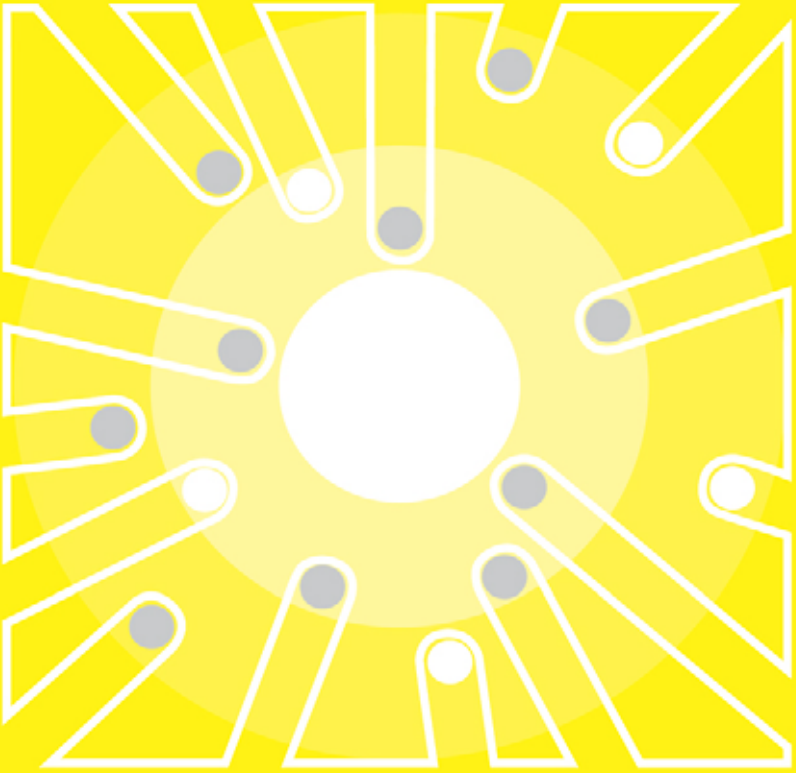
WHO FOOD ADDITIVES SERIES: 62

Safety evaluation of certain food additives

Prepared by the
Seventy-first meeting of the Joint FAO/WHO
Expert Committee on Food Additives (JECFA)

Food and Agriculture Organization of the United Nations

World Health Organization



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World Health Organization, Geneva, 2010

WHO Library Cataloguing-in-Publication Data

Safety evaluation of certain food additives / prepared by the Seventy-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(WHO food additives series ; 62)

1.Food additives - toxicity. 2.Food contamination. 3.Flavoring agents - analysis. 4.Flavoring agents - toxicity. 5.Risk assessment. I.Joint FAO/WHO Expert Committee on Food Additives. Meeting (71st : 2009 : Geneva, Switzerland). II.International Programme on Chemical Safety. III.Series.

ISBN 978 92 4 166062 4 (NLM Classification: WA 712)
ISSN 0300-0923

Typeset in India
Printed in India

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PREFACE

The monographs contained in this volume were prepared at the seventy-first meeting of the Joint Food and Agriculture Organization of the United Nations (FAO)/ World Health Organization (WHO) Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 16–24 June 2009. These monographs summarize the data on selected food additives reviewed by the Committee.

The seventy-first report of JECFA has been published by the World Health Organization as WHO Technical Report No. 950. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The monographs contained in this volume are based on working papers that were prepared by temporary advisers. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by M. Sheffer, Ottawa, Canada.

Many unpublished proprietary reports are unreferenced. These were voluntarily submitted to the Committee by various producers of the food additives under review and in many cases represent the only data available on those substances. The temporary advisers based the working papers they wrote on all the data that were submitted, and all these reports were available to the Committee when it made its evaluations.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in WHO concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

SPECIFIC FOOD ADDITIVES

BRANCHING GLYCOSYLTRANSFERASE FROM RHODOTHERMUS OBAMENSIS *EXPRESSED IN* BACILLUS SUBTILIS

First draft prepared by

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1. EXPLANATION

At the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008), the Committee evaluated the enzyme branching glycosyltransferase (1,4-α-glucan branching enzyme; Enzyme Commission number 2.4.1.18), which it had not evaluated previously. Branching glycosyltransferase catalyses the transfer of a segment of a 1,4-α-D-glucan chain to a primary hydroxy group in a similar glucan chain to create 1,6-linkages. The enzyme is intended for

use in starch processing to obtain modified starch with an increased number of branch points and improved functional properties.

1.1 Genetic modification

Branching glycosyltransferase is manufactured by pure culture fermentation of a genetically modified strain of *Bacillus subtilis* containing a synthetic gene coding for branching glycosyltransferase from *Rhodothermus obamensis*. *Bacillus subtilis* is a Gram-positive bacterium that is widely distributed in nature and is considered to be non-pathogenic and non-toxigenic. It has a long history of use in the production of enzymes used in food processing, including enzymes from genetically engineered strains. It has also been granted a Qualified Presumption of Safety status by the European Food Safety Authority (2008).

The gene encoding branching glycosyltransferase was originally cloned from *R. obamensis*, a thermophilic bacterium that was isolated from a marine hydrothermal vent. Based on the amino acid sequence of branching glycosyltransferase translated from the *R. obamensis* gene, a synthetic gene was designed that encodes branching glycosyltransferase with the same amino acid sequence as that of the native *R. obamensis* enzyme. The gene was subsequently placed under deoxyribonucleic acid (DNA) regulatory sequences derived from several *Bacillus* species and introduced into the *B. subtilis* host strain JA1343 by transformation. The chloramphenicol resistance gene (*cat*) was used in transformation as a selectable marker, but it was subsequently deleted to make the production strain marker free.

1.2 Chemical and technical considerations

Branching glycosyltransferase is secreted during fermentation into the fermentation broth and is subsequently purified and concentrated. The final product is formulated with sorbitol, glycerol and water and standardized to a desired activity. The total organic solids (TOS) content of the branching glycosyltransferase preparation is approximately 4%. The branching glycosyltransferase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

The branching glycosyltransferase preparation is intended for use in the production of modified starch with improved functional properties, such as higher solubility, lower viscosity and reduced retrogradation (undesirable structural changes). The recommended use levels range from 0.4 to 40 kg of the enzyme preparation per tonne of starch dry substance. The branching glycosyltransferase is likely to be inactivated and/or removed during starch processing steps. The enzyme is not added directly to food, and any carryover to food products formulated with modified starch is expected to be very low.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

Branching glycosyltransferase has been evaluated for potential allergenicity using bioinformatics criteria recommended in the report of the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization

(WHO) Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001). An amino acid sequence homology search between branching glycosyltransferase and known allergens listed in the allergen database at http://fermi.utmb.edu/SDAP/index.html was conducted. No homology was found for sequence fragments of six contiguous amino acids. However, when using a sliding window of 80 amino acids, a 35% match was found to sequences of Asp o 21 allergen, which is the α-amylase from *Aspergillus oryzae* (TAKA amylase A). However, the sequence alignment of the two enzymes showed that there are large differences in the loop regions, and the overall identity is only about 32%. As the two enzymes belong to the same family of glycosylhydrolases (Family 13; http://www.cazy.org/fam/GH13_3D.html), some homology is not surprising.

Although α-amylase from *A. oryzae* is an occupational allergen (Skamstrup Hansen et al., 1999), allergy symptoms after ingestion of the enzyme were reported only for four individuals. Three of these individuals consumed bread baked with the enzyme (Baur & Czuppon, 1995; Kanny & Moneret-Vautrin, 1995; Moreno-Ancillo et al., 2004), and one had a positive response to the oral challenge with α-amylase (Losada et al., 1992). In other studies conducted with patients with documented occupational or other allergies, no cases of food allergy to α-amylase from *A. oryzae* or other commercial enzymes used in food were identified (Skamstrup Hansen et al., 1999; Bindslev-Jensen et al., 2006). Thus, food allergy to α-amylase from *A. oryzae* is extremely rare. Moreover, branching glycosyltransferase is a bacterial protein, whereas nearly all known allergens listed in allergen databases are eukaryotic proteins. Therefore, despite certain homology to α-amylase from *A. oryzae,* branching glycosyltransferase does not seem to have the characteristics of a potential food allergen.

2.2 Toxicological studies

Bacillus subtilis is a non-pathogenic and non-toxigenic bacterium that has been utilized as a source of enzymes used in food for many years.

Toxicological studies were performed with branching glycosyltransferase using a representative batch (PPY 27209), which was produced according to the procedure used for commercial production. The liquid enzyme preparation used in the toxicological studies was a mixture of three preparations from fermentation sub-batches. The final preparation (specific gravity 1.065 g/ml) had an activity of 89 200 branching enzyme units (BEU) per gram and a TOS value of 7.3%.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

In a study conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents) and Good Laboratory Practice (GLP) requirements, a 10-ml aqueous suspension of branching glycosyltransferase (batch PPY 27209,

activity 89 200 BEU/g) was administered daily at 0, 77, 256 or 769 mg TOS/kg body weight (bw) by gavage to groups of 20 Wistar WU [Crl:WI(WU), outbred] rats (10 per sex) for 13 weeks. Stability testing of the prepared preparations at weeks 1, 6 and 13 indicated that the enzyme activity was similar to that predicted. The experimental parameters determined were clinical signs, body weight, food and water consumption, neurobehavioural testing (WHO/International Programme on Chemical Safety functional observational battery), ophthalmic end-points, haematological parameters, clinical chemical end-points, gross and microscopic appearance, and organ weights. Blood for haematology and clinical chemistry was collected during necropsy from male rats on day 91 of treatment and from female rats on day 92. Ophthalmoscopy was performed before treatment in all rats and then only in the control and high-dose groups during the last week of treatment. All other measurements were performed on day 91/92 only.

No treatment-related effects were observed for mortality, clinical signs, body weight gain, food and water consumption, clinical chemistry, neurobehavioural effects or ophthalmic end-points. A small, but statistically significant, reduction in the mean corpuscular haemoglobin concentration, which was observed only in high-dose males, was considered to have no toxicological significance, because it was not corroborated by other related haematological parameters, such as packed cell volume and haemoglobin concentration. A reduction in absolute and relative weights of the epididymides in low-dose and mid-dose males was considered to be unrelated to treatment because of the absence of any effects at a 3-fold higher dose. The slightly increased relative liver weight (5%) and reduced absolute brain weight (4%) in high-dose males together with the absence of corresponding histopathological lesions identified in these organs were not considered to be toxicologically relevant. In both sexes, macroscopic pathology and histopathology were unaffected by treatment.

Overall, it can be concluded that no toxicologically relevant effects were seen in this 13-week study of general toxicity in rats when branching glycosyltransferase was administered daily by gavage at doses up to 769 mg TOS/kg bw per day. This dose, the highest dose tested, was therefore taken to be the no-observed-adverse-effect level (NOAEL) (Appel & Van den Hoven, 2008).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

The results of two studies of genotoxicity with branching glycosyltransferase (batch PPY 27209) are summarized in Table 1. The first study was conducted in accordance with OECD Test Guideline 471 (Bacterial Reverse Mutation Test), whereas the second complied with OECD Test Guideline 487 (In Vitro Mammalian Cell Micronucleus Test; draft). Both studies were certified for compliance with GLP and quality assurance.

Table 1. Genotoxicity of branching glycosyltransferase in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrApKM101	156–5000 µg/ml (liquid culture method), ±S9	Negative	Pedersen (2008)
Clastogenicity/ aneuploidy	Human lymphocytes	1st and 2nd experiments: 2813, 3750 or 5000 µg/ml, ±S9	Negative	Whitwell (2008)

S9, 9000 × *g* supernatant from rat liver.

2.2.5 Reproductive toxicity

(a) Multigeneration studies

No multigeneration studies were available.

(b) Developmental toxicity

No developmental toxicity studies were available.

2.3 Observations in humans

No information was available.

3. DIETARY EXPOSURE

Branching glycosyltransferase can be used in a wide range of foodstuffs, but it is not expected to be present in the final product. The following estimation is based on the worst-case assumption that the enzyme is used in all processed food and beverages and remains in the products consumed. The maximum amount of TOS added to food is 48 mg/kg. Assuming a daily consumption of 750 g of food (50%) and 1500 g of beverages (25%), according to the budget method, the amount of TOS ingested would be about 2 mg/kg bw per day for an adult weighing 60 kg.

4. COMMENTS

4.1 Assessment of potential allergenicity

Branching glycosyltransferase was assessed for potential allergenicity by comparing its amino acid sequence with the sequences of known allergens according to the bioinformatics criteria recommended in the report of the Joint

FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology. A 35% homology within a sliding window of 80 amino acids to α-amylase from *Aspergillus oryzae* was identified. *Aspergillus oryzae* is recognized as the occupational allergen Asp o 21 and was also reported to cause allergy symptoms in a few individuals after ingestion. However, no homology between branching glycosyltransferase and α-amylase from *A. oryzae* was found at the level of six contiguous amino acid sequences. In addition, branching glycosyltransferase is a bacterial protein, whereas nearly all known allergens are of eukaryotic origin. Thus, branching glycosyltransferase does not seem to have the characteristics of a potential food allergen.

4.2 Toxicological data

Toxicological studies were performed with branching glycosyltransferase using a representative batch (PPY 27209), which was produced according to the procedure used for commercial production. The liquid enzyme preparation used in the toxicological studies was a mixture of three preparations from fermentation sub-batches. The final preparation (specific gravity 1.065 g/ml) had an activity of 89 200 BEU/g and a TOS value of 7.3%.

In a 13-week study of general toxicity in rats, no toxicologically relevant effects were seen when branching glycosyltransferase was administered daily by gavage at doses up to 769 mg TOS/kg bw per day. This dose, the highest dose tested, was therefore taken to be the NOAEL.

Branching glycosyltransferase was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberrations in human lymphocytes in vitro.

4.3 Assessment of dietary exposure

Branching glycosyltransferase can be used in a wide range of foodstuffs, but it is not expected to be present in the final product. However, the following estimation is based on the worst-case assumption that the enzyme is used in all processed food and beverages and remains in the products consumed. The maximum amount of TOS added to food is 48 mg/kg. Assuming a daily consumption of 750 g of food (50%) and 1500 g of beverages (25%), according to the budget method, the amount of TOS ingested would be about 2 mg/kg bw per day for an adult weighing 60 kg.

5. EVALUATION

The Committee allocated an acceptable daily intake (ADI) “not specified” for branching glycosyltransferase from this recombinant strain of *B. subtilis* (JA1343) used in the specified applications and in accordance with Good Manufacturing Practice.

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CASSIA GUM

First draft prepared by

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1. EXPLANATION

At the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008), the Committee evaluated cassia gum, which it had not evaluated previously. Cassia gum is related to guar gum, locust (carob) bean gum and tara gum in terms of structure and chemical properties. The galactomannans of guar gum, locust (carob) bean gum and tara gum have mannose to galactose ratios of 2:1, 4:1 and approximately 3:1, respectively. Each of these three gums was previously allocated an acceptable daily intake (ADI) “not specified” (Annex 1, references *39*, *57* and *74*).

1.1 Chemical and technical considerations

Cassia gum is the purified flour from the endosperm of the seeds of *Cassia tora* and *Cassia obtusifolia*, which belong to the Leguminosae family. Cassia gum is composed of at least 75% high relative molecular mass (approximately

200 000–300 000) polysaccharide, consisting primarily of a linear chain of 1,4-β-D-mannopyranose units with 1,6-linked α-D-galactopyranose units. The saccharides are composed of mannose (77.2–78.9%), galactose (15.7–14.7%) and glucose (7.1–6.3%). The ratio of mannose to galactose is 5:1.

The manufacture of cassia gum includes cleaning of the source material, by which the content of *Cassia occidentalis* (which is a naturally occurring contaminant) is reduced to less than 0.05%, de-husking and de-germing by thermal mechanical treatment, followed by milling and screening of the endosperm. The ground endosperm is further purified by extraction with isopropanol. The concentration of anthraquinones in cassia gum is below the 0.5 mg/kg detection limit. The food additive under evaluation is cassia gum that is refined and complies with the specifications established at the current meeting.

Cassia gum is used as a thickener, emulsifier, foam stabilizer, moisture retention agent and/or texturizing agent in processed cheese, frozen dairy desserts and mixes, meat products and poultry products.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

No specific absorption, distribution, metabolism or excretion data were available on the galactomannans from cassia gum. However, from studies on guar gum, locust (carob) bean gum and tara gum reviewed by the Committee at its nineteenth, twenty-fifth and thirtieth meetings, respectively (Annex 1, references *39*, *57* and *74*), it appears that other galactomannans in related gums undergo no or only minimal hydrolysis by digestive juices or enzymes, independent of the specific mannose to galactose ratio. They can be partially fermented by large gut microflora, but are largely excreted unchanged in faeces. The Committee concluded that cassia gum will be largely excreted unchanged as well, although fermentation by gut microflora may occur to some extent. If hydrolysis of cassia gum occurs, the resulting oligosaccharides or monosaccharides would be expected to be absorbed and metabolized in normal biochemical pathways.

2.2 Toxicological studies

Most available toxicological studies were performed with semi-refined cassia gum. Semi-refined cassia gum is produced similarly to the cassia gum currently under evaluation, with the exception of an additional isopropanol extraction step to significantly reduce the level of anthraquinones in the latter. Anthraquinones are impurities that occur naturally in the seeds from which cassia gum is produced, some of which may display muscle-toxic, genotoxic or carcinogenic properties. Semi-refined cassia gum contains approximately 70 mg total anthraquinones/kg.

2.2.1 Acute toxicity

Two studies of acute oral toxicity were available. In a limit test, five male Wister-Han-Schering rats were given in total 5000 mg semi-refined cassia gum/kg

body weight (bw) by oral gavage in two doses at a 2-h interval. The oral median lethal dose (LD_{50} value) in this study was >5000 mg/kg bw. The study was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA) (Schöbel, 1986). In another limit test, 10 male and 10 female KM mice were given in total 10 000 mg cassia gum/kg bw by oral gavage in four doses over 24 h. The oral LD_{50} value in this study was >10 000 mg/kg bw. Statements regarding compliance with GLP and QA were lacking (Weidu, 2006).

2.2.2 Short-term studies of toxicity

In a 28-day study of toxicity (Zühlke, 1990), groups of five male and five female Crl:CD (SD)BR Sprague-Dawley rats (aged 5–6 weeks) were administered semi-refined cassia gum at dietary concentrations of 0, 2500, 10 000, 25 000 or 50 000 mg/kg feed (equal to doses of 0, 250, 1030, 2590 and 4960 mg/kg bw per day for males and 0, 230, 1110, 2360 and 4590 mg/kg bw per day for females). A sixth group received semi-refined cassia gum by gavage (in distilled water) 2 times a day, at a total dose of 1000 mg/kg bw per day. The study was certified for compliance with GLP and QA and was essentially performed as described in Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (Repeated Dose 28-day Oral Toxicity Study in Rodents), although weekly detailed clinical investigations and measurements of sensory reactivity were omitted. Observations included mortality, clinical signs, behaviour, body weight, food consumption, haematology, clinical chemistry, organ weights (adrenals, brain, heart, kidneys, liver, ovaries and testes), macroscopic examination and histopathology (on major organs of the animals in the control group, the 50 000 mg/kg feed group and the group treated by gavage).

Five animals died during the experiment, but these deaths were incidental or due to an intubation error or blood sampling procedure and were not accompanied by signs of systemic target organ toxicity. No clinical changes that could be attributed to the treatment were observed. Body weight gain was statistically significantly reduced (–20%) in males of the 50 000 mg/kg feed group, possibly related to a small (–11%) decrease in food intake in these animals. In females, body weight gain was statistically significantly reduced (–17%) in the 10 000 and 25 000 mg/kg feed group and in the 1000 mg/kg bw per day group. These changes are considered to be related to the viscous nature of cassia gum and not considered to be of toxicological relevance.

