and 2002). $SnCl_2$ was also able to induce chromosomal aberrations in cultured human peripheral lymphocytes (Ganguly *et al*, 1992). $SnCl_2$ produced extensive DNA damage, detected as single strand breaks by alkaline sucrose gradient analysis in Chinese hamster cells (McLean *et al*, 1983). Tin (II) produced about 200 times more DNA damage, on equimolar basis, than did Cr (IV). $SnCl_4$ did not produce such damage. DNA damage was also induced by $SnCl_2$ in plasmid DNA (De Mattos *et al*, 2000) as well as in the K562 cell line, resistant to reactive oxygen species (ROS) (Dantas *et al*, 2002). $SnCl_2$ did not induce micronuclei in the bone marrow cells when given by intraperitoneal injections to mice at 0, 26.3, 52.5, 105 or 210 mg/kg body weight/day for 3 days (Shelby *et al*, 1993). Similarly, SnF_2

was unable to induce micronuclei in bone marrow cells of mice at the intraperitoneal doses of 0, 9.8, 19.6 or 39.5 mg/kg body weight given 24 hours apart (Gocke *et al*, 1981).

Based on the presently available data, $SnCl_2$ appears to be an *in vitro* genotoxic agent, able to induce gene mutations in bacterial cells, chromosome aberrations, sister chromatid exchanges and single strand breaks (SSBs) in mammalian cells. $SnCl_2$, as well as SnF_2, were unable to induce micronuclei in bone marrow cells of mice treated *in vivo*. Overall, there is a limited evidence of genotoxicity for soluble tin salts, likely due to generation of reactive oxygen species.

3.1.6. Studies on calcium metabolism

In a study in which groups of male weanling Wistar rats were given drinking water containing 0, 50, 150, 300, or 600 mg $SnCl_2$ per litre for a period of 28 days together with a diet which contained 52.4 mg Sn/kg diet, the compressive strength of the distal epiphysis was significantly reduced in the groups receiving 300 and 600 mg $SnCl_2$/litre. The NOAEL for this study calculated from the intake via drinking water and the amount which was present in the diet corresponds to 17.5 mg/kg body weight $SnCl_2$ (Ogoshi *et al*, 1981). Daily oral dosage of male Wistar rats with $SnCl_2$ at a dose level of 1.0 mg Sn^{2+}/kg body weight twice daily for 28 or 90 days reduced the calcium content of the femoral epiphysis, but without significantly altering serum calcium, intestinal calcium uptake or calcium excretion (Yamaguchi and Okada, 1980; Yamaguchi *et al*, 1982). In a 90-day study, the effects on weanling Wistar rats of $SnCl_2$ in the diet at 0, 10, 50, 100 and 250 mg Sn/kg diet were investigated. At the 50 mg Sn/kg level (equivalent to 2.5 mg Sn/kg body weight) and above there were significant reductions of the calcium content of serum and the femoral epiphysis (Yamaguchi *et al*, 1981). The authors suggested that these effects could be due to a systemic effect of absorbed tin; however, they did not consider the possible involvement of trace element depletion in these effects.

3.1.7. In vitro toxicity

Stannous and stannic oxides were not cytotoxic to fibroblasts cultivated from human gingival tissue (Hanawa *et al*, 1992).

3.2. Human toxicity data

3.2.1. Acute and short-term toxicity

Acute gastrointestinal effects such nausea, vomiting and diarrhoea, abdominal pain and sickness have been reported in humans from the ingestion of tin dissolved from internal surfaces of tin cans or saucepans since the late 19th century (Sedgwick, 1988; Luff and Metcalfe, 1890; Davidson, 1927; Savage, 1939).

Illness was reported in a group of 38 women who attended a banquet, of whom 37 completed a questionnaire and 31 reported symptoms. The symptoms included nausea (96.7%), abdominal cramps (86.7%), vomiting (70.0%), headache (56.7%), chills (36.7%) and diarrhoea (33.3%). The onset of symptoms was within 2 hours of the meal and they were reported to have persisted for 2-48 hours. Tests on food items did not reveal any pathogenic bacteria. The response data implicated a vodka punch as the causative agent, and analysis revealed that it contained 2000 mg tin/L; tests for copper, zinc and cadmium were negative. The punch had a pH of approximately 3 and had been stored in a re-tinned 5-gallon milk churn in which there were signs of corrosion (Warburton *et al*, 1962).