Haematology and clinical chemistry findings included several statistically significant changes that for the most part were small, were not dose related or occurred in one sex only. They were also claimed to be within the normal range for the species tested, but historical control data were not provided. The only changes that were outside the historical control range and could have been related to treatment were increased mean concentrations of glucose and triglyceride in both sexes of the 10 000 mg/kg feed group (males 41% and 149% and females 56% and 46%, respectively) and 25 000 mg/kg feed group (males 53% and 168% and females 74% and 67%, respectively). These findings were not dose related, however, as they were not observed in the 50 000 mg/kg feed group or in the group treated by gavage.

No treatment-related effects were observed at necropsy or during histopathological examination. In males, group mean absolute kidney weights were statistically significantly reduced in the 10 000 mg/kg feed (–8%), 50 000 mg/kg feed (–15%) and 1000 mg/kg bw per day (–7%) groups, but group mean relative kidney weights were not affected. In females, in contrast, no changes were observed in group mean absolute kidney weights, whereas the group mean relative kidney weight was statistically significantly increased (+11%) in the 50 000 mg/kg feed group. These inconsistent changes were not considered to be treatment related, given also the absence of histopathological changes in the kidneys.

Overall, it can be concluded that, in the absence of dose relationships and histopathological findings, the effects observed were of no toxicological relevance. The no-observed-adverse-effect level (NOAEL) was 50 000 mg/kg feed, equal to 4590 mg/kg bw per day, the highest dose tested (Zühlke, 1990).

In a limitedly reported 30-day study of toxicity, groups of 10 male and 10 female SD rats were administered cassia gum via the diet at levels corresponding to intakes of 0, 250, 500 and 1000 mg/kg bw per day. Statements regarding compliance with GLP and QA were lacking. No treatment-related effects on mortality, body weight gain, food consumption or food utilization were observed. The investigated haematological (red and white blood cell counts, haemoglobin) and biochemical parameters (albumin, cholesterol, creatinine, alanine aminotransferase, aspartate aminotransferase, glucose, total protein, triglyceride and urea nitrogen) were not affected. No gross findings were observed, and investigated organ weights (liver, kidney, spleen, ovaries and testes) were not affected. Histopathological examination of liver, kidney, spleen, stomach and intestines, ovaries and testes also did not show treatment-related effects. It seems, therefore, that no adverse effects were observed at doses up to and including 1000 mg/kg bw per day, the highest dose tested (Weidu, 2006).

Groups of four male and four female Beagle dogs were given semi-refined cassia gum mixed into canned dog food at a dietary concentration of 7500 or 25 000 mg/kg for 90 days (equal to doses of 980 and 3290 mg/kg bw per day for males and 1130 and 3890 mg/kg bw per day for females). A control group of the same size was administered the canned dog food with 2300 mg/kg locust (carob) bean gum. The study was essentially performed as described in OECD Test Guideline 409 (Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents) and was certified for compliance with GLP and QA. The only treatment-related effect observed was a dose-dependent increase in water consumption. However, as this was most likely associated with water retention in the gastrointestinal tract by colloidally dissolved semi-refined cassia gum, it was not considered to be of toxicological relevance. All other effects observed (for several haematological, blood coagulation and biochemical parameters and some organ weights) were not considered treatment related because they lacked a dose or time relationship, occurred in one sex only and/or remained within the historical reference range. Overall, it can be concluded that the NOAEL was 25 000 mg/kg feed, equal to 3290 mg/kg bw per day, the highest dose tested (Schuh, 1990).

In a 13-week study of toxicity (certified for compliance with GLP and QA), groups of five male and five female cats were given semi-refined cassia gum as part of a canned food diet at a concentration of 0, 5000 or 25 000 mg/kg (equal to doses of 0, 520 and 2410 mg/kg bw per day for males and 0, 530 and 2740 mg/kg bw per day for females). The study was essentially performed according to OECD Test Guideline 409, with some slight deviations. No adverse or treatment-related effects on mortality, behaviour, clinical signs, body weight gain, food and water consumption, haematology, clinical biochemistry, organ weights, macroscopy or microscopy were observed. The no-observed-effect level (NOEL) was 25 000 mg/kg feed, equal to 2410 mg/kg bw per day, the highest dose tested in this study (Virat, 1984).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available for cassia gum.

In a limited long-term study of toxicity with guar gum reviewed by the Committee at its nineteenth meeting (Annex 1, reference *39*), no adverse effects were observed in rats administered guar gum at a dietary concentration of 5% for 24 months. In carcinogenicity studies reviewed by the Committee at its twenty-fifth and thirtieth meetings (Annex 1, references *57* and *74*), no significant adverse effects were observed in rats and mice administered locust (carob) bean gum or tara gum at dietary concentrations up to 5% for 103 weeks.

2.2.4 Genotoxicity

The results of five studies of genotoxicity in vitro with cassia gum and/or semi-refined cassia gum (three bacterial reverse mutation assays, one chromosomal aberration assay and one gene mutation assay) are summarized in Table 1. The first bacterial reverse mutation study (Verspeek-Rip, 1998a) was conducted with semi-refined cassia gum, the second with purified semi-refined cassia gum (8.6 mg total anthraquinones/kg; Meerts, 2003) and the third with cassia gum (Weidu, 2006). The first two studies followed OECD Test Guideline 471 (Bacterial Reverse Mutation Test) and were certified for compliance with GLP and QA. For the third, limitedly reported study, no statements regarding compliance with GLP and QA were available. The positive results obtained for semi-refined cassia gum in strain TA100 at precipitating concentrations in the first reverse mutation study (Verspeek-Rip, 1998a) were not observed in the second reverse mutation study. The chromosomal aberration assay (Bertens, 1998) was performed according to OECD Test Guideline 473 (In Vitro Mammalian Chromosome Aberration Test), and the gene mutation assay (Verspeek-Rip, 1998b) was conducted according to OECD Test Guideline 476 (In Vitro Mammalian Cell Gene Mutation Test). Both studies were certified for compliance with GLP and QA and used semi-refined cassia gum.

Table 1. Results of studies of genotoxicity in vitro with cassia gum

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* strain TA100, *Escherichia coli* WP2uvrA	Range-finding study: 3–5000 µg/plate, ±S9	Positive/ negative[a]	Verspeek-Rip (1998a)
	S. typhimurium strains TA98, TA1535 and TA1537	1st experiment: 4–1000 µg/plate, ±S9		
	S. typhimurium strains TA98, TA100, TA1535 and TA1537, *E. coli* WP2uvrA	2nd experiment: 1.6–1000 µg/plate, ±S9		
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537, *E. coli* WP2uvrA	1st experiment: 0.3–100 µg/plate, ±S9 2nd experiment: 0.3–100 µg/plate, ±S9	Negative[b]	Meerts (2003)
	S. typhimurium strain TA100, *E. coli* WP2uvrA	3rd experiment: 100–5000 µg/plate, ±S9		
Reverse mutation	*S. typhimurium* strains TA97, TA98, TA100 and TA102	1st experiment: 0.05–5 mg/plate, ±S9 2nd experiment: 0.05–5 mg/plate, ±S9	Negative[c]	Weidu (2006)
Gene mutation	Mouse lymphoma L5178Y $TK^{+/-}$ cells	1st experiment: 0.003–10 µg/ml, ±S9 2nd experiment: 0.003–10 µg/ml, ±S9	Negative[d]	Verspeek-Rip (1998b)
Chromosomal aberration	Human lymphocytes	1st experiment: 1–10 µg/ml, ±S9 2nd experiment: 1–10 µg/ml, ±S9	Negative[e]	Bertens (1998)

S9, 9000 × *g* supernatant from rat liver.

[a] With semi-refined cassia gum, with and without metabolic activation (S9), by the plate incorporation method, using dimethyl sulfoxide (DMSO) as a vehicle. Slight precipitation occurred from 100 µg/plate upwards in the dose range–finding study and from 62.5 and 200 µg/plate upwards in the first and second experiments, respectively. In none of the experiments was toxicity observed. In two independent experiments, *S. typhimurium* strain TA100 showed a dose-related increase in the number of revertants at precipitating concentrations in the presence and absence of metabolic activation, whereas *E. coli* WP2uvrA showed negative responses. *Salmonella typhimurium* strains TA98, TA1535 and TA1537 showed negative responses in the first experiment, but in the second experiment they showed dose-related increases in the number of revertants at precipitating concentrations in

Table 1 (contd)

the presence (TA1537 only) and absence of metabolic activation. However, the increases observed in strains TA98, TA1535 and TA1537 were within the historical control range.

[b] With purified semi-refined cassia gum (8.6 mg total anthraquinones/kg), with and without metabolic activation (S9), by the plate incorporation method. In the first and second experiments, ultrapure water was used as a vehicle; slight precipitation occurred at 33 and 100 µg/plate, but no toxicity was observed. In the third experiment, DMSO was used as a vehicle; slight precipitation occurred at all concentrations, but no toxicity was observed.

[c] With cassia gum, with and without metabolic activation (S9), by the plate incorporation method, using sterilized distilled water as a vehicle. The study was reported in a very limited manner, and no information was provided on the occurrence of precipitation or toxicity.

[d] With semi-refined cassia gum, with and without metabolic activation (S9), with DMSO as vehicle. In both experiments, precipitation occurred at 10 µg/ml. In experiment 1, cells were exposed for 3 h and harvested 3 days following exposure. No toxicity was observed. In experiment 2, in the absence of metabolic activation, cells were exposed for 24 h and harvested 2 days later, whereas in the presence of metabolic activation, cells were exposed for 3 h and harvested 3 days later. Without metabolic activation, the cell count and cloning efficiency were reduced by 42% and 81% at the highest concentration tested, respectively, but with metabolic activation, no toxicity was observed.

[e] With semi-refined cassia gum, with and without metabolic activation (S9), with DMSO as vehicle. In both experiments, precipitation occurred at 10 µg/ml. In the first experiment, the cells were exposed for 3 h and harvested 21 h later. The highest tested concentration induced mitotic inhibition (22%) in the presence, but not in the absence, of metabolic activation. In the second experiment, cells were exposed for 24 or 48 h without S9 and harvested immediately after exposure. With S9, the cells were treated for 3 h and harvested another 45 h later. The highest tested concentration induced mitotic inhibition (33%) in the presence, but not in the absence, of metabolic activation.

Table 2. Results of studies of genotoxicity in vivo with cassia gum

End-point	Test system	Concentration	Result	Reference
Micronucleus test	Bone marrow of KM mice (males and females)	625–2500 mg/kg bw, by oral gavage (divided over 2 doses in 30 h)	Negative[a]	Weidu (2006)
Sperm abnormality test	Male KM mice	625–2500 mg/kg bw per day, by oral gavage for 5 days	Negative[b]	Weidu (2006)

[a] Study was performed with cassia gum and was reported in a very limited manner. Bone marrow was collected 6 h after second gavage, and micronuclei of 1000 polychromatic erythrocytes (PCE) per animal were counted, followed by determination of the ratio of PCE to normal chromatic erythrocytes (NCE).

[b] Study was performed with cassia gum and was reported in a very limited manner. Sperm was collected 30 days after last administration, and aberrations were counted in 1000 sperm cells per animal.

The results of two limitedly reported studies of genotoxicity in vivo (a sperm abnormality test and a micronucleus test in mice) are summarized in Table 2. These studies were performed with cassia gum. No statements regarding compliance with GLP and QA were available (Weidu, 2006).

Overall, the Committee concluded that cassia gum is not genotoxic.

2.2.5 Reproductive toxicity

In a two-generation study of reproductive toxicity, groups of 25 male and 25 female Ico:OFA.SD Sprague-Dawley rats were given diets containing 0, 5000, 20 000 or 50 000 mg semi-refined cassia gum/kg. These dietary concentrations were equal to doses of 0, 510, 2060 and 5280 mg/kg bw per day for males and 0, 510, 2090 and 6120 mg/kg bw per day for females (calculated using the mean food intake and mean body weights in weeks 1–10). An additional group received a diet containing 50 000 mg of purified semi-refined cassia gum (resulting from an additional isopropanol extraction step) per kilogram (equal to a dose of 5430 mg/kg bw per day for males and 6230 mg/kg bw per day for females). All parental animals (P) were treated for approximately 10 weeks before mating and during mating, gestation and lactation. Pregnant females were allowed to rear their offspring (F_{1a}) to weaning. Rats in both 50 000 mg/kg diet groups exhibited low pregnancy rates, and the non-pregnant rats were mated again with the same males. They were allowed to litter, and the subsequent offspring (F_{1b}) were terminated on days 5–7 postpartum. Selected F_{1a} offspring were treated for a 10-week period of maturation and during mating, gestation and lactation. Pregnant F_{1a} females were allowed to rear their offspring (F_2) to weaning. The study was performed according to OECD Test Guideline 416 (Two-Generation Reproduction Study) and was certified for compliance with GLP and QA. The only significant findings were a slightly reduced pregnancy rate in the 50 000 mg semi-refined cassia/kg diet group (pregnancy rate

17/25 compared with 24/25 in the control group) and the 50 000 mg purified semi-refined cassia/kg diet group (pregnancy rate 18/25 compared with 24/25 in the control group) of the P generation and a slightly, but not statistically significantly, reduced mean pup weight of the F_{1a} (–11%) and the F2 (–14%) generations in the 50 000 mg purified semi-refined cassia/kg diet group on day 21 postpartum. When both 50 000 mg/kg diet treatment groups of the P generation were mated again (resulting in the F_{1b} generation), however, a reduced pregnancy rate was no longer observed. Therefore, 50 000 mg/kg diet (equal to a dose of 5280 mg/kg bw per day) was taken to be the NOEL (McIntyre, 1990).

There was also a one-generation study of reproductive toxicity with cats available, in combination with a short-term study of toxicity. In this study, groups of 10 male and 20 female cats were given semi-refined cassia gum as part of a canned food diet at a dietary concentration of 0, 7500 or 25 000 mg/kg for at least 83 weeks. These dietary concentrations were equal to 0, 690 and 2470 mg/kg bw per day for males and 0, 860 and 2950 mg/kg bw per day for females (calculated using the mean food intake and mean body weights in weeks 1–83). The F_1 generation was sacrificed after at least 13 weeks. The study was certified for compliance with GLP and QA and was performed according to OECD Test Guidelines 415 (One-Generation Reproduction Toxicity Study) and 409 (Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents). An unusually high mortality in the control group resulted in a high litter loss, impairing appropriate comparison between control and treatment groups. In general, no obvious effects on short-term toxicity parameters were observed in both parental generation and offspring. Reproductive performance was not affected, nor were growth and development of the offspring. Only at the highest dose were some effects observed that were possibly related to treatment: a slight decrease in food consumption during late gestation and a slight increase in absolute and relative ovarian weights in parental females, together with a significantly higher combined incidence of stillborns and neonatal deaths (Virat, 1989). As a result of the unusually high mortality in the control group, the Committee considered this study not suitable for use for the evaluation.

In a developmental toxicity study that was reported in a limited manner, groups of 12 pregnant female SD rats were administered 0, 250, 500 or 1000 mg cassia gum/kg bw per day by oral gavage from day 7 until day 16 of gestation. No treatment-related effects on maternal body weight, number of resorptions or dead embryos, or weight and length of the fetuses were observed. No abnormalities were detected upon observation of the skeleton and visceral examination of the fetuses. It seems, therefore, that no adverse effects were observed in dams or offspring at doses up to and including 1000 mg/kg bw per day, the highest dose tested (Weidu, 2006).

In a developmental toxicity study, groups of 28 pregnant Crl:CD (SD)BR Sprague-Dawley rats were administered semi-refined cassia gum in distilled water by gavage twice daily at a total dose of 0, 350 or 1000 mg/kg bw per day from days 6 to 19 post-coitum. A fourth group of 29 pregnant rats was administered 1000 mg purified semi-refined cassia gum/kg bw per day following the same dosing regimen. All animals were sacrificed and examined on day 20 post-coitum. The study was certified for compliance with GLP and QA and was conducted in accordance with OECD Test Guideline 414 (Prenatal Development Toxicity Study). Two animals in

the purified semi-refined cassia gum group died as a result of improper gavaging. No compound-related adverse effects were reported for pregnancy incidence, implantations, post-implantation loss or fetal defects upon necropsy. A statistically significant reduction in mean daily food consumption and mean body weight gain was observed in the pregnant animals of both 1000 mg/kg bw per day groups. These effects are probably related to the viscous nature of cassia gum and were not considered to be of toxicological relevance. The only finding in the offspring was a statistically significantly increased fetal weight in the purified semi-refined cassia gum group. No evidence of embryotoxicity or teratogenicity was observed in any of the treated groups. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Müller, 1989a).

In a related developmental toxicity study (certified for compliance with GLP and QA), groups of 20 pregnant New Zealand White rabbits were administered semi-refined cassia gum or purified semi-refined cassia gum following the same study protocol and dosing regimen, the only difference being that the rabbits were treated from days 6 to 27 post-coitum and were sacrificed and examined on day 28 post-coitum. Eleven animals (four controls, one animal of the 350 mg semi-refined cassia gum/kg bw per day group, four animals of the 1000 mg semi-refined cassia gum/kg bw per day group and two animals of the 1000 mg purified semi-refined cassia gum/kg bw per day group) died during the treatment period or were killed moribund. All deaths could be attributed to improper gavaging and/or were incidental. A reduction (not statistically significant) in mean daily food consumption (–19%) was reported in animals administered 1000 mg semi-refined cassia gum/kg bw per day. Possibly secondary to this effect on food consumption, a slight, not statistically significant reduction in mean fetal weights (–16%) was observed in this group. These effects are probably related to the viscous nature of cassia gum and were not considered to be of toxicological relevance. No compound-related adverse effects were reported for pregnancy incidence, implantations, post-implantation loss or fetal defects upon necropsy. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Müller, 1989b).

2.3 Observations in humans

The only human data available relate to hypersensitivity or allergenicity. Studies in industrially exposed and pharmaceutical workers showed that *Cassia* and cassia powder may act as sensitizers following dermal and inhalatory exposure (Satheesh et al., 1994; Steget et al., 1999). No information is available on food allergies or food intolerance following oral intake in humans.

3. DIETARY EXPOSURE

3.1 Use in foods

Cassia gum is used as a thickener, emulsifier, foam stabilizer, moisture retention agent and/or texturizing agent in processed cheese, frozen dairy desserts and mixes, meat products and poultry products. Maximum use levels for cassia gum range from 2500 mg/kg food in frozen desserts and 3000 mg/kg food in cheeses to 3500 mg/kg food in meat and poultry products (Lubrizol, 2008). In a submission to

the European Commission, use levels for cassia gum only up to 2500 mg/kg food were considered, with a maximum of 1500 mg/kg food for processed meat and poultry products (European Food Safety Authority, 2006).

3.2 Dietary exposure estimates

The Committee received an assessment of dietary exposure to cassia gum and additionally accessed data on dietary exposure from the European Food Safety Authority (EFSA) web site.

An EFSA opinion published in 2006 (European Food Safety Authority, 2006) contained an assessment of dietary exposure to cassia gum. Per capita food consumption figures for yogurt and yogurt drinks, ice cream, desserts, processed cream cheese, and canned/preserved meat and poultry items were combined with cassia gum concentrations at the suggested maximum use levels, resulting in an estimated dietary exposure of approximately 195 mg/person per day. Assuming a default body weight of 60 kg, dietary exposure was 3.3 mg/kg bw per day. In the opinion, it was noted that the use of per capita food consumption did not allow an analysis of high-percentile consumers of these foods.

The EFSA opinion also contained a dietary exposure estimate prepared using individual dietary records for USA consumers of foods that might contain cassia gum. Maximum use levels in nine broad food categories were combined with reported food consumption as measured in the 1994–1996 Continuing Survey of Food Intakes by Individuals (CSFII). This yielded an estimated mean dietary exposure of 2.1 mg/kg bw per day (actual body weights are accessible in the CSFII). Dietary exposure at the 90th percentile was 4.9 mg/kg bw per day. In the EFSA opinion, these figures were considered to be sufficiently conservative based on the assumptions and data used in making the estimates.

A sponsor supplied an estimate of dietary exposure to cassia gum from its proposed use in four broad food categories: processed cheese at a maximum cassia gum level of 3000 mg/kg food; frozen desserts at up to 2500 mg/kg food; and meat products and poultry products at up to 3500 mg/kg food. This assessment used individual dietary records from the USA's 2003–2004 National Health and Nutrition Examination Survey (NHANES), with modification to the interim results using a 30-day food frequency questionnaire that was a part of NHANES III. The sponsor noted that dietary exposures derived using food consumption survey data from 2 non-consecutive days would likely overestimate exposure, especially for infrequently consumed foods. By using the number of days on which a food was reported consumed over the 30-day survey period, the sponsor proportionally adjusted the 2-day average intakes to 30-day averages. The typical conservative assumption that all foods that might contain cassia gum would contain it at the highest allowed levels was retained in the analysis, ensuring that the final result would be conservatively high. The dietary exposure to cassia gum from each broad food category was reported separately, as was the total dietary exposure from all foods. Per capita estimates were also included in the analysis. The results are reported in Table 3 (all ages) and Table 4 (2- to 5-year-olds). The adjusted mean dietary exposure was 2.7 mg/kg bw per day; at the 90th percentile, dietary exposure was 5.4 mg/kg bw per day.

Table 3. Frequency-adjusted 30-day average daily intake of cassia gum by the population in the USA[a,b,c]

	Intake per consumer only (mg/kg bw per day)		Intake per capita (mg/kg bw per day)	
Food category	Mean	90th percentile	Mean	90th percentile
Cheese (processed only)	0.5	1.1	0.4	1.0
Frozen dairy desserts	1.3	3.0	0.9	2.3
Meat products	1.4	3.2	1.3	3.1
Poultry products	0.2	0.5	0.2	0.5
Total intake	2.7[d]	5.4[e]	2.7	5.4[e]

[a] Food consumption (mg/kg bw per day): 2003–2004 NHANES data, USA population.
[b] Food frequency (typical frequency) for NHANES III participants (weighted).
[c] 30-day average adjusted for each category.
[d] It is not statistically valid to sum intake estimates per consumer only at the category level to derive total intake.
[e] It is not statistically valid to sum the upper-percentile intake estimates at the category level to derive total intake.

Table 4. Frequency-adjusted 30-day average daily intake of cassia gum by children 2–5 years of age in the USA[a,b,c]

	Intake per consumer only (mg/kg bw per day)		Intake per capita (mg/kg bw per day)	
Food category	Mean	90th percentile	Mean	90th percentile
Cheese (processed only)	1.3	3.0	1.3	3.0
Frozen dairy desserts	2.2	5.2	1.9	4.8
Meat products	3.7	7.5	3.6	7.5
Poultry products	0.3	0.6	0.3	0.6
Total intake	6.8[d]	12.1[e]	6.8	12.1[e]

[a] Food consumption (mg/kg bw per day): 2003–2004 NHANES data, children 2–5 years of age.
[b] Food frequency (typical frequency) for NHANES III participants (weighted).
[c] 30-day average adjusted for each category.
[d] It is not statistically valid to sum intake estimates per consumer only at the category level to derive total intake.
[e] It is not statistically valid to sum upper-percentile intake estimates at the category level to derive total intake.

The Committee concluded that the estimated 90th-percentile dietary exposure to cassia gum from the proposed uses would be less than 6 mg/kg bw per day.

4. COMMENTS

4.1 Toxicological data

Most available toxicological studies have been performed with semi-refined cassia gum, which is produced similarly to the cassia gum currently under evaluation, with the exception of an additional isopropanol extraction step to significantly reduce the level of anthraquinones in the latter. Semi-refined cassia gum contains approximately 70 mg total anthraquinones/kg.

Although specific absorption, distribution, metabolism and excretion data were not available for cassia gum, the Committee concluded, based on data on related galactomannans, that cassia gum will be largely excreted unchanged, although fermentation by gut microflora may occur to some extent. If hydrolysis of cassia gum occurs, the resulting oligosaccharides or monosaccharides would be expected to be absorbed and metabolized in normal biochemical pathways.

Cassia gum is of low acute oral toxicity in rats and mice. It was also of low oral toxicity in a 28-day study in rats, a 90-day study in dogs and a 90-day study in cats. In these studies, administration of semi-refined cassia gum at dietary concentrations up to 50 000 mg/kg in rats (equal to doses of 4960 mg/kg bw per day for males and 4590 mg/kg bw per day for females), 25 000 mg/kg in dogs (equal to doses of 3290 mg/kg bw per day for males and 3890 mg/kg bw per day for females) and 25 000 mg/kg in cats (equal to doses of 2410 mg/kg bw per day for males and 2740 mg/kg bw per day for females) did not result in adverse effects. The decrease in food consumption and accompanying decrease in body weight gain noted in the 28-day rat study and the increase in water consumption observed in the 90-day dog study were considered to be related to the viscous nature of cassia gum and not considered to be of toxicological relevance. Therefore, the NOAELs in rats and dogs were 4590 and 3290 mg/kg bw per day, respectively, the highest doses tested. The NOEL in cats was 2410 mg/kg bw per day, the highest dose tested.

Cassia gum and/or semi-refined cassia gum were tested in reverse mutation assays in bacteria and in a chromosomal aberration assay and a gene mutation assay in mammalian cells. Cassia gum was also tested in an in vivo sperm abnormality test and an in vivo micronucleus test in mice. From these studies, the Committee concluded that cassia gum is not genotoxic. Cassia gum was not tested in a carcinogenicity study, but, given the lack of genotoxicity and the negative results obtained in assays of carcinogenicity of locust (carob) bean gum and tara gum, the Committee did not consider a study of long-term toxicity and/or carcinogenicity necessary for the safety evaluation of cassia gum.

Semi-refined cassia gum was tested in reproductive toxicity studies in the rat (two-generation study) at dietary concentrations up to 50 000 mg/kg (equal to a

dose of 5280 mg/kg bw per day for males and 6120 mg/kg bw per day for females) and in the cat (one-generation study) at dietary concentrations up to 25 000 mg/kg (equal to a dose of 2470 mg/kg bw per day in males and 2950 mg/kg bw per day in females). In the cat study, high mortality in the control group resulted in a high litter loss, impairing appropriate comparison between control and treatment groups. Therefore, this cat study was considered not suitable for use in the evaluation. In the two-generation reproductive toxicity study in rats, the only effects observed were a slightly reduced pregnancy rate (which was not observed in a subsequent second mating resulting in an F_{1b} generation) and a slight, but not significant, decrease in pup weights of the F_{1a} and F_2 generations at the highest dose level. Therefore, 50 000 mg/kg feed (equal to 5280 mg/kg bw per day), the highest dose tested, was taken to be the NOEL.

Semi-refined cassia gum was also tested in studies of developmental toxicity in the rat and the rabbit at doses up to 1000 mg/kg bw per day. In the rat study, food intake was statistically significantly reduced in the pregnant animals of the highest dose group, accompanied by a statistically significant reduction in mean body weight gain. In the rabbit study, a reduction in mean daily food consumption was reported, as well as a slight reduction in mean fetal weights at the highest dose level, but these reductions were not statistically significant. These effects are probably related to the viscous nature of cassia gum and were not considered to be of toxicological relevance. No embryotoxicity or teratogenicity was observed. The NOAELs were 1000 mg/kg bw per day, the highest dose tested, in both rats and rabbits.

The findings of overall low toxicity for cassia gum are in line with the findings for the related food additives guar gum, locust (carob) bean gum and tara gum. The Committee noted that in the toxicological studies available on cassia gum and semi-refined cassia gum, no indications for anthraquinone-related toxicity were found.

4.2 Assessment of dietary exposure

The Committee received an assessment of dietary exposure to cassia gum and additionally accessed data on dietary exposure from the EFSA web site.

An EFSA opinion published in 2006 contained an assessment of dietary exposure to cassia gum. Per capita food consumption figures for yogurt and yogurt drinks, ice cream, desserts, processed cream cheese, and canned/preserved meat and poultry items were combined with cassia gum concentrations at the suggested maximum use levels, resulting in an estimated dietary exposure of approximately 195 mg/person per day. Assuming a default body weight of 60 kg, dietary exposure was 3.2 mg/kg bw per day. The EFSA opinion also contained a dietary exposure estimate prepared using individual dietary records for consumers of foods that may contain cassia gum in the USA. Maximum use levels in nine broad food categories were combined with reported food consumption, yielding an estimated mean dietary exposure of 2.1 mg/kg bw per day. Dietary exposure at the 90th percentile was 4.9 mg/kg bw per day.

A sponsor supplied an estimate of dietary exposure to cassia gum from its proposed use in four broad food categories: processed cheese at a maximum cassia

gum level of 3000 mg/kg food; frozen desserts at up to 2500 mg/kg food; and meat products and poultry products at up to 3500 mg/kg food. Food consumption data from the USA (the 2003–2004 NHANES) were used for this analysis. As this survey contains dietary records for 2 non-consecutive days of food consumption, it likely overestimates exposure. To better estimate "usual" consumption, the sponsor proportionally adjusted the 2-day average intakes to 30-day averages using a factor calculated from the number of days on which a food was reported to be consumed over an additional 30-day survey period. The adjusted mean dietary exposure was 2.7 mg/kg bw per day; at the 90th percentile, dietary exposure was 5.4 mg/kg bw per day.

The Committee concluded that the estimated 90th-percentile dietary exposure to cassia gum from the proposed uses would be less than 6 mg/kg bw per day.

5. EVALUATION

Comparing the conservative exposure estimate of 6 mg/kg bw per day with the lowest reported NOAEL of 1000 mg/kg bw per day (the highest dose tested) derived from the developmental toxicity studies in rats and rabbits, the margin of exposure is at least 160. The Committee noted that in a 28-day study in rats, in 90-day studies in dogs and cats and in a two-generation study in rats, no adverse effects were observed at doses up to, respectively, 4590, 3290, 2410 and 5280 mg/kg bw per day, the highest doses tested in these studies.

Considering the low toxicity and the negative genotoxicity results, the Committee allocated an ADI "not specified" for cassia gum that complies with the tentative specifications established at the current meeting, when used in the applications specified and in accordance with Good Manufacturing Practice.

As the method for determination of anthraquinones at a level of 0.5 mg/kg and below was not considered to be suitable for inclusion in the specifications, the Committee decided to make the specifications tentative pending submission of data on a suitable and validated method by the end of 2010.

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CYCLAMIC ACID AND ITS SALTS: DIETARY EXPOSURE ASSESSMENT

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1. EXPLANATION

Cyclamic acid and its sodium and calcium salts are food additives commonly termed "cyclamates". Cyclamates are used in over 50 countries as intense sweeteners in a range of food categories.

The fortieth session of the Codex Committee on Food Additives (CCFA) in 2008 (FAO/WHO, 2008a) requested an evaluation by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) of the impact on dietary exposures to cyclamates of different maximum levels of use of cyclamates in the Codex General Standard for Food Additives (GSFA) Food Category 14.1.4, Water-based flavoured drinks, including "sport", "energy" or "electrolyte" drinks and particulated drinks, which includes all carbonated and non-carbonated varieties and concentrates, products based on fruit and vegetable juices[1] and coffee-, tea- and herbal-based drinks. The different use levels to be considered were 250, 500, 750 and 1000 mg/kg. While there are provisions for the use of cyclamates in the GSFA in a wide range of food categories, the GSFA does not currently have a provision for the use of cyclamates in Food Category 14.1.4.

Cyclamates were evaluated at the eleventh, fourteenth, twenty-first, twenty-fourth and twenty-sixth meetings of the Committee (Annex 1, references *14*, *22*, *44*, *53* and *59*).

2. PREVIOUS SAFETY EVALUATION

Cyclamates have very low acute toxicity. However, the metabolite, cyclohexylamine, which is formed by bacterial fermentation in the colon, causes testicular atrophy in a number of animal species. Cyclohexylamine can be formed from unabsorbed cyclamates by the intestinal flora in certain individuals. An acceptable daily intake (ADI) of 0–11 mg/kg body weight (bw) was established at the twenty-sixth meeting (Annex 1, reference *59*), based on testicular atrophy induced by the metabolite cyclohexylamine in rats, with a no-observed-adverse-effect level (NOAEL) of 100 mg/kg bw per day.

A critical factor in the establishment of the ADI was the level of conversion of cyclamates to cyclohexylamine in the gastrointestinal tract, as this varies considerably between and within individuals. In deriving the ADI for cyclamates, it was assumed that 37% was absorbed in the body and not metabolized. Sixty-three per cent was not absorbed, and it was assumed that 30% of this unabsorbed material was converted to cyclohexylamine (overall conversion rate of 18.9%). Applying the conversion rate factor and the ratio of molecular weights for cyclamate and cyclohexylamine of 2 to the NOAEL for cyclohexylamine of 100 mg/kg bw per day resulted in a calculated NOAEL for cyclamates of 1058 mg/kg bw per day. A safety factor of 100 was assumed to derive the ADI of 0–11 mg/kg bw for cyclamates.

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[1] Fruit and vegetable juices per se are found in Codex GSFA Food Categories 14.1.2.1 and 14.1.2.2, respectively.

New data on the metabolism of cyclamates in humans have been published since the twenty-sixth meeting of JECFA. In 1995, the European Commission's Scientific Committee on Food (SCF) evaluated the data available at that time and retained the ADI of 0–11 mg/kg bw (Scientific Committee on Food, 1997). In 2000, the SCF published a revised opinion on cyclamic acid and its salts, establishing an ADI of 0–7 mg/kg bw (Scientific Committee on Food, 2000). The 2000 SCF evaluation was based on the same toxicological data used by JECFA and more recent data on the metabolism of cyclamates (Renwick et al., 2004). The Renwick et al. (2004) study indicated that individuals exhibit a range of rates of conversion from cyclamates to cyclohexylamine, some people being non-converters and others having high conversion rates. The SCF assumed the highest reported conversion rate of 85%, but used a lower safety factor of 32; these factors were applied to the NOAEL for cyclohexylamine of 100 mg/kg bw per day to derive the ADI of 0–7 mg/kg bw (Scientific Committee on Food, 2000). Food Standards Australia New Zealand (2007) assessed these data and other recent studies on cyclamate toxicity in 2007 and determined that the ADI established by JECFA in 1982 was adequately protective of consumers.

In this dietary exposure assessment, the predicted dietary exposures to cyclamates are compared with the ADI of 0–11 mg/kg bw, established at the twenty-sixth meeting of JECFA.

3. USE OF CYCLAMATES

Cyclamates were first synthesized in 1937 and are now available for use as intense sweeteners in a range of food categories in over 50 countries (Bopp & Price, 2001). Cyclamates are approximately 30 times sweeter than sucrose, but this ratio can vary according to the sucrose solution with which cyclamates are compared and the food matrix in which they are used (Bopp & Price, 2001; Henin, 2001).

Cyclamates have commonly been used in conjunction with saccharin in non-alcoholic beverages to replace sugar, as there is a synergistic effect on sweetness when the two sweeteners are used together, reducing the total amount of additives needed. The ratio of 1 part saccharin to 10 parts cyclamates is used to achieve the desired sweetness profile to replace sugar in these products (Bopp & Price, 2001). However, mixtures of cyclamates and other intense sweeteners are now used in a variety of food products.

There are provisions for the use of cyclamic acid, calcium cyclamate and sodium cyclamate in the Codex GSFA (FAO/WHO, 2008b) in a wide range of food categories, with maximum use levels as given in Table 1. The GSFA does not currently have a provision for the use of cyclamates in Food Category 14.1.4, Water-based flavoured drinks. There are GSFA provisions for use of cyclamates in fruit and vegetable nectars and aromatized beverages—for example, beer, wine and spirit-based cooler-type beverages and low-alcoholic refreshers.

In the European Union (EU), cyclamates (E952, cyclamic acid and its sodium and calcium salts) were approved as sweeteners for a variety of food products in 1994 (European Parliament, 1994). In 2004, an amendment to Directive 94/35/EC

Table 1. Codex GSFA provisions for cyclamates

Food class	Food category	Maximum use level (mg/kg)
1.1.2	Dairy-based drinks, flavoured and/or fermented	250
1.7	Dairy-based desserts	250
2.4	Fat-based desserts excluding 1.7	250
3.0	Edible ices, including sherbet and sorbet	250
4.1.2.4	Canned or bottled (pasteurized) fruit	1000
4.1.2.5	Jams, jellies and marmalades	1000
4.1.2.6	Fruit-based spreads excluding 4.1.2.5	2000
4.1.2.8	Fruit preparations including pulp, purees, fruit toppings and coconut milk	250
4.1.2.9	Fruit-based desserts including fruit-flavoured water-based desserts	250
4.2.2.6	Vegetable (including mushrooms, fungi, roots, tubers, pulses, legumes, aloe vera), seaweed, nut and seed pulp preparations excluding 4.2.2.5	250
5.1.2	Cocoa mixes (syrup)	250
5.1.3	Cocoa-based spreads including fillings	500
5.1.4	Cocoa and chocolate products	500
5.1.5	Imitation chocolate, chocolate substitute products	500
5.2	Confectionery including hard and soft candy, excluding 5.1, 5.3, 5.4	500
5.3	Chewing gum	3000
5.4	Decorations, toppings and sweet sauces	500
6.5	Cereal- and starch-based desserts	250
7.2	Fine bakery wares and mixes	1600
10.4	Egg-based desserts	250
11.4	Other sugars and syrups	500
11.6	Tabletop sweeteners including those containing high-intensity sweeteners	GMP
12.6.1	Emulsified sauces	500
12.7	Salads and sandwich spreads excluding cocoa- and nut-based spreads in 4.2.2.5, 4.1.3	500
13.3	Dietetic foods intended for special medical purposes	400
13.4	Dietetic formulae for slimming purposes and weight reduction	400

Table 1 (contd)

Food class	Food category	Maximum use level (mg/kg)
13.5	Dietetic foods excluding 13.1–13.4, 13.6	400
13.6	Food supplements	1250
14.1.3.1	Fruit nectar	400
14.1.3.2	Vegetable nectar	400
14.1.3.3	Concentrates for fruit nectar	400
14.1.3.4	Concentrates for vegetable nectar	400
14.2.7	Aromatized beverages	250

GMP, Good Manufacturing Practice.

produced a reduction in the maximum usable dose of cyclamates in water-based flavoured drinks from 400 to 250 mg/l, along with deletions of some food categories (European Parliament, 2004). Table 2 gives the current European Commission permissions for use of cyclamates.

Table 2. Cyclamate permissions for food in the EU[a]

Food	Maximum usable dose (mg/kg)[b]
Non-alcoholic drinks	
Water-based flavoured drinks, energy reduced or with no added sugar	250 mg/l
Milk- and milk derivative–based or fruit juice–based drinks, energy reduced or with no added sugar	250 mg/l
Desserts and similar products	
Water-based flavoured desserts, energy reduced or with no added sugar	250
Milk- and milk derivative–based preparations, energy reduced or with no added sugar	250
Fruit- and vegetable-based desserts, energy reduced or with no added sugar	250
Egg-based desserts, energy reduced or with no added sugar	250
Cereal-based desserts, energy reduced or with no added sugar	250
Fat-based desserts, energy reduced or with no added sugar	250

Table 2 (contd)

Food	Maximum usable dose (mg/kg)[b]
Confectionery	
Cocoa-, milk-, dried fruit– or fat-based sandwich spreads, energy reduced or with no added sugar	500
Other	
Canned or bottled fruit, energy reduced or with no added sugar	1000
Energy-reduced jams, jellies and marmalades	1000
Energy-reduced fruit and vegetable preparations	250
Fine bakery products for special nutritional uses	1600
Foods intended for use in energy-restricted diets for weight reduction as referred to in Directive 96/8/EC	400
Dietary foods for special medical purposes as defined in Directive 1992/21/EC	400
Food supplements as defined in Directive 2002/46/EC supplied in a liquid form	400
Food supplements as defined in Directive 2002/46/EC supplied in a solid form	500
Drinks consisting of a mixture of a non-alcoholic drink and beer, cider, perry, spirits or wine	250 mg/l
Food supplements as defined in Directive 2002/46/EC, based on vitamins and/or mineral elements and supplied in a syrup-type or chewable form	1250

[a] From European Parliament (2004). The consolidated document, Directive 94/35/EC amended by Directive 2003/115/EC on 29 January 2004, deleted permissions for cyclamates in tabletop sweeteners included in starch-based confectionery, energy reduced or with no added sugar; confectionery with no added sugar; cocoa- and dried fruit–based confectionery, energy reduced or with no added sugar; chewing gum with no added sugar; breath-freshening micro-sweets with no added sugar; and edible ices, energy reduced or with no added sugar.