Benoy *et al* (1971) cited an unpublished report of the Metal Box Co. Ltd (1967) as recording that nausea, vomiting and diarrhoea were observed in a large, unspecified number of persons in Kuwait who had consumed formulated orange juice and apple juice containing 250-385 mg tin/kg. Omori and colleagues (1973) report several outbreaks of poisoning with limited data in Japan associated with canned orange juice; the main symptoms were nausea, vomiting, diarrhoea, fever and headache. The toxicity was attributed to tin on the basis of exclusion of microbiological contamination and the detection of 425 mg tin/kg in a toxicity-associated sample compared with concentrations ranging from 84-337 mg tin/kg in other purchased samples. Other published case reports of toxicity associated with canned orange or tomato drinks containing tin in concentrations ranging from 100 to 494 mg/kg were reviewed by JECFA (JECFA, 1982; Omori, 1966a and b; Horio *et al*, 1967a and b; EGVM, 2002). In well-documented cases severe abdominal bloating, vomiting, diarrhoea, and headache were noted after the consumption of canned tomato juice with tin levels ranging from 141 to 405 mg tin/kg; the mean concentrations ranged from 245 to 363 mg tin/kg in the various lots implicated as the cause of the intoxication. The cans were visibly de-tinned, an effect which was attributed to unusually high nitrate levels on the tomatoes used to prepare the juice (Barker and Runte, 1972).

In an early study that Schryver (1909) performed on himself, he ingested sodium tin tartrate for a period of 3 weeks at total daily doses of approximately 1, 2 and 3 mg Sn/kg body weight in successive weeks, no symptoms were reported. Analyses of faecal and urinary total nitrogen, and urinary ammonia, urea and uric acid were reported as indicating that no disturbance of metabolism had occurred (EGVM, 2002).

Five volunteers were given orange juice containing tin at concentrations of 0-1400 mg/kg on various occasions. The volunteers were unaware of the nature of the test substance. All experienced either nausea (3 individuals), diarrhoea (1 individual) or both (1 individual) when they first drank the juice containing 1400 mg tin/kg, which corresponded to a dose of tin of 4.4-6.7 mg/kg body weight; administration 1 month later resulted in only one case of nausea (Benoy *et al*, 1971).

Solomons *et al* (1983) reported noxious gastrointestinal symptoms (nausea, cramps and loose stools) in 4 subjects given a single dose of 100 mg Sn as stannous chloride together with 12.5 mg zinc (as zinc sulphate) in 100 mL soft drink. Symptoms were not observed when single doses of 25 or 50 mg Sn were given.

A limited number of case-reports of acute gastrointestinal disorders after consumption of food containing 100-500 mg tin/kg have been reported. Controlled clinical studies on acute effects of tin migrated from packaging suggest a threshold concentration for adverse effects of >730 mg/kg. Two separate randomised, single-centre, double-blind, cross-over investigations of the tolerability of tin added as stannous chloride at concentrations of <0.5, 161, 264 and 529 mg tin/kg in 250 mL juice in 20 volunteers (study 1) and tin migrated from packaging at concentrations of <0.5, 201 and 267 mg tin/kg in 250 mL tomato soup in 24 volunteers (study 2) were carried out. A clear dose-response relationship was only observed when tin was added as stannous chloride in tomato juice (study 1). No clinically significant adverse effects were reported in study 2 and comparison of the incidence of tin-related adverse effects showed no difference between the dose levels (including control). Studies on the distribution of low molecular weight (<1000 Da) tin species in the beverage showed that the chemical form of tin, and not the elemental concentration *per se,* determined the severity of the adverse effects in the gastrointestinal tract. Tin species of low molecular weight in supernatant represented 31-32% of total tin in canned tomato soup versus 56-61% in juice freshly spiked with stannous chloride. The differences in the incidence of adverse effects following administration of tomato juice with 161 and 264 mg of tin per kg and tomato soup with 201 and 267 mg of tin per kg probably resulted from differences in the concentration of low molecular weight tin species and in the nature of tin complexes formed. According to the investigators the results of this work demonstrated that tin concentrations up to 267 mg/kg in canned food cause no adverse effects in healthy adults (Boogaard *et al*, 2003). Regulatory limits of 200 mg/kg for the concentration of tin in canned foods and 100 mg/kg in canned beverages have been established to protect against the occurrence of episodes of acute human poisoning by tin (EC, 2004).