[b] Unless otherwise specified.

In Australia and New Zealand, permissions for the use of cyclamates are given for a variety of food categories (Food Standards Australia New Zealand, 2009a). Maximum permitted levels for cyclamates in beverages covered by Food Category 14.1.4 were lowered from 1200 to 600 mg/kg in 2000 and further lowered to 350 mg/kg in 2007 (Food Standards Australia New Zealand, 2007). Cyclamates have been banned in the USA from use in all foods, beverages and drugs since

1970 after earlier being approved. Their status in other countries in 2001 is summarized by Bopp & Price (2001).

It is noted that at the currently permitted levels of cyclamates in beverages covered by Food Category 14.1.4 in the EU (250 mg/l) and in Australia and New Zealand (350 mg/kg) and in nectars in the Codex GSFA (400 mg/kg), it is necessary to use other sweeteners in conjunction with cyclamates in these products to achieve complete sugar replacement and the desired sweetness profile. For example, 4000 mg cyclamates/kg would be required in beverages covered by Food Category 14.1.4 containing 12% sugar, assuming a relative sweetness of 30.

4. DIETARY EXPOSURE TO CYCLAMATES

4.1 Concentrations of cyclamates in food

In most dietary exposure assessments, it has been assumed that cyclamates are present in the food either at the maximum use level as permitted in the relevant food legislation or at typical use levels as reported by manufacturers. In many cases, the typical use level was close to the maximum use level.

In some studies, detailed databases of sweetener content reported by the manufacturers or as analysed were available by brand and/or flavour, so that concentrations could be matched to individual foods consumed (Bär & Biermann, 1992; National Food Authority, 1995; Leclercq et al., 1999; Arcella et al., 2004; Food Standards Australia New Zealand, 2004).

Few reports in the literature publish the concentrations used in the dietary exposure calculations. Analytical levels for cyclamate-containing beverages covered by Food Category 14.1.4 were reported by Leth et al. (2007, 2008); carbonated beverages containing cyclamates had mean levels of 245 mg/l in 1999 and 151 mg/l in 2007, and non-carbonated drinks containing cyclamates had mean levels of 333 mg/l in 1999 and 165 mg/l in 2007. The decrease in reported concentration of cyclamates between 1999 and 2007 in Denmark followed a change to the permitted level of cyclamates in beverages covered by Food Category 14.1.4 for the EU in 2004 (European Parliament, 2004). A mean level of 282 mg cyclamates/l (range 84–1010 mg/l) in beverages was reported for Brazil (De Carmargo & Toledo, 2006), and a weighted mean concentration of 359 mg/kg was reported for Italy (Leclercq et al., 1999).

A cyclamate concentration of 90 mg/kg was reported for one sample of jam available in Italy in 1999; samples of candies, chewing gum and yogurt analysed did not contain cyclamates (Leclercq et al., 1999). A concentration range of 846–1225 mg/l was reported for yogurts available in Brazil (De Carmargo & Toledo, 2006).

Available analytical data for cyclamates are summarized in Table 3.

Table 3. Reported concentration data for cyclamates

Food category (reference)	Mean concentration	Range (number of samples)	Maximum permitted use level at time of survey (reference)
Beverages (Leclercq et al., 1999)	359[a] mg/kg	356, 400 mg/kg (2)	400 mg/l (European Parliament, 1994)
Beverages, carbonated (Leth et al., 2007)	245 mg/l	203–404 mg/l (13)	400 mg/l (European Parliament, 1994)
Beverages, still (Leth et al., 2007)	333 mg/l	84–454 mg/l (55)	400 mg/l (European Parliament, 1994)
Beverages, carbonated (Leth et al., 2007)	151 mg/l	21–240 mg/l (22)	250 mg/l (European Parliament, 2004)
Beverages, still (Leth et al., 2007)	165 mg/l	87–222 mg/l (16)	250 mg/l (European Parliament, 2004)
Beverages (De Camargo & Toledo, 2006)	282 mg/l	84–1010 mg/l	1300 mg/l (Brazil Ministry of Health, 2001)
Yogurts (De Camargo & Toledo, 2006)	Not reported	846–1225 mg/l	1300 mg/l (Brazil Ministry of Health, 2001)
Jam (Leclercq et al., 1999)	90 mg/kg	– (1)	100 mg/kg (European Parliament, 1994)

[a] Concentrations have been weighted to allow for the market share of the two brands.

4.2 Screening methods

The budget method can be used to estimate the theoretical maximum level of cyclamates in those foods and beverages that are likely to contain the food additive that would not result in the ADI being exceeded by the population (Hansen, 1979; World Health Organization, 2001). Assuming an ADI of 0–11 mg/kg bw, that 50% of cyclamates are used in solid food and 50% in beverages and that only 25% of the total amount of solid food and beverages in the food supply contain the food additive, the theoretical maximum level of cyclamates would be 880 mg/kg for solid food and 220 mg/kg for beverages. These theoretical maximum levels for solid food and beverages were lower than the relevant Codex GSFA maximum use level of 3000 mg/kg for solid food and 400 mg/kg for beverages. Hence, data on dietary exposures were required.

An alternative screening method for intense sweeteners has been used in which it is assumed that the sweetener replaces all sucrose in the diet (Renwick, 2008). The population in the USA has one of the highest reported sugar intakes, 62 kg/person per year (170 g/person per day); if this sugar were to be completely replaced by cyclamates, it would result in a dietary exposure of 5.7 g/day, or 95 mg/kg bw per day, assuming a 60-kg body weight and relative sweetness of 1:30

(Haley & Jerardo, 2009). As with the budget method, this approach is also very conservative, because it is very unlikely that one sweetener will replace all sucrose; additionally, it ignores other technological functions of sucrose in food. The overestimation of dietary exposures to intense sweeteners that are predicted by the sugar replacement method is illustrated by the fact that dietary exposures to cyclamates for consumers only reported in the literature were equivalent to less than 150 mg sucrose/kg bw per day (9 g sucrose/day, assuming a 60-kg body weight) (Renwick, 2008). The sucrose equivalent predicted for intakes of other more widely used intense sweeteners, such as aspartame, saccharin and sucralose, for the general population was higher, at 255 mg sucrose/kg bw per day (range from 100 to 300 mg sucrose/kg bw per day or from 6 to 18 g sucrose/day, assuming a 60-kg body weight). All these predictions were much lower than the actual amount of sucrose consumed per day from the diet.

4.3 Dietary exposure estimates based on individual dietary records

Dietary exposure estimates for cyclamates were available from a number of countries, either as estimates based on national nutrition survey data or from specialized surveys that were designed to evaluate exposures to intense sweeteners. A comprehensive summary of the data available on dietary exposures to a number of intense sweeteners, including cyclamates, has been provided in reviews by Renwick (2006, 2008).

4.3.1 Dietary exposure estimates for the whole population

Information on dietary exposure estimates for cyclamates for the whole population is summarized in Table 4, including details on the source of the cyclamate concentration used in the calculations. Where dietary exposures to cyclamates were reported in these studies for consumers only as well as for all respondents, the consumer-only results are included in Table 5 in section 4.3.2.

Table 4. Dietary exposure estimates for cyclamates for the whole population

Country (reference)	Survey type	Source of concentration data[a]	Population group	Dietary exposure to cyclamates (mg/kg bw per day)
Germany (Bär & Biermann, 1992)	1988–1989 survey, 24-h recall, 2291 respondents, 31% cyclamate users	TUL, labels, some analysis	Mean (all)	0.9
			90th percentile (all)	3.2

Table 4 (contd)

Country (reference)	Survey type	Source of concentration data[a]	Population group	Dietary exposure to cyclamates (mg/kg bw per day)
Spain (Serra-Majem et al., 1996)	1992 survey, 2450 respondents aged 6–75 years, two 24-h recalls, food frequency, two different seasons, 18% cyclamate users	TUL	Mean (all)	0.4
			90th percentile (all)	1.3
Denmark (Leth et al., 2007)	1995 NNS, 7-day record, 3098 respondents aged 1–80 years	Analysed soft drinks (1999), mean and high content (only drinks included in estimates)	Median (all)	0.1 (0.1 high)
			90th percentile (all)	1.6 (1.8 high)
			Median (children 1–3 years)	1.6 (1.8 high)
			90th percentile (children 1–3 years)	6.3 (7.1 high)
Denmark (Leth et al., 2008)	1995 NNS, 7-day record, 3098 respondents aged 1–80 years	Analysed soft drinks (2005–2006), mean and high content (only drinks included in estimates)	Median (all)	0.04 (0.06 high)
			90th percentile (all)	0.8 (0.9 high)
			Median children (1–3 years)	0.6 (0.9 high)
			90th percentile (children 1–3 years)	2.4 (3.7 high)
Italy (Arcella et al., 2004)	2000–2001 survey, 270 respondents aged 14–17 years, 6% cyclamate users (17% females)	TUL matched by brand and flavour	Mean (all)	0.01
			95th percentile (all)	0.05

NNS, National Nutrition Survey; TUL, typical manufacturers' use level.

[a] The typical manufacturers' use level is usually a mean value or matched to brand and flavour. Where products were analysed, mean values for product categories or values for individual products by brand and flavour were used.

4.3.2 Dietary exposure estimates for users of products containing cyclamates

Some dietary exposure analyses used screening surveys to identify consumers of products containing intense sweeteners, then continued with a more detailed study of those consumers (National Food Authority, 1995; Toledo & Ioshi, 1995; Food Standards Australia New Zealand, 2004). Several studies focused on young children or teenagers (National Food Authority, 1995; Leclercq et al., 1999; Food Standards Agency, 2003a) as a result of concerns about the potential for high exposure to intense sweeteners in these age groups. Most of these detailed studies either analysed concentrations of cyclamates in individual drinks (Toledo & Ioshi, 1995; Leclercq et al., 1999; Food Standards Agency, 2003a; for dilutable drinks only) or matched consumption records with typical use levels reported by manufacturers by brand and flavour (National Food Authority, 1995; Food Standards Agency, 2003a; Food Standards Australia New Zealand, 2004; for foods and drinks other than dilutable drinks). These consumer studies are summarized in Table 5.

Table 5. Dietary exposure estimates for consumers of cyclamates

Country (reference)	Survey type	Source of concentration data[a]	Population group	Dietary exposure to cyclamates (mg/kg bw per day)
Australia (National Food Authority, 1995)	1993 intense sweetener survey, 128 respondents, 7-day diary	TUL, matched by brand and flavour	Mean (12–39 years, consumers)	2.5
			90th percentile (12–39 years, consumers)	11.8
			Mean (12–17 years, consumers)	3.8
			90th percentile (12–17 years, consumers)	14.6
Australia (Food Standards Australia New Zealand, 2007)	1995 National Nutrition Survey, 24-h recall, 1921 respondents aged 2–11 years, 10% consumers	TUL, except for 14.1.4 beverages, for which MUL of 350 mg/kg used	Mean (2–11 years, consumers)	3.6–4.1
			90th percentile (2–11 years, consumers)	8.1–8.9
			95th percentile (2–11 years, consumers)	10.1–11.0

Table 5 (contd)

Country (reference)	Survey type	Source of concentration data[a]	Population group	Dietary exposure to cyclamates (mg/kg bw per day)
Australia (Food Standards Australia New Zealand, 2004)	2003 intense sweetener survey, 263 respondents, 7-day diary	TUL, matched by brand and flavour	Mean (12+ years, consumers)	3.1
			90th percentile (12+ years, consumers)	8.2
			95th percentile (12+ years, consumers)	9.9
Brazil (Toledo & Ioshi, 1995)	1990–1991 survey across two seasons, 673 consumers of intense sweeteners selected from two regions, 67% cyclamate users	Analysis and labels	Mean (consumers)	4.0
			Maximum (consumers)	17.9
Germany (Bär & Biermann, 1992)	1988–1989 survey, 24-h recall, 2291 respondents, 31% cyclamate users	TUL, labels, some analysis	Mean (consumers)	3.0
			90th percentile (consumers)	6.4
Italy (Leclercq et al., 1999)	1996 survey, teenagers aged 13–19 years, 212 respondents, 6% cyclamate users	TUL, matched by brand and flavour	Mean (consumers)	0.2
			Maximum (consumers)	0.6
Italy (Arcella et al., 2004)	2000–2001 survey, 270 respondents aged 14–17 years, 6% cyclamate users (17% females)	TUL, matched by brand and flavour	Mean (consumers)	0.05
			Maximum (consumers)	1.2

Table 5 (contd)

Country (reference)	Survey type	Source of concentration data[a]	Population group	Dietary exposure to cyclamates (mg/kg bw per day)
New Zealand (Food Standards Australia New Zealand, 2004)	2003 intense sweetener survey, 137 respondents, 7-day diary	TUL, matched by brand and flavour	Mean (12+ years, consumers)	2.2
			90th percentile (12+ years, consumers)	7.5
			95th percentile (12+ years, consumers)	8.8
Spain (Serra-Majem et al.,1996)	1992 survey, 2450 respondents aged 6–75 years, two 24-h recalls, food frequency, two different seasons, 18% cyclamate users	TUL	Mean (consumers)	2.4
			90th percentile (consumers)	4.7
United Kingdom (Food Standards Agency, 2003a)	2003 survey, 7-day diary, children aged 1.5–4.5 years, 1110 consumers of drinks with intense sweeteners only	TUL, analysis of dilutable drinks, matched by brand and flavour	Mean (consumers)	4.5
			97.5th percentile (consumers)	14.1

MUL, maximum use level; TUL, typical manufacturers' use level.

[a] The typical manufacturers' use level is usually a mean value or matched to brand and flavour. Where products were analysed, mean values for product categories or values for individual products by brand and flavour were used.

Following the increased use of intense sweeteners after the establishment of a European directive on sweeteners (European Parliament, 1994), the United Kingdom's Food Standards Agency undertook a survey of intense sweeteners in beverages covered by Food Category 14.1.4 for young children (aged 1.5–4.5 years) in 2003 (Food Standards Agency, 2003a). This study followed a survey of intense sweetener levels in dilutable drinks prepared for young children, where the reported range of dilution was from 1.6 to 36, with a mean of 9.5 (Food Standards Agency, 2003b). For this age group, Food Category 14.1.4 beverages containing intense sweeteners (including cyclamates) were the only products included in the

survey, as it was presumed that this age group did not use tabletop sweeteners or other low-joule (low-energy) products. The estimated mean dietary exposure to cyclamates for children 1.5–4.5 years old was 4.5 mg/kg bw per day, and the estimated dietary exposure for high consumers in this group (97.5th percentile) was 14.1 mg/kg bw per day.

The Italian study focused on teenagers living in Rome, again following the implementation of the 1994 European directive, as a result of concern that teenagers were likely to have above-average consumption of these products. Results indicated that teenage girls were more likely than boys to consume products with intense sweeteners, although all dietary exposures to cyclamates were low compared with those found in other studies. The mean dietary exposure for consumers was 0.2 mg/kg bw per day, and the maximum reported dietary exposure was 0.6 mg/kg bw per day (Leclercq et al., 1999). Similar low dietary exposures to intense sweeteners were reported in a follow-up study of Italian teenagers in 2003; mean dietary exposures to cyclamates were 0.01 mg/kg bw per day for the whole population and 0.05 mg/kg bw per day for consumers, with a maximum of 1.2 mg/kg bw per day (Arcella et al., 2004; see Tables 4 and 5).

Dietary exposure estimates reported from the 1995 intense sweetener survey in Australia were 2.5 mg/kg bw per day for the 12- to 39-year age group studied and 11.8 mg/kg bw per day at the 90th percentile of consumption. Dietary exposures were higher for the youngest age group studied (12–17 years old), with a mean dietary exposure of 3.8 mg/kg bw per day and 90th-percentile exposure of 14.6 mg/kg bw per day (National Food Authority, 1995). In the 2003 survey in Australia and New Zealand, the mean dietary exposure for the group studied (12 years of age and over) was slightly higher than that in 1995 (3.1 mg/kg bw per day), but the 90th-percentile consumption level was lower (8.2 mg/kg bw per day). Similar results were obtained for the New Zealand population, with a mean dietary exposure of 2.2 mg/kg bw per day and a 90th-percentile exposure of 7.5 mg/kg bw per day (Food Standards Australia New Zealand, 2004).

The issue of consumer loyalty to one branded product was also explored in the Leclercq et al. (1999) and Arcella et al. (2003) studies of Italian teenagers, with theoretical estimates indicating that if consumers always chose a product with the highest level of cyclamates, potential dietary exposure to cyclamates would be higher than those predicted using a more conventional approach: 1.7 mg/kg bw per day for consumers of beverages and 1.5 mg/kg bw per day for consumers of tabletop sweeteners. This issue is also discussed in *Principles and methods for the risk assessment of chemicals in food* (FAO/WHO, 2009). In the Arcella et al. (2003) analysis, probabilistic modelling was used to evaluate the impact of the inclusion of indicators of market share and consumer loyalty on predicted dietary exposures by conducting a sensitivity analysis. Results for female teenagers consuming Food Category 14.1.4 beverages with cyclamates indicated that consumer loyalty had an impact on dietary exposure for intense sweeteners including cyclamates, and only models that took the market share and consumer loyalty indicators into account met the validation criteria.

4.3.3 Dietary exposure estimates for specific groups

Various studies have investigated the potential dietary exposure to intense sweeteners for people with diabetes or on weight control diets, as there have been concerns that these groups may have higher exposures than the rest of the population because of a wider use of low-joule products (Bär & Biermann, 1992; National Food Authority, 1995; Toledo & Ioshi, 1995; Serra-Majem et al., 1996; Ilbäck et al., 2003; Food Standards Australia New Zealand, 2004). Results are summarized in Table 6. In some studies, reported mean dietary exposures for people with diabetes and on weight control diets were higher than that for the whole population (Serra-Majem et al., 1996). In others, however, although the proportion of the population of people with diabetes or on weight control diets who used low-joule products was higher than the proportion of users in the whole population, the actual dietary exposure to intense sweeteners for consumers was similar to that for consumers from the whole population (Bär & Biermann, 1992; Toledo & Ioshi, 1995; Food Standards Australia New Zealand, 2004).

Table 6. Dietary exposure estimates for people with diabetes and/or on weight control diets who consume cyclamates

Country (reference)	Survey type	Source of concentration data[a]	Population group	Dietary exposure to cyclamates (mg/kg bw per day)
Australia (Food Standards Australia New Zealand, 2004)	2003 intense sweetener survey, 263 respondents, 7-day diary, people with diabetes or glucose intolerance	TUL, matched by brand and flavour	Mean (12+ years, consumers)	3.6
			90th percentile (12+ years, consumers)	9.2
			95th percentile (12+ years, consumers)	11.9
Brazil (Toledo & Ioshi, 1995)	1990–1991 survey across two seasons, 673 consumers of intense sweeteners selected from two regions, 67% of these were consumers of products containing cyclamates	Analysis and labels	*Diabetics (35% of consumers of intense sweeteners)*	
			Mean	3.0
			Median	2.0
			Weight control (59% of consumers of intense sweeteners)	
			Median	1.5

Table 6 (contd)

Country (reference)	Survey type	Source of concentration data[a]	Population group	Dietary exposure to cyclamates (mg/kg bw per day)
Germany (Bär & Biermann, 1992)	1988–1989 survey, 24-h recall, 2291 respondents representative of population, 36% consumed intense sweeteners	TUL, labels, some analysis	*Diabetics (2% of population)*	
			Mean (consumers)	2.5
			90th percentile (consumers)	6.4
			Weight control (3% of population)	
			Mean (consumers)	3.1
			90th percentile (consumers)	6.4
New Zealand (Food Standards Australia New Zealand, 2004)	2003 intense sweetener survey, 137 respondents, 7-day diary, people with diabetes or glucose intolerance	TUL, matched by brand and flavour	Mean (12+ years, consumers)	2.1
			90th percentile (12+ years, consumers)	3.5
			95th percentile (12+ years, consumers)	8.8
Spain (Serra-Majem et al., 1996)	1992 survey, 2450 respondents aged 6–75 years, two 24-h recalls, food frequency, two different seasons	TUL	Mean (diabetics, 3% population)	1.56
Sweden (Ilbäck et al., 2003)	1999 survey, questionnaire, 790 respondents aged 0–90 years, diabetics only	MUL (400 mg/l drinks)	Worst case, diabetics only	5

MUL, maximum use level; TUL, typical manufacturers' use level.

[a] The typical manufacturers' use level is usually a mean value or matched to brand and flavour. Where products were analysed, mean values for product categories or values for individual products by brand and flavour were used.

5. PROPOSED CHANGES FOR CYCLAMATES IN THE CODEX GSFA

The CCFA in 2008 requested an evaluation by JECFA of the impact of introducing different maximum levels of use for cyclamates in Food Category 14.1.4, Water-based flavoured drinks, on dietary exposures to cyclamates. The different use levels to be considered were 250, 500, 750 and 1000 mg/kg.

In the one submission from Australia that modelled the impact of different maximum use levels for beverages covered by Food Category 14.1.4 on dietary exposure to cyclamates, three analyses were performed (Food Standards Australia New Zealand, 2009b):

1) The first assumed Codex GSFA maximum use levels in the low-joule version of all food categories including beverages covered by Food Category 14.1.4 (where identified) and in the whole food category where the version was not identified. Individual body weights were used in the calculations.
2) The second assumed that the above was true, except for beverages covered by Food Category 14.1.4, where it was assumed that cyclamates were added to all these beverages.
3) The third assumed typical use levels for cyclamates in Australia for the low-joule versions of all food categories and the proposed GSFA use levels for low-joule beverages covered by Food Category 14.1.4.

Dietary exposures were presented for the three analyses for the baseline (i.e. no cyclamates in beverages covered by Food Category 14.1.4) and maximum use levels of 250, 500, 750 and 1000 mg/kg, as well as for a level of 350 mg/kg, which is the current permitted level of use in Australia and New Zealand for this category of beverages. The same food consumption data derived from individual records from the 1995 Australian National Nutrition Survey were used for estimating dietary exposures to cyclamates for the three analyses. For the whole population aged 2 years and over who were consumers of beverages covered by Food Category 14.1.4, mean beverage consumption amounts ranged from 375 to 560 g/day, and 90th-percentile amounts ranged from 625 to 1040 g/day. For children aged 2–6 years who were consumers of beverages covered by Food Category 14.1.4, mean beverage consumption amounts ranged from 230 to 420 g/day, and 90th-percentile amounts ranged from 320 to 900 g/day.

Results for the first and second analyses are given in Table 7 and Table 8, respectively, for the Australian population. For two food categories in these models, “fruit juice and fruit nectars” and “fine bakery wares and mixes”, the Codex GSFA levels were assigned to the whole category, as the low-joule versions of these products were not identified in the 1995 Australian National Nutrition Survey.

Table 7. Estimated dietary exposure to cyclamates for consumers in Australia (Codex GSFA maximum use levels in all low-joule versions of food, including Food Category 14.1.4 beverages)[a]

a. Australia: 2 years and above (baseline, *n* = 10 898; scenarios, *n* = 11 167)

		Use level scenarios				
	Baseline (0 mg/kg)	250 mg/kg	350 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
Mean						
mg/day	139	151	157	166	182	197
mg/kg bw per day	3	3	3	3	3	3
Intake as % of ADI	25	25	25	25	30	30
Median						
mg/day	103	110	114	118	119	120
mg/kg bw per day	2	2	2	2	2	2
Intake as % of ADI	15	15	15	15	20	20
90th percentile						
mg/day	305	330	347	372	410	450
mg/kg bw per day	6	6	6	7	7	8
Intake as % of ADI	55	55	55	60	65	75

b. Australia: 2–6 years (baseline, *n* = 856; scenarios, *n* = 862)

		Use level scenarios				
	Baseline (0 mg/kg)	250 mg/kg	350 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
Mean						
mg/day	116	120	122	124	128	133
mg/kg bw per day	6	7	7	7	7	7
Intake as % of ADI	60	60	60	60	65	65
Median						
mg/day	87	93	94	96	98	99
mg/kg bw per day	5	5	5	5	5	5

b. Australia: 2–6 years (contd)

		Use level scenarios				
	Baseline (0 mg/kg)	250 mg/kg	350 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
Intake as % of ADI	45	45	45	45	45	45
90th percentile						
mg/day	249	252	258	265	272	293
mg/kg bw per day	13	14	14	14	15	16
Intake as % of ADI	120	120	120	130	140	150

[a] Consumers only: includes only those people who have consumed a food with GSFA permissions, based on 1995 National Nutrition Survey food consumption data. The ADI is 0–11 mg/kg bw (Annex 1, reference *59*).

Table 8. Estimated dietary exposure to cyclamates for consumers in Australia (Codex GSFA maximum use levels in all Food Category 14.1.4 beverages and in low-joule versions of other foods)[a]

a. Australia: 2 years and above (baseline, *n* = 10 898; scenarios, *n* = 12 105)

		Use level scenarios				
	Baseline (0 mg/kg)	250 mg/kg	350 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
Mean						
mg/day	139	181	204	238	295	351
mg/kg bw per day	3	3	4	4	5	6
Intake as % of ADI	25	30	35	40	45	55
Median						
mg/day	103	136	152	174	200	221
mg/kg bw per day	2	2	2	3	3	4
Intake as % of ADI	15	20	20	25	30	35
90th percentile						
mg/day	305	388	439	525	675	832
mg/kg bw per day	6	7	8	10	12	15
Intake as % of ADI	55	65	75	90	110	140

b. Australia: 2–6 years (baseline, *n* = 856; scenarios, *n* = 937)

		Use level scenarios				
	Baseline (0 mg/kg)	250 mg/kg	350 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
Mean						
mg/day	116	144	160	182	220	258
mg/kg bw per day	6	8	9	10	12	14
Intake as % of ADI	60	70	80	90	110	130
Median						
mg/day	87	115	131	151	181	204
mg/kg bw per day	5	6	7	8	10	11
Intake as % of ADI	45	55	65	75	90	100
90th percentile						
mg/day	249	302	330	380	467	566
mg/kg bw per day	13	15	17	20	24	29
Intake as % of ADI	120	140	150	180	220	270

[a] Consumers only: includes only those people who have consumed a food with GSFA permissions, based on 1995 National Nutrition Survey food consumption data. The ADI is 0–11 mg/kg bw (Annex 1, reference *59*).

5.1 Predicted dietary exposures assuming that the low-joule versions of beverages covered by Food Category 14.1.4 contain cyclamates (first analysis)

For consumers of products with Codex GSFA permissions for cyclamates, mean dietary exposures for consumers in the Australian population were 139 mg/day (3 mg/kg bw per day), assuming current GSFA permissions, and ranged from 151 to 197 mg/day (3 mg/kg bw per day) across the scenarios (Table 7). For high consumers, 90th-percentile dietary exposures to cyclamates were predicted to be 305 mg/day (6 mg/kg bw per day) at baseline and ranged from 330 to 450 mg/day (6–8 mg/kg bw per day) across the scenarios.

For children aged 2–6 years who were consumers of products assigned Codex GSFA permissions for cyclamate, mean dietary exposures were 116 mg/day (6 mg/kg bw per day), assuming current GSFA permissions, and ranged from 120 to 133 mg/day (6–7 mg/kg bw per day) across the scenarios (Table 7). For high consumers aged 2–6 years, 90th-percentile dietary exposures to cyclamates were predicted to be 249 mg/day (13 mg/kg bw per day) at baseline and ranged from 252 to 293 mg/day (14–16 mg/kg bw per day) across the scenarios.

5.2 Predicted dietary exposures assuming that all beverages covered by Food Category 14.1.4 contain cyclamates (second analysis)

For consumers of products with Codex GSFA permissions for cyclamates, mean dietary exposures for consumers in the Australian population were 139 mg/day (3 mg/kg bw per day), assuming current GSFA maximum use levels, and ranged from 181 to 351 mg/day (3–6 mg/kg bw per day) across the scenarios (Table 8). For high consumers, 90th-percentile dietary exposures to cyclamates were predicted to be 305 mg/day (6 mg/kg bw per day) at baseline and ranged from 388 to 832 mg/day (7–15 mg/kg bw per day) across the scenarios.

For children aged 2–6 years who were consumers of products assigned Codex GSFA permissions for cyclamate, mean dietary exposures were 116 mg/day (6 mg/kg bw per day), assuming current GSFA permissions, and ranged from 144 to 258 mg/day (8–14 mg/kg bw per day) across the scenarios (Table 8). For high consumers aged 2–6 years, 90th-percentile dietary exposures to cyclamates were predicted to be 249 mg/day (13 mg/kg bw per day) at baseline and ranged from 302 to 566 mg/day (15–29 mg/kg bw per day) across the scenarios.

5.3 Predicted dietary exposures assuming typical use levels for cyclamates in Australia and Codex GSFA proposed levels in beverages covered by Food Category 14.1.4 (third analysis)

As the 1995 and 2003 Australian intense sweetener surveys did not include younger children, an estimate of potential dietary exposure to cyclamates for children aged 2–11 years was later undertaken, assigning typical use levels for low-joule products only, except for beverages covered by Food Category 14.1.4, for which a maximum use level of 350 mg/kg was used for the low-joule version. Beverages covered by Food Category 14.1.4 contributed 70–90% of total dietary exposure to cyclamates. Estimated mean dietary exposures ranged from 3.6 to 4.1 mg/kg bw per day for this age group, and 90th-percentile exposures ranged from 8.1 to 8.9 mg/kg bw per day (Table 5; Food Standards Australia New Zealand, 2007).

6. ASSESSMENT OF DIETARY EXPOSURE

The Committee noted two important considerations in the analysis of the impact of the variable maximum use levels for cyclamates in beverages covered by Food Category 14.1.4. First, the current uses of cyclamates in beverages (although not in the Codex GSFA, many countries allow the use of cyclamates in the beverages covered by Food Category 14.1.4) are at or near their maximum levels. This may be a result of their relatively low intensity of perceived sweetness (30 times that of sucrose) compared with other intense sweeteners (200 times that of sucrose for aspartame and 600 times that of sucrose for sucralose). It is noted that maximum use levels for cyclamates are not sufficient to allow complete sugar replacement in beverages covered by Food Category 14.1.4 and that it is necessary to use other

intense sweeteners in conjunction with cyclamates to achieve the desired sweetness profile. Second, in countries where regulated cyclamate levels in beverages covered by Food Category 14.1.4 have been reduced in the past decade, published analyses have shown that overall dietary exposure has decreased. For example, dietary exposures to cyclamates decreased in Denmark following a change in EU legislation in 2004 that reduced the maximum use level for beverages covered by Food Category 14.1.4 from 400 to 250 mg/kg; dietary exposures to cyclamates also decreased in Australia when the maximum use level for these beverages was reduced from 1200 to 600 mg/kg in 1999 and further lowered to 350 mg/kg in 2007.

6.1 Assessment of published dietary exposure estimates for cyclamates

Published dietary exposure analyses for cyclamates were available for several countries, as reported in Tables 4, 5 and 6 for the whole population, consumers only and special groups, respectively. In all these studies, the beverages covered by Food Category 14.1.4 had provisions for cyclamates.

The evaluation and comparison of dietary exposures to cyclamates reported in different studies are difficult, as many factors need to be considered, and results are often not directly comparable (Renwick, 2006). Potential dietary exposures are directly influenced by the maximum use levels in legislation at the time of the study and the number and type of food categories for which permissions to add cyclamates are given. The reason for this is that typical use levels for cyclamates as an intense sweetener to replace sugar in products tend to be close to maximum use levels, owing to the low sucrose equivalence of cyclamates compared with other intense sweeteners (30 times sucrose for cyclamates, 200 times sucrose for aspartame, 600 times sucrose for sucralose). In addition, the definition of respondents and consumers in each study must be taken into account. Some studies are representative of the whole population and report dietary exposures for all respondents as well as for consumers of products containing cyclamates. Other studies have specifically recruited consumers of products containing intense sweeteners and may report dietary exposures to different sweeteners for that group and/or for consumers of specific sweeteners in the group. It would, of course, be expected that studies that recruited users of products containing intense sweeteners would tend to report higher dietary exposures to cyclamates.

In general, reported mean and high consumer dietary exposures to cyclamates were below the ADI of 0–11 mg/kg bw, although for some younger age groups, consumers at the 90th or higher percentiles of dietary exposure had exposures close to or in exceedance of the ADI. Earlier studies tended to report higher dietary exposures to cyclamates compared with those reported in more recent studies, probably reflecting the increased use of alternative intense sweeteners in low-joule products over time (Renwick, 2006, 2008) and the lowering of provisions for cyclamates in each food supply as a result of legislative changes. Although some studies reported that people with diabetes or on weight control diets had higher dietary exposures to cyclamates than did other consumers (Serra-Majem et al., 1996), other studies reported that there were no differences in actual dietary exposures compared with those of other consumers but that a higher

proportion of these groups were consumers of products containing intense sweeteners (Bär & Biermann, 1992; Toledo & Ioshi, 1995; Food Standards Australia New Zealand, 2004). In two studies of Italian teenagers, the tendency for consumers to be loyal to one brand and the size of the market share for beverages covered by Food Category 14.1.4 that contain cyclamates were shown to influence dietary exposure, indicating a greater probability of higher dietary exposures for those consumers loyal to a given brand (Leclercq et al., 1999; Arcella et al., 2003, 2004).

In countries where there is wide use of cyclamates across a number of food categories, major contributors to dietary exposure tend to be beverages covered by Food Category 14.1.4 for the younger age groups and tabletop sweeteners for the older age groups (Leclercq et al., 1999; Food Standards Australia New Zealand, 2004), with females tending to consume more low-joule products than males of a similar age (Leclercq et al., 1999; Arcella et al., 2004; Food Standards Australia New Zealand, 2004). However, there are some exceptions, with Ilbäck et al. (2003) reporting a surprising number of young children using tabletop sweeteners in Sweden, noting that these products tended to be based on cyclamates. Dietary exposure also varied with season for some age groups, as beverages covered by Food Category 14.1.4 tend to be consumed in higher amounts in warmer months (Bär & Biermann, 1992).

6.2 Assessment of dietary exposure to cyclamates following legislative changes

In some cases, evidence of dietary exposure to cyclamates exceeding the ADI for specific population subgroups has been used to support legislative changes to lower maximum use levels for cyclamates in beverages covered by Food Category 14.1.4 (Leclercq et al., 1999; Arcella et al., 2003, 2004; Food Standards Agency, 2003a; Food Standards Australia New Zealand, 2007; Leth et al., 2007, 2008). It was noted that the current maximum use levels for beverages covered by Food Category 14.1.4 in the EU of 250 mg/l and in Australia and New Zealand of 350 mg/kg are too low for cyclamates to be used alone to replace sugar in a given product, and likely too low for a cyclamate/saccharin mix to be used without the addition of other intense sweeteners.

The studies by Leth et al. (2007, 2008) indicated a decrease in dietary exposure to cyclamates from consumption of beverages covered by Food Category 14.1.4 in the Danish population and a reduction in the number of young children likely to exceed the ADI of 0–7 mg/kg bw established in the EU following the implementation of the amendment to EU legislation in 2004. This amendment lowered the maximum use level for cyclamates in beverages covered by Food Category 14.1.4 from 400 to 250 mg/l (Leth et al., 2007, 2008). A similar decrease in reported dietary exposure to cyclamates and the proportion of the population exceeding the ADI was seen for the Australian population (aged 12–39 years) between 1994 and 2003 following a lowering of the maximum use level for cyclamates in beverages covered by Food Category 14.1.4 from 1200 to 600 mg/kg in 2000 (Food Standards Australia New Zealand, 2004). A further lowering of the maximum use level for cyclamates in beverages covered by Food Category 14.1.4 was implemented in 2007 (applies to Australia and New Zealand), with maximum

use levels changing from 600 to 350 mg/kg, owing to continuing concerns that some high consumers in younger age groups (2–17 years) had the potential to exceed the ADI of 0–11 mg/kg bw when the maximum use level for cyclamates in beverages covered by Food Category 14.1.4 was 600 mg/kg, as reported in the 2003 intense sweetener survey for Australia and New Zealand (Food Standards Australia New Zealand, 2004, 2007).

6.3 Assessment of predicted dietary exposure to cyclamates following proposed changes to Codex GSFA use levels for cyclamates in beverages covered by Food Category 14.1.4

The Committee received a submission from Australia containing an analysis of the impact of various maximum use levels for cyclamates in beverages covered by Food Category 14.1.4 on overall dietary exposure to cyclamates.

For the first analysis, for the whole population aged 2 years and over, maximum Codex GSFA use levels were assumed in all low-joule versions of foods, including beverages in Food Category 14.1.4. Mean and 90th-percentile dietary exposures for consumers of products containing cyclamates were below the ADI of 0–11 mg/kg bw (25–30% of the ADI for mean consumers, 55–75% of the ADI for 90th-percentile consumers). For children aged 2–6 years, mean dietary exposures to cyclamates for consumers were also below the ADI (60–65% of the ADI). However, in this age group, dietary exposures to cyclamates exceeded the ADI at the 90th percentile of exposure at baseline and for all optional levels of cyclamates in beverages covered by Food Category 14.1.4 (120–150% of the ADI). The higher dietary exposures expressed per kilogram body weight for young children compared with adults are to be expected, owing to relatively higher levels of consumption of food per kilogram body weight; additionally, members of this age group are relatively high consumers of fine bakery wares, juices and juice nectars, which were all assumed to contain cyclamate, as the low-joule version could not be identified. In reality, very few of these products do contain the sweetener. Hence, the predicted dietary exposures to cyclamates based on GSFA levels were considered by the Committee to be overestimates.

In many populations, the proportion of people consuming beverages covered by Food Category 14.1.4 that contain high-intensity sweeteners is increasing compared with those consuming sugar-sweetened drinks. As the Australian food consumption data were collected in 1995, patterns of consumption of these drinks are likely to be out of date. For this reason, the second analysis was a more conservative dietary exposure analysis for the Australian population, assuming that cyclamates were added to all beverages covered by Food Category 14.1.4. In this analysis, for the whole population aged 2 years and over, mean dietary exposures for consumers remained below the ADI at all maximum use levels for beverages covered by Food Category 14.1.4 (30–55% of the ADI), but dietary exposures for 90th-percentile consumers exceeded the ADI at the 750 and 1000 mg/kg maximum use levels. For children aged 2–6 years, predicted mean dietary exposures to cyclamates for consumers were 70–130% of the ADI; exposures were below the ADI at use levels for cyclamates up to 500 mg/kg in beverages covered by Food Category 14.1.4, but exceeded the ADI at higher use levels. In this age group,

exposures of 90th-percentile consumers exceeded the ADI for cyclamates at all use levels (140–270% of the ADI).