A few short-term human studies indicate that high intakes of tin (30-50 mg tin/day) may reduce the absorption of e.g. zinc (Johnson *et al*, 1982; Valberg and Chamberlain, 1984), while no effect was seen in one (Solomons *et al*, 1983). The long-term effects on zinc status and effects on other minerals are, however, not known.

3.2.2. Long-term toxicity and carcinogenicity

No carcinogenic effects of orally ingested inorganic tin in humans have been reported. Stannosis, a benign pneumoconiosis consequent of prolonged industrial exposure to tin oxide dusts, has been described from several countries (Barnes and Stoner, 1959; Bartak *et al*, 1948; Pendergrass and Pryde, 1948; Robertson *et al*, 1961).

4. DOSE-RESPONSE ASSESSMENT

4.1. Gastrointestinal acute effects

Acute toxicity of stannous compounds results from irritation of the mucosa of the gastrointestinal tract. Vomiting and diarrhoea were reported in cats given soluble salts of tin, but there was no clear dose-relationship, and the vehicles in which the tin was administered may have affected its toxicity.
Episodes of human poisoning resulting from the consumption of foods and drinks contaminated with tin have resulted in abdominal distension and pain, vomiting, diarrhoea, and headache. These symptoms commonly start within 0.5-3 hours, and recovery occurs within 48 hours. The doses of tin ingested in such episodes of poisoning were generally not estimated. In one study five volunteers experienced symptoms when they ingested juice containing 1400 mg Sn/kg. Administration of the same dose to

these individuals one month later resulted in symptoms in only one person. In another human study with in total 44 volunteers the lowest dose of $SnCl_2$ in canned beverage (tomato juice) without acute gastrointestinal effects was 200 mg tin/kg. In an experimental study Solomons *et al* (1983) reported adverse gastrointestinal symptoms (nausea, cramps and loose stools) in 4 adults given a single dose of 100 mg Sn as stannous chloride together with 12.5 mg zinc (as zinc sulphate) in 100 mL soft drink. Symptoms were not observed when single doses of 25 or 50 mg Sn were given. The balance of evidence suggests that the concentration of tin in contaminated foods is critical to the development of acute gastrointestinal effects, and that tin concentrations of 250 mg/kg in canned foods and 150 mg/kg in canned beverages are more likely to be associated with this (SCF, 2001).

4.2. Inhibition of trace element absorption

There is evidence of reduced status of iron, zinc and copper when rats are fed diets containing 50 mg Sn/kg diet or greater. This appears to be due to reduced absorption of trace elements, particularly zinc, but also iron and copper, as a result of formation of insoluble complexes (probably with phosphates) in the gastrointestinal tract. Such effects probably explain growth depression, loss of appetite and reduced feed conversion efficiency observed in rats at doses of stannous chloride greater than 150 mg Sn/kg diet. It is likely that other effects of tin e.g. pancreatic atrophy at a dose level of 2000 mg tin/kg diet or reduced compressive bone strength at a dose level above 50 mg Sn/kg diet are not systemic effects of absorbed tin but manifestations of deficiency of one or more trace elements.

In human adults a diet containing 50 mg tin/day (compared to 0.1 mg/day) reduced apparent absorption of dietary zinc by 16% but had no effect on absorption of copper, iron, manganese or magnesium, while inclusion of 36 mg tin as stannous chloride reduced the absorption of zinc from a test meal by 29%.