In the third, more realistic analysis of dietary exposure assessment for cyclamates for 2- to 11-year-old children using typical use levels for cyclamates in low-joule products in the Australian analysis, two food categories ("fine bakery wares and mixes" and "juices and fruit nectars") were excluded, as they do not contain cyclamates in Australia. Beverages covered by Food Category 14.1.4 contributed 70–90% of total dietary exposure to cyclamates. In this analysis, 90th-percentile consumers in this age group were predicted to have dietary exposures to cyclamates that exceeded the ADI only at maximum use levels for cyclamates of 400 mg/kg and above in beverages covered by Food Category 14.1.4; at 350 mg/kg, dietary exposures were less than the ADI.

7. EVALUATION

Potential dietary exposures to cyclamates are directly influenced by the maximum use levels in legislation, the number and type of food categories for which provisions to add cyclamates are given, as well as food consumption patterns. The reason for this is that typical use levels for cyclamates as an intense sweetener to replace sugar in products tend to be close to maximum use levels, because of the low sucrose equivalence of cyclamates compared with other intense sweeteners. As beverages covered by Food Category 14.1.4 are major contributors to dietary exposure to intense sweeteners, the concentration of cyclamates in these products can considerably influence total dietary exposures.

Most reported mean dietary exposures to cyclamates were below the ADI of 0–11 mg/kg bw; however, several studies reported population subgroups for which exposures for high consumers approached or exceeded the ADI when cyclamate levels in beverages covered by Food Category 14.1.4 were 400 mg/kg or over, particularly for children and in one study for people with diabetes. Theoretical models for the Australian population indicated that maximum use levels for cyclamates of 500 mg/kg and under in all foods with Codex GSFA provisions and in beverages in Food Category 14.1.4 would be protective of all populations, except for young children who were high consumers. However, these estimates were likely to overestimate dietary exposure, as it was assumed that all low-joule soft drinks contained cyclamates and that all fruit juices, juice nectars and fine bakery wares contained cyclamates, which in reality would not be the case. A more accurate estimate for the Australian population using typical use levels for cyclamates indicated that maximum use levels for cyclamates of 400 mg/kg and above in beverages covered by Food Category 14.1.4 would result in dietary exposures to cyclamates that exceeded the ADI of 0–11 mg/kg bw for children up to 11 years of age who were high consumers.

7.1 Conclusion

Of the four maximum use levels (250, 500, 750 and 1000 mg/kg) that the Committee considered at the request of CCFA for cyclamates in beverages covered by Codex GSFA Food Category 14.1.4, only the lowest level of 250 mg/kg was not likely to lead to dietary exposures exceeding the ADI for high consumers, including children. Moreover, it was noted that a maximum use level of 350 mg/kg also resulted in dietary exposures for high consumers, including children, that were less than the ADI.

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FERROUS AMMONIUM PHOSPHATE

First draft prepared by

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1. EXPLANATION

At the present meeting, the Committee evaluated the safety of and established specifications for ferrous ammonium phosphate ($FeNH_4PO_4$; Figure 1) for use in food fortification, at the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008). The Committee had not previously evaluated ferrous ammonium phosphate. The Committee had, however, at its ninth and twenty-third meetings, evaluated a large number of food acids and salts and was of the opinion that acceptable daily intakes (ADIs) for ionizable salts should be based on previously accepted recommendations for the constituent cations and anions (Annex 1, references *11* and *50*).

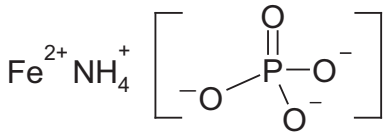

Figure 1. Chemical structure of ferrous ammonium phosphate

Ferrous ammonium phosphate consists of iron(II), ammonium and phosphate ions in a 1:1:1 molar ratio, with the iron content ranging between 24% and 30%. Ferrous ammonium phosphate is intended for use as an alternative to currently permitted iron fortification compounds. Ferrous ammonium phosphate is stable in foods but readily dissociates to iron(II), ammonium and phosphate ions when subject to the low pH conditions of the stomach.

Iron was evaluated at the twenty-seventh meeting (Annex 1, reference *62*) and assigned a group provisional maximum tolerable daily intake (PMTDI) of 0.8 mg/kg body weight (bw), which applies to iron from all sources except for iron oxides used as colouring agents, supplemental iron taken during pregnancy and lactation, and supplemental iron for specific clinical requirements. The sodium iron salt of ethylenediaminetetraacetate (EDTA) was evaluated by the Committee at its forty-first and fifty-third meetings (Annex 1, references *107* and *144*). At the forty-first meeting, the Committee provisionally concluded that sodium iron EDTA that met the tentative specifications prepared at the meeting would not present a safety concern when used in supervised food fortification programmes in iron-deficient populations. The Committee emphasized that its evaluation pertained only to the use of sodium iron EDTA as a dietary supplement to be used under supervision. The Committee requested the results of additional studies to assess the site of deposition of iron administered in this form and to assess the metabolic fate of sodium iron EDTA after long-term administration. Several studies were submitted at the fifty-third meeting, and the Committee considered that the data satisfied its concerns about the use of sodium iron EDTA in food fortification programmes. At that evaluation, the Committee concluded that sodium iron EDTA could be considered safe for use in supervised food fortification programmes when public health officials had determined the need for iron supplementation of the diet of a population. Such programmes would provide daily iron intakes of approximately 0.2 mg/kg bw. At the sixty-first meeting (Annex 1, reference *167*), the Committee evaluated the safety of ferrous glycinate (processed with citric acid) as a source of iron for dietary supplementation. The Committee concluded that ferrous glycinate was suitable for use as a source of iron for supplementation and fortification, provided that total intake of iron does not exceed the PMTDI of 0.8 mg/kg bw. Additionally, the metabolic fate and toxicity of iron compounds have been reviewed by a number of other scientific bodies, including the International Nutritional Anemia Consultative Group (1993), the Institute of Medicine (2001), the Expert Group on Vitamins and Minerals (2003) and the World Health Organization (2006).

Phosphoric acid and phosphate salts were evaluated by the Committee at its sixth, seventh, eighth, ninth, thirteenth, fourteenth, seventeenth and twenty-sixth

meetings (Annex 1, references *6*, *7*, *8*, *11*, *19*, *22*, *32* and *59*). A group maximum tolerable daily intake (MTDI) of 70 mg/kg bw, expressed as phosphorus, was established at the twenty-sixth meeting and applies to the sum of phosphates present naturally in food and those present as additives. In addition, the Institute of Medicine (1997) and the Expert Group on Vitamins and Minerals (2003) have previously reviewed the metabolic fate and toxicity of phosphorus (as phosphate).

The Committee has also previously evaluated ammonium salts. At its twenty-sixth meeting (Annex 1, reference *59*), the Committee evaluated the safety of ammonium carbonate and ammonium hydrogen carbonate and allocated an ADI "not specified", while noting that although toxicological data for these ammonium salts were limited, extrapolation of results from studies with ammonium compounds (primarily ammonium chloride) and with sodium or potassium carbonate provided a basis for evaluation. At its twenty-ninth meeting (Annex 1, reference *70*), the Committee prepared a table giving the ADIs for a large number of combinations of cations and anions, including ammonium salts. No restriction was placed on the intake of ammonium from ammonium salts, provided that the contribution made to food is assessed and considered acceptable. Ammonia has also previously been evaluated by the International Programme on Chemical Safety (1986), which concluded that ammonia does not present a direct threat to humans except as a result of accidental exposure, particularly in industry. The World Health Organization (2006) has also previously assessed the safety of ammonia in its Guidelines for Drinking-water Quality and concluded that ammonia is not of direct importance for health in the concentrations expected in drinking-water; therefore, a health-based guideline was not derived.

1.1 Chemical and technical considerations

Ferrous ammonium phosphate is manufactured by mixing phosphoric acid, iron powder and ammonium hydroxide. Iron powder and phosphoric acid are combined in demineralized water with stirring to form a suspension. The mixture is heated until no further gas is evolved. Ammonia solution is added to the resultant slurry to yield ferrous ammonium phosphate. The product is then spray dried and milled to obtain a greyish-green fine powder.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

The biochemistry of each of the constituent ions of ferrous ammonium phosphate is briefly discussed below, as ferrous ammonium phosphate dissociates to iron, ammonium and phosphate ions in the stomach following oral consumption. The in vitro data indicate that ferrous ammonium phosphate is highly soluble and readily releases free iron under conditions that mimic those of the stomach (Nestlé Research and Development, 2000).

The solubility of ferrous ammonium phosphate was assessed in an in vitro solubility study. In a standard hydrochloric acid dissolution test, ferrous ammonium

phosphate in an amount providing 20 mg of iron was added to 250 ml of diluted hydrochloric acid (pH 1.93) at 37 °C. By 3 h, 67% of the ferrous ammonium phosphate had dissolved. In comparison, 100% of ferrous sulfate, the reference compound, was dissolved under the same conditions (Nestlé Research Center, 2007a).

The amount of iron released from ferrous ammonium phosphate was studied in vitro. Solutions were prepared in deionized water at 37 °C containing 100 mg ferrous ammonium phosphate/l. The pH was adjusted to 2.0 or 3.5 using hydrochloric acid to mimic the conditions of infant and adult stomachs, respectively. At 15 min of incubation, 95% of the total iron from ferrous ammonium phosphate was released as free iron; a maximum release of 97% occurred at 30 min (Nestlé Research and Development, 2000).

2.1.1 Absorption, distribution and excretion

(a) Iron from organic and inorganic sources

Iron is an essential element required nutritionally in order to maintain a variety of physiological functions. Dietary iron exists in two forms: haem (organic) iron and non-haem (inorganic) iron. Haem iron in the diet is predominantly derived from haemoglobin and myoglobin found in meat, poultry and fish, whereas non-haem iron is derived from iron salts contained in plant and dairy products as well as from foods fortified with iron salts. Iron salts can occur naturally (e.g. iron oxalate, iron citrate, iron phytate) and as supplements (e.g. iron EDTA, iron gluconate, iron sulfate). The absorption of iron in general is dependent on the physiological requirement for iron, which is largely dependent on the status of tissue iron stores as well as on the rate of erythropoiesis. The absorption of iron is also dependent on the source of iron (haem or non-haem iron), the iron content of the diet and other dietary ingredients. The amount of iron absorbed in normal subjects is subject to mucosal regulation, so that excessive iron is not stored in the body, unless there is considerable dietary overload, as can occur in certain disease states such as haemochromatosis and anaemia, which results in a breakdown in the normal control of iron absorption and distribution. The result of such iron storage disorders is a massive increase in body burden of iron due to an increased rate of iron absorption (Annex 1, reference *63*; International Nutritional Anemia Consultative Group, 1993; Institute of Medicine, 2001; Expert Group on Vitamins and Minerals, 2003).

Dietary iron is composed of 10% haem iron and 90% non-haem iron. Haem iron is highly bioavailable and is absorbed approximately 2- to 3-fold more readily than non-haem iron. Unlike non-haem iron, the absorption of haem iron is not greatly affected by other dietary constituents. Haem iron is absorbed into intestinal mucosal cells as the intact porphyrin complex, which occurs via a specific haem receptor, and is subsequently rapidly released from the complex by the enzymatic activity of haem oxygenase. In contrast, the absorption of non-haem iron requires prior solubilization of the iron salts in the upper gastrointestinal tract. Ferrous salts (chloride, fumarate, gluconate, glycerophosphate, succinate, sulfate) are more readily absorbed than ferric salts. Succinate and sulfate salts are the most common and most easily absorbed. The absorption of non-haem iron is also dependent on

other dietary constituents. Such constituents can either facilitate (e.g. ascorbic acid) or inhibit (e.g. polyphenols, phytic acid) the absorption of iron. The mechanism of absorption of non-haem iron into intestinal mucosal cells is not clearly established, but may involve the divalent metal transporting protein (Annex 1, reference *63*; International Nutritional Anemia Consultative Group, 1993; Institute of Medicine, 2001; Expert Group on Vitamins and Minerals, 2003). Carbonyl iron is a pure (>98%) form of elemental iron, in the zero valence state. When ingested, carbonyl iron requires gastric acid to convert it to the soluble ferrous (Fe^{2+}) iron for absorption (Gordeuk et al., 1986; Huebers et al., 1986). Because of the slow rate of solubilization, carbonyl iron is absorbed much more gradually than other types of iron. Carbonyl iron is used as a dietary supplement to treat iron deficiency.

Following absorption into the intestinal mucosal cells, intracellular iron (with no distinction between haem and non-haem sources) is released into the bloodstream, where it is oxidized into the ferric (Fe^{3+}) form and reversibly bound to the iron transport protein transferrin. Iron is then distributed throughout the body and is taken up by cells through the binding of transferrin to the transferrin receptor present on cell membranes, with subsequent internalization. Iron is incorporated into several different types of proteins for utilization in a variety of biological functions. Proteins that require iron for functionality include haemoglobin and myoglobin for the transfer of oxygen from the lungs to tissues, cytochromes within the mitochondrial electron transport chain for cellular energy production and cytochrome P450 enzymes for the metabolism of endogenous and exogenous compounds. Excess iron that is not utilized is stored in the liver, bone marrow and reticuloendothelial cells in the form of the iron storage proteins, ferritin and haemosiderin (Annex 1, reference *63*; International Nutritional Anemia Consultative Group, 1993; Institute of Medicine, 2001; Expert Group on Vitamins and Minerals, 2003). In weanling male CD-1 mice fed diets containing up to 8000 mg iron/kg for 7 days, the liver and spleen were the primary storage organs for excess iron absorbed from the diet. Iron supplementation increased liver weight ($P < 0.01$), the iron deposition index of the liver ($P < 0.01$) and the concentration of iron in the liver ($P < 0.001$), all in a dose-dependent manner. The concentration of iron in the liver was positively correlated with dietary iron levels ($r = 0.989$). Histological examination revealed the accumulation of iron in the hepatocytes, Kupffer cells and splenic macrophages, with the accumulation dependent on dietary levels of iron. This study is described in further detail in section 2.2.2(a) (Omara et al., 1993).

The excretion of iron is largely dependent on the production and degradation of erythrocytes, as most iron in the body is contained within erythrocytes. However, virtually all of the iron from erythrocytes is recycled for incorporation into haemoglobin. Thus, only a small amount of iron is excreted daily, with the exception of circumstances involving large amounts of blood loss. Specifically, daily basal iron losses are reported to be 0.2 mg in infants, 0.5 mg in children, 1.0 mg in men, 0.64 mg in non-menstruating women and 1.3 mg in menstruating women. Excretion of iron occurs predominantly via faeces, although trace amounts of iron are also excreted via urine, desquamated gastrointestinal cells and bile (Annex 1, reference *63*; International Nutritional Anemia Consultative Group, 1993; Institute of Medicine, 2001; Expert Group on Vitamins and Minerals, 2003).

(b) Ammonium from ammonium salts

Ammonium ions (NH_4^+) form upon dissolution of ammonia (NH_3) in water, and the two forms exist in equilibrium. Within the human body, approximately 4 g of ammonia is produced within the intestinal tract per day through the biological degradation of nitrogenous matter (International Programme on Chemical Safety, 1986). Almost all of the ammonia produced is absorbed from the gastrointestinal tract, with the ileal segment of the intestine identified as the major site of absorption (Health Canada, 1987). Absorption of free ammonia from the intestine is reported to occur rapidly, whereas absorption of the ammonium ion occurs more slowly as a result of the lower permeability of the cell membrane to ammonium ions (Milne et al., 1958; Vitti et al., 1964). Once absorbed, ammonia is transported to the liver for incorporation into urea as part of the urea cycle; thus, very little of the absorbed ammonia reaches the systemic circulation (International Programme on Chemical Safety, 1986; World Health Organization, 2003). As distribution is pH dependent, ammonia diffuses more freely than do ammonium ions (International Programme on Chemical Safety, 1986). Ammonia is present in all tissues and constitutes a metabolic pool from which it is taken up by glutamic acid and utilized in various transamination reactions. Ammonia in the liver is utilized in protein synthesis. Ammonia is excreted in the urine in the form of urea or as free ammonia to aid in the secretion of hydrogen ions for the purpose of maintaining pH balance (International Programme on Chemical Safety, 1986; Health Canada, 1987).

(c) Phosphate from phosphate salts

Phosphate is an essential nutrient that plays a critical role in skeletal development, mineral metabolism, nucleic acid synthesis and diverse cellular functions involving intermediary metabolism and energy transfer mechanisms. Dietary sources of phosphates include foods, such as meat, dairy products, eggs and legumes, containing naturally occurring phosphorus, as well as inorganic phosphate salts that are used in foods and drinks as additives. It is the inorganic form that is predominantly absorbed as a result of the presence of inorganic phosphate in foods or as liberated from organic phosphorus–containing compounds. Phosphate is rapidly absorbed mainly from the duodenum and jejunum, with smaller amounts absorbed by the ileum, colon and stomach. Phosphate enters enterocytes by active transport and is effluxed via the basolateral membrane into the circulation (Nordin, 1988; Institute of Medicine, 1997; Expert Group on Vitamins and Minerals, 2003).

Phosphorus is a pentavalent element that generally occurs in the body as the inorganic phosphate ion (PO_4^{3-}), but it can also occur as pyrophosphate ($P_2O_7^{4-}$) or as the organic form ($R\text{-}PO_4^{2-}$). Approximately 85% of the body's phosphorus is present in bone and 14% in soft tissues, with the remainder distributed in teeth, blood and extravascular fluid. About 70–75% of the phosphorus in the blood is present in phospholipids, whereas 25–30% occurs as free (80–85%) or protein-bound (15–20%) inorganic phosphate. The inorganic phosphate fraction in serum varies throughout the day according to the circadian rhythm and is under the endocrine control of parathyroid hormone and 1,25-dihydroxyvitamin D, the activated form of vitamin D. An increase in circulating phosphate concentrations increases the secretion of parathyroid hormone from the parathyroid glands.

Parathyroid hormone, in turn, acts to stabilize circulating phosphate levels by inhibiting tubular reabsorption of phosphates by the kidney, thus increasing urinary excretion of phosphates. Parathyroid hormone also acts on the bone matrix by bringing about demineralization of bone tissue through activation of osteoclasts, resulting in an increase in calcium release into the circulation. Conversely, 1,25-dihydroxyvitamin D levels are decreased in response to high serum phosphate levels. As 1,25-dihydroxyvitamin D acts to stimulate calcium and phosphate absorption from the small intestine as well as calcium reabsorption from the proximal tubule, a decrease in 1,25-dihydroxyvitamin D levels reduces calcium and phosphate absorption and also reduces calcium reabsorption (Nordin, 1988; Institute of Medicine, 1997; Expert Group on Vitamins and Minerals, 2003).

The amount of parathyroid hormone that enters the circulation is regulated by the calcium level in the blood. As a result, high dietary phosphorus intakes, combined with low calcium intakes, can increase the secretion of parathyroid hormone, thus decreasing serum phosphate levels. Phosphate ions are filtered by the glomeruli in the kidney, and the majority (80–90%) are reabsorbed in the proximal renal tubules, with the remainder excreted in the urine (Nordin, 1988; Institute of Medicine, 1997; Expert Group on Vitamins and Minerals, 2003).

2.1.2 Bioavailability

(a) Bioavailability of iron from ferrous ammonium phosphate

The bioavailability of iron from ferrous ammonium phosphate, ferrous sulfate and ferric pyrophosphate was assessed in two groups of 19 healthy women between the ages of 18 and 30 years in a randomized, double-blind crossover trial. Subjects were given test meals consisting of 250 ml of reconstituted full cream instant milk powder supplemented with [^{57}Fe]ferrous ammonium phosphate, [^{58}Fe]ferrous sulfate or [^{57}Fe]ferric pyrophosphate at a fortification level of 2.5 mg of iron per serving. In the first trial, subjects consumed two servings of either ferrous ammonium phosphate or ferrous sulfate test meals on day 1 and then two servings of the other test meal on day 2. In the second trial, subjects consumed two servings of either ferric pyrophosphate or ferrous sulfate test meals on day 1 and then two servings of the other test meal on day 2. Baseline measurements for haemoglobin, serum ferritin and C-reactive protein were taken from blood on day 1 before test meal administration to determine iron status. Iron absorption was determined in fasting subjects 14 days after test meal intake using a double stable isotope technique assessing erythrocyte incorporation of isotopic labels. The geometric means of fractional iron absorption and 95% confidence intervals [CI] from ferrous sulfate, ferrous ammonium phosphate and ferric pyrophosphate were 10.4% (95% CI = 7.8–13.8), 7.4% (95% CI = 5.5–10) and 3.3% (95% CI = 2.4–4.5), respectively, equivalent to 0.52, 0.37 and 0.17 mg of iron, respectively. The amount of iron absorbed from the ferrous ammonium phosphate–fortified meal was significantly less (P = 0.0002) than that from the meal fortified with ferrous sulfate, yet significantly greater (P < 0.0001) than that from the meal fortified with ferric pyrophosphate. Eight subjects experienced symptoms of the common cold during the test period, including runny nose, blocked nose, cough and sore throat. Iron

absorption was not affected in these subjects. Baseline ferritin concentrations had a statistically significant (P = 0.0007) inverse impact on iron absorption; in other words, an increase of baseline plasma ferritin by 20 ng/ml led to a 5.3% decrease of iron absorption. The authors noted that the absorption and detection of radiolabelled iron in plasma following ingestion of ferrous ammonium phosphate indicated that the iron compound dissociates in the gut, releasing free iron (Nestlé Research Center, 2007a,b).

(b) Bioavailability of iron from ferrous sulfate

The absorption and bioavailability of iron from a wide variety of food types fortified with ferrous sulfate have been extensively studied in humans. Ferric salts are typically less soluble, and therefore iron from these iron formulations is less readily absorbed than iron from ferrous salts. Iron absorption values from ferrous sulfate–fortified foods vary widely (from <1% to 26%) (Ashworth & March, 1973; Rios et al., 1975; Martínez-Torres et al., 1979, 1991; MacPhail et al., 1981; Stekel et al., 1986; Fomon et al., 1989; Davidsson et al., 1997, 1998, 2001, 2002, 2005; Hurrell et al., 2000; Layrisse et al., 2000; Mendoza et al., 2001, 2004; Fidler et al., 2003; Pérez-Expósito et al., 2005). These iron absorption values are consistent across all age groups (infants, children, adolescents and adults). Iron absorption from ferrous sulfate–fortified foods is very sensitive to absorption-inhibiting substances present in certain food vehicles. Higher iron absorption values are frequently the result of concomitant ascorbic acid supplementation of the food vehicle, whereas very low values are sometimes observed in foods with high polyphenol and/or phytic acid content. The iron absorption value of 10.4% from a ferrous sulfate–fortified milk product described above in section 2.1.2 is consistent with the high-end range reported in other studies (Nestlé Research Center, 2007a,b). Based on this absorption measurement as well as on the in vitro solubility data described above, it can be expected that the absorption of iron from ferrous ammonium phosphate–fortified cereal-based foods and milk-based products will be slightly less than that reported for ferrous sulfate–fortified foods.

2.1.3 Biotransformation

Under acidic conditions, such as those in the human stomach, ferrous ammonium phosphate dissociates to its constituents, iron(II), ammonium and phosphate ions. As discussed in section 2.1.1, the iron, ammonium and phosphate ions are metabolized via normal metabolic processes.

2.1.4 Effects of high-dose iron overload

At high doses such as in iron overload, iron can exert a range of adverse effects by facilitating oxidative reactions or by interacting with other transition metals. As iron, zinc and copper have similar physicochemical characteristics and share chemically similar absorption and transport mechanisms, they are thought to compete for uptake and transport pathways. Consequently, iron supplementation can impair the uptake and use of zinc and copper by the body (Sandstrom, 2001). Studies in rats have shown that iron supplementation impairs the absorption of zinc, and this has raised concerns that iron supplements may have adverse effects on

zinc nutrition in humans. However, the magnitude of such an effect is less apparent in human studies than in rat studies (Expert Group on Vitamins and Minerals, 2003).

Iron overload can result in increased lipid peroxidation at the cellular level, with consequent membrane damage to mitochondria, microsomes and other cellular organelles. The ability of iron to gain or lose single electrons makes it an efficient catalyst for free radical reactions, and both haem and iron ions can stimulate lipid peroxidation. Free radical damage is produced by the hydroxyl radical, which is generated in vivo by an iron-dependent reduction of hydrogen peroxide. Iron-mediated lipid peroxidation and its role in disease have been reviewed by Britton et al. (1987), Minotti (1993) and Cheng & Li (2007).

2.2 Toxicological studies

The toxicological data related specifically to ferrous ammonium phosphate are limited. The Committee received a submission including new toxicological data on iron, phosphate and ammonium salts that have become available subsequent to the latest evaluations conducted by the Committee, as well as older studies not previously reviewed by the Committee. The safety of ferrous ammonium sulfate was discussed primarily in terms of the iron component. However, the latest toxicological information on ammonium and phosphate salts was compared with the results of previously evaluated studies in order to identify whether the new information would indicate a need to revise the Committee's previous opinions on these components.

2.2.1 Acute toxicity

(a) Iron

The results of acute oral toxicity studies conducted on iron in the form of ferrous sulfate and carbonyl iron, published since the review by the Committee at its twenty-seventh meeting (Annex 1, reference *62*) or not previously evaluated by the Committee, are summarized in Table 1. Consistent with the previously reported median lethal doses (LD_{50} values), the oral LD_{50} of iron derived from ferrous sulfate was approximately 250 mg/kg bw in mice, 300–1100 mg/kg bw in rats, 200 mg/kg bw in rabbits and greater than 200 mg/kg bw in dogs; and the oral LD_{50} of elemental iron (carbonyl iron) in rats was greater than 50 000 mg/kg bw. Adverse effects following acute exposure to ferrous sulfate as reported in the studies summarized in Table 1 largely consisted of diarrhoea, the intensity of which increased with the dose, as well as mucosal lesions and distension of the stomach and proximal segment of the small intestine and mortality.

Groups of 20 prepubertal (3 weeks old) and adult Wistar rats of each sex were administered a single dose of 750 mg iron/kg bw in the form of ferrous sulfate or distilled water by gavage. A group of 40 female and 30 male pubertal rats (6–8 weeks old) was similarly treated with ferrous sulfate. Mortalities, which all occurred within the first 24 h of ferrous sulfate administration, were statistically significantly different between sexes; rates were 40% for males and 80% for females of the prepubertal group ($P < 0.01$) and 47% for males and 60% for females of the pubertal group ($P = 0.3$). Among adult rats, mortalities were similar (95% for both males and

Table 1. Acute toxicity of iron

Compound	Species (strain)	Sex	Route	LD_{50} (mg elemental iron/ kg bw)	Reference
Ferrous sulfate	Mouse (Swiss)	Male and female	Oral	247–250	Boccio et al. (1998)
	Rat (albino)	Male and female	Oral	344	Keith (1957)
	Rat (Sprague-Dawley)	Male	Oral	>320	Nayfield et al. (1976)
	Rat (Sprague-Dawley)	Male	Oral	1 100	Whittaker et al. (2002)
	Rabbit (albino)	Male and female	Oral	206	Keith (1957)
	Dog	Not specified	Oral	>200	Keith (1957)
Carbonyl iron	Rat (Sprague-Dawley)	Male	Oral	>50 000	Whittaker et al. (2002)

females). In both the pubertal and adult rats, females died earlier than males (Berkovitch et al., 1997).

Male Sprague-Dawley rats were administered a single oral dose of 200 mg iron/kg bw as ferrous sulfate or distilled water. Seven animals from each group were killed 3 h after treatment, and the remainder (unspecified number) were killed 24 h after treatment. Sections of stomach and duodenum were fixed in formalin and examined histologically. Moderate mucosal necrotic lesions were observed in the stomachs and villi of the duodenum, with iron deposition in the necrotic tissue of treated rats at 3 h. At 24 h, minor necrotic lesions were observed in the stomachs and duodenum of treated rats (Benoni et al., 1993).

(b) Ammonium

Median lethal oral doses for ammonium sulfamate or sulfate were in the range 3–5 g/kg bw in both rats and mice (Frank, 1948; International Programme on Chemical Safety, 1986). Acute pulmonary oedema, nervous system dysfunction, metabolic acidosis, and renal hypertrophy and other kidney effects were reported to occur in rodents following administration of various ammonium salts, primarily ammonium chloride, at doses ranging from 500 to 1000 mg/kg bw for periods of 1–8 days (International Programme on Chemical Safety, 1986).

(c) Phosphate

There are a limited number of studies on the oral toxicity of inorganic phosphate salts in experimental animals (Expert Group on Vitamins and Minerals, 2003) and no new studies published subsequent to the Committee's last evaluation.

Kidney lesions have been reported in rats following the administration of acute doses of phosphates (5000 mg/kg bw per day, equivalent to 1200 mg phosphorus/kg bw per day) in the diet for 24–72 h (Craig, 1957). Median lethal oral doses for phosphates were in the range 2–4 g/kg bw in experimental animals, depending on the species and phosphate compound (Annex 1, reference *60*).

2.2.2 Short-term studies of toxicity

(a) Iron

(i) Mice

Three groups of five SPF female BALB/c infant (2 weeks old) and three groups of five SPF female BALB/c young adult (4 weeks old) mice were administered iron-free and protein-free diets supplemented with iron at a concentration of 0, 25 or 250 mg/kg diet (equivalent to 0, 3.75 and 37.5 mg/kg bw per day, respectively), prepared from ferrous sulfate, for 1 or 2 weeks. The parameters evaluated included body weight, haematocrit and haemoglobin concentrations, hepatic iron content and electron microscopic examination of the intestinal tract. There were no significant differences in the mean values of body weight or of haematocrit and haemoglobin concentrations among the treated groups. In infant mice, hepatic iron levels increased with the duration of feeding and were significantly increased ($P < 0.01$) in the high-dose group compared with the control group after 1 and 2 weeks of feeding. No similar significant increase in hepatic iron levels in the young adult mice was observed. Electron microscopy of fixed intestinal tissue samples revealed an increase in the frequency of intercellular junction openings between intestinal epithelial cells as well as an increase in the occurrence of eosinophilic leukocytes outside the basement membrane in young adult mice fed 250 mg/kg diet for 2 weeks. After 1 week of feeding, the frequency of intercellular junction openings between intestinal epithelial cells was greater in the infant mice than in the young adult mice; however, after 2 weeks of feeding, the frequency was greater in the young adult mice. Eosinophilic leukocytes outside the basement membranes of epithelial cells were also observed in infant mice fed 250 mg/kg diet for 1 or 2 weeks. The authors suggested that the continuous intake of iron in large amounts may be beyond the capacity of the cells to store iron, resulting in a disruption of the normal intercellular environment and subsequent formation of hydroxyl radicals and initiation of lipid peroxidation, with resultant loss of membrane structure and function. The authors concluded that excess iron in the diet of mice triggered the opening of cell junctions and the appearance of eosinophilic leukocytes in the intestinal tract, indicating an immunological reaction (Hirohata et al., 1998).

Groups of eight male weanling CD-1 mice were administered ferrous sulfate–supplemented diets ad libitum for 7 weeks to provide 120 (control), 5000 or 8000 mg iron/kg diet (equivalent to 18, 750 and 1200 mg iron/kg bw per day, respectively). Body weight gain, water consumption and haematocrit levels were monitored during the treatment period. On day 49, blood was collected by direct cardiac puncture, and mice were killed by carbon dioxide. The liver, spleen, kidneys,

heart and pancreas were removed, weighed, examined macroscopically and then fixed in 10% phosphate-buffered formalin for microscopic analysis. The concentration of iron in the liver and the deposition and distribution of iron in the liver and spleen were determined. There were no mortalities, and haematocrit values, water consumption and relative spleen, heart and kidney weights were not affected by iron supplementation. In mice fed diets containing 8000 mg iron/kg, a significant reduction ($P < 0.05$) in body weight was observed compared with the control group. A dose-dependent increase in liver weight was observed, significant ($P < 0.01$) at 8000 mg/kg diet, accompanied by a dose-dependent increase in hepatic iron concentration, significant ($P < 0.0001$) at 5000 and 8000 mg/kg diet. The weight of the liver and increase in hepatic iron were correlated with the amount of iron in the diet ($r = 0.902$ and $r = 0.989$, respectively). Iron accumulation was also observed in the splenic macrophages. There were no gross macroscopic lesions and no effects on serum glucose, urea, total protein or albumin concentrations in any of the groups. The activities of serum alanine aminotransferase (ALT) and alkaline phosphatase (AP) were increased by dietary iron supplementation. AP activity increased dose dependently and was 75% and 170% higher in mice fed 5000 and 8000 mg/kg diet, respectively ($P < 0.01$). ALT was increased only in mice fed 8000 mg/kg diet (by 68%, $P < 0.05$). The authors concluded that excess dietary iron can cause liver damage, as depicted by elevated serum activities of liver enzymes (Omara et al*.,* 1993).

(ii) Rats

Groups of 40 young adult male Sprague-Dawley (Crl:CD BR) rats were fed diets supplemented with ferrous sulfate at 35, 70 or 140 mg iron/kg diet for 31 or 61 days (mean iron intakes for each treatment group were calculated to be equal to 2.84, 5.69 and 11.54 mg/kg bw per day, respectively). Separate groups of rats were fed sodium iron EDTA similarly for comparison. Twenty rats from each group were sacrificed after 31 days or 61 days. No statistically significant differences in plasma iron concentrations were observed among the groups. After 61 days, a statistically significantly higher ($P < 0.05$) total iron-binding capacity was observed in the low-dose group compared with the other two treatment groups; this was not evident at 31 days. At both time points, statistically significant increases ($P < 0.05$) in non-haem iron concentration in the liver, spleen and kidneys were found with increasing dietary iron concentrations, with the exception of kidney concentrations at 61 days, which were increased, but not significantly. Macroscopic examinations did not reveal any treatment-related changes. The authors concluded that feeding ferrous sulfate at dietary concentrations up to 140 mg iron/kg diet did not result in excessive iron loading or any other significant toxicological effects (Appel et al., 2001). An unpublished report (Appel, 1999) of this study was reviewed by the Committee when evaluating sodium iron EDTA (Annex 1, reference *144*).

Three groups of male Sprague-Dawley rats (two experimental animals and one control animal per group) were administered a diet supplemented with 0 (control) or 20 000 mg carbonyl iron/kg (equivalent to 0 or 1000 mg iron/kg bw per day) for 4, 6 or 15 months, respectively. Biochemical analysis and microscopy showed increased iron content in all organs tested (liver, spleen, intestinal

mucosa, pancreas and heart), with the highest concentrations in the liver and spleen (4-fold and 7- to 10-fold increases over the controls, respectively). Periportal distribution of stored iron was seen in the liver, similar to that observed in human haemochromatosis. Animals treated beyond 6 months showed cytosiderosis in Kupffer cells and sinusoidal lining cells. No evidence of cirrhosis was observed. The authors concluded that the iron-overloaded rat is a suitable experimental model for human haemochromatosis (Iancu et al., 1987).

Groups of weanling male Sprague-Dawley rats were fed diets supplemented with carbonyl iron at 35 (control), 350, 3500 or 20 000 mg iron/kg diet (11, 10, 10 and 18 rats per group, respectively), equivalent to 1.75, 17.5, 175 and 1000 mg/kg bw per day, for a period of 12 weeks. Hepatic and cardiac iron content and lipid peroxidation and apoptosis in the liver were assessed. At the end of the 12-week treatment period, the rats were fasted and then anaesthetized by intramuscular injection of ketamine hydrochloride, decapitated and subjected to a full necropsy. Samples of liver, heart, spleen and pancreas were collected and fixed for microscopy. Seven animals died during the study, two from the middle dose group and five from the top dose group. Five of these seven animals had heart damage, which included iron in the cytoplasm of the myocardial fibres, haemorrhagic necrosis, epicardial damage and clot formation. There was a dose-dependent and significant increase in hepatic ($P < 0.001$) and cardiac ($P = 0.001$) iron content. Iron deposition (haemosiderosis) was evident in the periportal hepatocytes and the sinusoidal macrophages of the spleen of all treatment groups, with the intensity increasing with the dose. Cardiomyopathy was evident in the three dose groups, but particularly in the top two dose groups (70% and 71% incidence, respectively). Splenic atrophy and pancreatic atrophy were evident in animals fed 3500 and 20 000 mg/kg diet. The authors suggested that the effects observed in the liver, heart, spleen and pancreas may be due to peroxidative injury to cellular membranes caused by iron-induced oxidative stress (Whittaker et al., 1996). Based on the occurrence of death and observed organ atrophy and cardiomyopathy at the two highest dose levels, the no-observed-adverse-effect level (NOAEL) for iron was 350 mg/kg diet, equivalent to 17.5 mg/kg bw per day, although it should be noted that increased iron deposition was evident at this dose.

(b) Ammonium

(i) Rats

In a series of experiments to determine the effect of ammonium chloride on the susceptibility of the kidney to bacterial infection, groups of 6 or 12 male Sprague-Dawley rats were administered ammonium chloride in the drinking-water ad libitum for up to 21 days. Initially a dose of 2% was chosen, but this was found to interfere with food and water consumption and so was reduced to 1.6% (equivalent to 1600 mg ammonium chloride/kg bw per day or 540 mg ammonium/kg bw per day). Inocula of *Escherichia coli* were injected intravenously into the renal medulla of rats, subsequent to ammonium chloride administration. Urinalysis, measurement of glutaminase activity and histological examinations of the kidneys were performed. There was a steady rise in glutaminase activity, which was

associated with increasing length of treatment with ammonium chloride. Urinalysis did not reveal any evidence of renal injury (proteinuria, haematuria, pyuria or cylindruria). Bacterial infection was evident in the form of bacteriological colonies and gross lesions (abscesses) in the medulla and cortex. Histological examinations did not reveal any renal injury. The authors concluded that ammonium chloride increased the susceptibility of the rat kidney to infection (Freedman & Beeson, 1961).

Groups of adult female albino rats and weanling male and female albino rats were orally administered ammonium sulfamate dissolved in distilled water (100 mg/ml; not specified whether given by gavage). For each group of rats (female adult, male weanling and female weanling), there were four groups of 20 animals receiving doses of 0, 100, 250 or 500 mg ammonium sulfamate/kg bw per day (equivalent to 0, 16, 40 and 79 mg ammonium/kg bw per day, respectively), 6 days/week, for up to 90 days. Body weights and food and water consumption were recorded throughout the treatment period. At the end of 30, 60 and 90 days, six rats from each group were sacrificed under anaesthesia, and blood was collected for haematological analyses. All animals were subjected to an autopsy, and a range of organs were collected for histological examination. Two animals died during the study as a result of bronchopneumonia: one adult female (250 mg/kg bw per day) and one male weanling (500 mg/kg bw per day). Apart from these two animals, the general condition and health of the animals were good. There was a significant reduction ($P < 0.05$) in the body weight of adult rats receiving 250 and 500 mg/kg bw per day. Food intake decreased in all groups as the treatment progressed, whereas water intake increased, both being significant at 500 mg/kg bw per day in male and female weanlings ($P < 0.05$ for food intake and $P < 0.01$ for water intake). There were no significant differences in haematological analyses, organ weights or histological examinations, although the liver of one adult rat receiving 500 mg/kg bw per day showed slight fatty degenerative changes in the hepatic cytoplasm after 90 days. The authors concluded that administration of ammonium sulfamate to rats for a period of 90 days did not produce any toxic effects up to a dose of 500 mg/kg bw per day (equivalent to 79 mg ammonium/kg bw per day) (Gupta et al., 1979).