4.3. Systemic toxicity

There are no data on systemic toxicity of orally ingested tin salts in humans. Orally ingested inorganic tin compounds generally have low systemic toxicity in animals because of limited absorption from the gastrointestinal tract, limited accumulation, and rapid excretion, primarily in the faeces. Because soluble tin salts can cause depletion of essential trace elements, particularly zinc, copper and iron, as a result of gastrointestinal interactions, it is difficult to distinguish between the effects of such nutritional deficiencies and possible systemic effects of absorbed tin. Given the very low absorption of tin it is likely that adverse effects which occur at levels of 50 mg Sn/kg diet e.g. reduced bone compressive strength at 50 mg tin/kg diet or pancreatic atrophy at a dose level of 2000 mg tin/kg diet, are not systemic effects of absorbed tin but rather manifestations of deficiency of one or more trace elements.

Available evidence indicates that orally ingested tin salts are neither carcinogenic nor teratogenic.

Based on the results of *in vitro* and *in vivo* tests, the evidence of genotoxic activity of soluble tin salts is considered limited.

4.4. Conclusions

The absorption of inorganic compounds of tin from the gastrointestinal tract in humans and animals is very low with as much as 98% being excreted directly in the faeces. Because of their limited absorption, orally ingested inorganic tin compounds have low systemic toxicity in man and animals.

In man and animals, gastrointestinal effects are the main acute manifestation of toxicity associated with ingestion of tin. These are caused by the irritant action of soluble inorganic tin compounds on the mucosa of the gastrointestinal tract. The balance of evidence suggests that the concentration of tin in contaminated foods is critical to the development of acute gastrointestinal effects, and that tin concentrations above 250 mg/kg in canned foods and 150 mg/kg in canned beverages are more likely to be associated with this. The regulatory limits of 200 mg/kg for the concentration of tin in canned foods and 100 mg/kg in canned beverages have been established to protect against the occurrence of episodes of acute human poisoning by tin (EC, 2004).

In rats, growth depression, loss of appetite and reduced feed conversion efficiency are observed at doses of stannous chloride greater than 150 mg Sn/kg diet. This appears to be due to reduced absorption and status of trace elements, particularly zinc, but also iron and copper, as a result of formation of insoluble complexes (probably with phosphates) in the gastrointestinal tract. There is evidence of reduced status of iron, zinc and copper when rats are fed diets containing 50 mg Sn/kg diet or greater. It is likely that other effects which

occur at this or higher dietary levels of tin e.g. reduced calcium content of bone (femoral epiphysis) at 50 mg Sn/kg diet or pancreatic atrophy at 2000 mg tin/kg diet, are not systemic effects of absorbed tin but rather manifestations of deficiency of one or more trace elements.

In short term studies in human adults a diet containing 50 mg tin/day (compared to 0.1 mg/day) reduced apparent absorption of dietary zinc by 16% but had no effect on absorption of copper, iron, manganese or magnesium, while inclusion of 36 mg tin as stannous chloride reduced the absorption of zinc from a test meal by 29%.

CONCLUSIONS AND RECOMMENDATIONS

1. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The Panel considered that the available data from human or animal studies are insufficient to derive a tolerable upper intake level for tin.

2. RISK CHARACTERISATION

Occasional high intakes of tin are associated with high consumption of canned foods, and regulatory limits of tin content in canned foods (200 mg/kg) and beverages (100 mg/kg) have been established to protect against possible local acute effects on the gastrointestinal tract.

Short-term human studies indicate that high intakes of tin (about 30-50 mg tin/day or per meal) may reduce the absorption of zinc, but not other minerals such as iron, copper, manganese or magnesium. However, the possible long-term effects, if any, of such intake levels on status of zinc or other minerals have not been investigated.The current mean daily intake of tin in EU countries (e.g. ranging up to about 6 mg/day in the UK) appears to be well below the lowest intakes reported to cause adverse effects on zinc absorption.

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