In a series of experiments to assess silica urolithiasis in tetraethylorthosilicate-supplemented diets, groups of 24 male Sprague-Dawley rats were fed either a control diet or one of four diets supplemented with ammonium chloride or phosphorus for 8 weeks. The treatments included 0.98% of an equimolar phosphate mix containing monosodium phosphate and disodium phosphate; 0.75% ammonium chloride (equivalent to 375 mg ammonium chloride/kg bw per day or 127 mg ammonium/kg bw per day); 0.75% ammonium chloride and 0.98% of the equimolar phosphate mix; or 0.92% of dibasic ammonium phosphate (equivalent to approximately 460 mg dibasic ammonium phosphate/kg bw per day or 125 mg ammonium/kg bw per day). Measured parameters included body weight, water intake, urinalysis, urinary and plasma calcium, magnesium and phosphorus levels, and the presence of bladder and kidney uroliths. No significant effect of diet on body weight was observed. Water intake, urine volume, and urine and plasma mineral concentrations were not significantly altered with ammonium chloride treatment compared with the control diet. Ammonium chloride–associated effects included a

significant ($P < 0.01$) decrease in urinary pH. Phosphorus-associated effects included a significant ($P < 0.01$), but slight, increase in water intake, a significant ($P < 0.01$) decrease in urinary pH, a significant ($P < 0.01$) increase in plasma and urinary phosphorus concentrations, and a significant ($P < 0.01$) decrease in urinary calcium and magnesium concentrations. The addition of ammonium chloride and/or phosphorus to the diet was associated with a lower incidence ($P < 0.01$) of urolith formation compared with the control diet (Schreier & Emerick, 1986).

The effects of diet-induced acid–base disturbances were examined in 4- and 13-week toxicity studies and in an 18-month toxicity study and a 30-month carcinogenicity study (see section 2.2.3 for a description of the 18- and 30-month studies). Groups of 10 male and 10 female weanling SPF-bred Wistar rats were fed a diet supplemented with 0%, 2% or 4% ammonium chloride (equivalent to 0, 2000 and 4000 mg ammonium chloride/kg bw per day or 0, 679 and 1358 mg ammonium/kg bw per day, respectively) for 4 weeks. In the 13-week study, the 2% ammonium chloride level was raised to 2.1% (equivalent to 2100 mg ammonium chloride/kg bw per day or 713 mg ammonium/kg bw per day). Clinical signs and survival were monitored daily, and body weights were measured weekly. Food and water intakes were also recorded regularly. Haematology and urinalysis were conducted at regular intervals throughout the treatment period. Clinical chemistry analyses were performed at the end of the treatment periods. At the end of the treatment periods, all rats were killed by exsanguination under light ether anaesthesia and subjected to a postmortem examination and femur assessment. Gross and microscopic examinations were performed on an extensive range of organs.

No effects were noted in the condition or behaviour of the animals, and there were no deaths. Mean body weights were significantly reduced in males and females in both studies and at both doses (statistical data not provided). There was marked (around 20%) growth depression at 4%, and so lower dietary levels of ammonium chloride were administered for the chronic studies. Food intake was similar among groups in both studies, but water intake was increased. Ammonium chloride induced metabolic acidosis, as shown by a dose-dependent decrease ($P < 0.01$) in base excess associated with reduced blood pH and bicarbonate concentration in males and females at both doses and in both studies. Urinary pH was significantly ($P < 0.01$) decreased and net acid excretion significantly ($P < 0.01$) increased in both dose groups of both studies. Plasma chloride levels were significantly ($P < 0.01$) increased at 4% in both studies and were significantly ($P < 0.05$) increased in males only in the low-dose group in both studies. In the 13-week study, urinary calcium and phosphate levels were significantly ($P < 0.01$) increased in both sexes, but there were no indications that bone minerals were involved (femur weight, calcium content and total bone substance). Relative kidney weights of both sexes fed 2.1% and 4% and relative adrenal weights of males fed 4% were significantly ($P < 0.01$) increased in both studies. Histopathological analysis revealed a significant ($P < 0.01$) increase in the incidence of adrenal zona glomerulosa hypertrophy due to acidosis in animals treated with 4% ammonium chloride for 13 weeks. The authors concluded that most of the observed changes could be regarded as physiological adaptations to an acid load (Lina & Kuijpers, 2004).

A group of 10 male Wistar-Hannover rats was administered ammonium chloride in the drinking-water at a concentration of 0.25 mol/l (13 000 mg ammonium chloride/l or 4415 mg ammonium/l) ad libitum for 10 days. A similar group of pair-fed animals served as the control. Volumes of water ingested were recorded. At the end of the 10-day treatment period, each animal received a tap water load by gavage (5% of body weight), followed by a second load of the same volume 1 h later; spontaneously voided urine was then collected. Blood samples were taken from the tail vein or cardiac puncture in anaesthetized rats, and the kidneys were removed. Plasma and urinary sodium, potassium and creatinine concentrations were determined. The volume of liquid intake was 34.8 ± 0.5 ml/day in treated rats and was comparable with that in control rats (equivalent to approximately 1131 mg ammonium chloride/kg bw per day or 384 mg ammonium/kg bw per day). On day 10 of treatment, metabolic acidosis was confirmed in ammonium chloride–treated rats by a blood pH of 7.16 ± 0.13 and a marked reduction in bicarbonate values compared with control rats. Body weight gain was not significantly different between the two groups. Kidney weight (not specified whether relative or absolute kidney weight) and serum potassium levels were significantly ($P < 0.01$) increased in the treated group compared with the control group. Ammonium chloride treatment resulted in a significant ($P < 0.05$) increase in renal fractional sodium excretion. The authors concluded that ammonium chloride–induced acidosis causes a disturbance in renal sodium handling (Bento et al., 2005).

(ii) Pigs

Young male pigs were fed basal diets restricted in non-essential amino acids and containing the minimum requirement of essential amino acids to evaluate the growth effect of dietary diammonium citrate. Four pigs were fed the basal diet supplemented with diammonium citrate at 37 500 mg/kg diet (equal to 2754 mg diammonium citrate/kg bw per day or 438 mg ammonium/kg bw per day) for 28 days. Two further groups of four pigs served as controls. Body weights and food consumption were recorded regularly, and faeces, urine and blood samples were collected. All animals were sacrificed by bleeding on the 29th day and autopsied. One pig receiving the diammonium citrate diet showed weak appetite in the final stage due to an injury to the hind legs from the wired floor and was thus omitted from the results. Diammonium citrate–treated pigs displayed significantly ($P < 0.05$) greater weight gain and slightly reduced food utilization efficiency than did four pigs fed the basal diet alone. Consumption of diammonium citrate did not result in any significant differences in packed cell volume, blood ammonia nitrogen levels or plasma total protein concentration compared with the pigs fed the unsupplemented diet. Towards the final stage (day 25 onwards), urinary urea nitrogen levels were significantly ($P < 0.05$) higher in treated pigs than in controls. No lesions or abnormalities were revealed upon autopsy (Kagota et al., 1979).

(c) Phosphate

Weanling female SPF-derived Wistar rats were maintained on a diet containing 4000 mg phosphorus/kg as sodium phosphate and 5000 mg calcium/kg for 10 days, prior to a pair of experiments. In the first experiment, a control group of

6 rats was fed a diet containing 4000 mg phosphorus/kg and a test group of 16 rats was fed a diet containing 6000 mg phosphorus/kg for 28 days (equivalent to 400 and 600 mg phosphorus/kg bw per day, respectively). The second experiment was very similar and involved 30 rats that were maintained on a diet containing 4000 mg phosphorus/kg and 5000 mg calcium/kg for 10 days. Six of these rats were killed after 10 days, and the remaining 24 rats were fed diets containing either 4000 or 6000 mg phosphorus/kg (12 rats per group). Animals were housed individually in metabolism cages, and urine and faeces were collected at regular intervals for analyses of mineral levels. At the end of the treatment periods, blood was collected and animals were killed by carbon dioxide asphyxiation. The kidneys, parathyroids, heart, liver, stomach, lungs and thoracic aorta were removed for histological processing.

No significant differences were found in body weight and body weight gains or food intake between the adequate-phosphorus (4000 mg/kg diet) and high-phosphorus (6000 mg/kg diet) groups in either experiment. In the first experiment, no significant change in faecal phosphorus excretion was observed between the two dietary groups. However, urinary phosphorus output was significantly ($P < 0.01$) increased in animals fed the 6000 mg/kg diet compared with rats fed the 4000 mg/kg diet, resulting in significantly ($P < 0.01$) greater whole-body phosphorus retention in animals fed the high-phosphorus diet. Urinary and faecal calcium and magnesium levels were unaffected by the dietary level of phosphorus. In the second experiment, plasma calcium and magnesium levels were significantly ($P < 0.05$) reduced in the 6000 mg phosphorus/kg diet group compared with the 4000 mg/kg diet group, but plasma phosphorus levels were unaffected. In both experiments, a significant ($P < 0.01$) reduction in urinary pH was observed in the 6000 mg phosphorus/kg diet groups compared with the 4000 mg/kg diet groups, and an increase ($P < 0.01$) in urinary albumin levels was observed in the high-phosphate groups. A significant ($P < 0.01$) increase in relative kidney weights was observed in animals fed the high-phosphorus diet compared with animals fed the adequate-phosphorus diet, although this was evident only in the first experiment. A significant ($P < 0.05$) increase in mineral (calcium, phosphate and magnesium) content of the kidney was observed in the high-phosphorus group in both experiments. Also observed in both experiments was the development of nephrocalcinosis in all animals fed the 6000 mg/kg diet. Animals fed the adequate-phosphate diet developed nephrocalcinosis to a milder degree. Histopathology conducted on the kidneys revealed calcium deposits in the corticomedullary junction. In severe cases of nephrocalcinosis, interstitial fibrosis and focal tubuli with regenerated epithelium were noted. No phosphorus-induced calcium deposits or microscopic changes were observed in the heart, liver, thoracic aorta, parathyroids, stomach or lungs. The authors concluded that dietary phosphorus–induced nephrocalcinosis is associated with impaired kidney function in rats (Ritskes-Hoitinga et al., 1989).

In a related study, groups of weanling female SPF-derived Wistar rats were fed diets containing either 2000 mg phosphorus/kg (18 rats) or 6000 mg phosphorus/kg (37 rats) as sodium phosphate for 28 days, following a 10-day pre-experimental diet containing 2000 mg phosphorus/kg. The test diets were equivalent to doses of 200 and 600 mg phosphorus/kg bw per day, respectively.

On day 29, six rats from each group were randomly selected for autopsy. Also on day 29, 19 rats fed the 6000 mg phosphorus/kg diet were switched to the 2000 mg phosphorus/kg diet to assess any regression in phosphorus-induced nephrocalcinosis. The remaining 12 rats fed the 6000 mg/kg diet remained on this diet and served as positive controls, alongside the remaining 12 rats fed the 2000 mg/kg diet (negative controls). Animals were housed in metabolism cages. Body weights were recorded at regular intervals. On days 57 and 92, six or seven rats from each group were killed by exposure to carbon dioxide and the kidneys removed for calcium analysis and histological analysis. All remaining rats were killed on day 120, and kidneys were removed. Throughout the treatment period, the animals were examined clinically for 14 variables to determine the degree of possible discomfort associated with nephrocalcinosis. The clinical assessment variables included general appearance, stance, alertness, development, behaviour on handling, hair coat, skin colour and palpation of the dorsolumbar region. Body weights were similar at all time points among groups. Of the variables clinically assessed, none differed between rats with or without nephrocalcinosis. The results of renal histopathology are provided in section 2.2.6(c). The authors concluded that phosphorus-induced nephrocalcinosis does not appear to regress after phosphorus restriction and that rats with nephrocalcinosis do not appear to experience discomfort (Soeterboek et al., 1991).

In a similarly designed experiment, weanling female rats of each of 10 strains were divided into two groups of six rats per strain. Rat strains included ACI, Brown Norway, Fischer, Long-Evans, Lewis, PVG, RP, Spontaneously Hypertensive Rat, WAG and Wistar Kyoto. Following a 10-day pre-experimental period as above, rats were fed diets containing either 2000 or 5000 mg phosphorus/kg as sodium phosphate (equivalent to 200 and 500 mg/kg bw per day, respectively) for 28 days. Following on from this experiment, a second experiment was performed with only two strains of rats: Long-Evans (nephrocalcinosis-insensitive) and RP (nephrocalcinosis-sensitive) rats. In the second experiment, there were two groups of six rats per strain per diet (2000 or 5000 mg phosphorus/kg diet as sodium phosphate), with half the animals being housed in metabolism cages. Body weights were recorded weekly, and food and water intakes were recorded. Urine and faeces of rats housed in metabolism cages were collected at regular intervals. After 4 weeks of treatment, the rats were anaesthetized, blood was collected and then the rats were killed by cervical dislocation. The kidneys were removed; the right one was frozen for chemical analysis, and the left one was fixed for histological analysis. Results were comparable between the two experiments. Growth rate was significantly ($P < 0.05$) elevated in the 5000 mg phosphorus/kg diet groups compared with the 2000 mg phosphorus/kg diet groups in six of the rat strains (ACI, Brown Norway, Fischer, Lewis, Spontaneously Hypertensive Rat and Wistar Kyoto). No nephrocalcinosis was detected in any of the rat strains fed 2000 mg phosphorus/kg diet, whereas all rats except the Brown Norway and Long-Evans strains fed the 5000 mg/kg diet developed nephrocalcinosis ($P < 0.01$). The results of urinary and faecal mineral levels and renal histopathology are provided in section 2.2.6(c). The authors concluded that there are considerable strain differences in nephrocalcinosis (Ritskes-Hoitinga et al., 1992).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Iron

Iron lactate was administered ad libitum to groups of 50 Fischer 344/DuCrj rats of each sex in the diet at levels of 0%, 1% or 2% for 104 weeks to determine carcinogenic potential. The iron contents of the diets were estimated to be 170, 1670 and 3560 mg/kg, respectively, and average iron intake was calculated to be equal to 0, 82 and 167 mg/kg bw per day for males and 0, 91 and 185 mg/kg bw per day for females, respectively, based on iron lactate intakes. Body weight and food consumption were recorded every week until week 13, then every 2 weeks until week 26 and every 4 weeks thereafter. Animals were fasted overnight prior to necropsy, following which an extensive range of organs and tissues were collected for histological analysis.

There were no significant differences in survival or food intake. Body weights of all treated animals were reduced in a dose-related manner compared with controls by the end of the treatment period, although these were significant ($P < 0.01$) only in males and females of the 2% group and males in the 1% group. At 2%, body weights were reduced by 8–9% compared with controls; in males of the 1% group, body weights were reduced by 5% compared with controls. Histological analysis revealed various neoplastic lesions in all groups, which were considered by the authors to occur spontaneously in this particular strain of rats. There were no treatment-related increases in tumour incidence. Incidences of mammary gland fibroadenomas and thyroid c-cell adenomas in female rats of the 2% group were significantly ($P < 0.05$) decreased compared with control rats. The incidences of focal pancreatic acinar cell and endometrium hyperplasias were increased ($P < 0.05$) in males and females, respectively, at the 2% dose level. The authors noted that the increased incidence of focal pancreatic acinar cell hyperplasia was in agreement with previous literature reports. Pituitary hyperplasias were more frequent in females receiving 1% iron lactate ($P < 0.05$), but this finding was without dose dependence. The authors suggested that the high incidence of endometrium hyperplasia indicated that iron lactate may possess estrogenic potential, but found negative results in in vitro assays for competitive binding to estrogen receptors in an estrogen-responsive rat pituitary cell line (MtT/Se) and an estrogen-responsive human breast cancer cell line (MCF-7). Brown pigmentation (accumulation of iron as haemosiderin) was observed in a number of organs, such as the liver, kidney, spleen and uterus of all animals. The authors concluded that iron lactate has no potential to induce tumours in either male or female rats at dietary levels of 1% or 2%, equal to iron doses of 82 and 167 mg/kg bw per day for males and 91 and 185 mg/kg bw per day for females, respectively (Imai et al., 2002). Based on the dose-related decreases in body weight, which were significant at 1% in males, the dose level of 1% iron lactate (equal to 82 mg iron/kg bw per day) was the lowest-observed-adverse-effect level (LOAEL).

(b) Ammonium

(i) Mice

Ammonium hydroxide was given as a 0.1%, 0.2% or 0.3% solution in the drinking-water to 5-week-old Swiss mice (49–50 per sex per group) and as a 0.1% solution to 7-week-old C3H mice (40 males and 40 females) for the remainder of their lifetime. Control groups were not included. These concentrations provided ammonium hydroxide doses of 460, 820 and 975 mg/kg bw per day in Swiss males and 415, 650 and 720 mg/kg bw per day in Swiss females; and 395 and 420 mg/kg bw per day for C3H males and females, respectively. Mortality, body weights, water intakes and tumour incidence were recorded. Animals were allowed to die or were killed humanely if found in poor condition. Necropsies were performed on all animals, and the liver, kidneys, spleen and lungs were examined microscopically. In Swiss mice, no effects on survival were reported, and minor tumour incidences were not considered to be treatment related. In C3H mice, 60% of treated mice developed adenocarcinomas of the mammary gland compared with 76% of control mice. The authors concluded that ammonium hydroxide administered in the drinking-water of Swiss and C3H mice was without carcinogenic effect and that it did not inhibit the development of breast adenocarcinomas in female C3H mice (Toth, 1972).

(ii) Rats

Groups of 15 male and 15 female weanling SPF-bred Wistar rats were fed a diet supplemented with 0%, 1% or 2.1% ammonium chloride (equivalent to 0, 500 and 1050 mg ammonium chloride/kg bw per day or 0, 170 and 357 mg ammonium/kg bw per day, respectively) for 18 or 30 months. Parallel experiments were conducted for 4 and 13 weeks (see section 2.2.2). Clinical signs and survival were monitored daily, and body weights were measured weekly. Visible or palpable masses and food and water intakes were recorded regularly. Haematology, clinical chemistry and urinalysis were conducted at regular intervals throughout the treatment periods. In week 53, six rats per sex per group were placed in metabolism cages. At the end of the treatment periods, all rats were killed by exsanguination under light ether anaesthesia and subjected to a postmortem examination and femur assessment. Gross and microscopic examinations were performed on an extensive range of organs.

No effects were noted in the condition or behaviour of the animals, and the mortality rate was deemed by the authors not to be affected by treatment. The incidence and type of palpable masses did not indicate any effects related to ammonium chloride. After 30 months, mean body weights were decreased by 2.5–5% in high-dose males and females. Food and water intakes were similar between ammonium chloride–treated animals and the control groups. Urinary pH was significantly ($P < 0.01$) decreased in ammonium chloride–treated rats at both doses and both times. Net acid excretion was significantly ($P < 0.01$) increased in both sexes treated with 2.1% and in males only treated with 1%, in both studies. Urinary excretion of calcium, phosphate and urea was significantly ($P < 0.01$) increased in both sexes in a dose-dependent manner. An increased incidence of

granular casts in the urinary sediment was observed in males and females of both studies treated with 1% ammonium chloride. In both studies, plasma chloride levels were significantly ($P < 0.05$) increased in males treated with 2.1% ammonium chloride and in females of both dose groups during the last few months ($P < 0.01$). Serum AP activity was significantly ($P < 0.01$) increased in males fed 2.1% ammonium chloride during the first few months of both studies. No significant differences were observed in red blood cell variables, clotting potential or white blood cell counts. Analysis conducted on the femur did not reveal any effects on femur weight, calcium content or total bone substance that were related to the dietary administration of ammonium chloride. No significant organ weight changes and no macroscopic abnormalities attributable to ammonium chloride were observed. Histopathological analysis revealed a significant increase in the incidence of adrenal zona glomerulosa hypertrophy at 30 months in males (both doses, $P < 0.01$) and females (2.1% only, $P < 0.01$). The authors attributed this effect to chronic stimulation of the adrenal cortex by ammonium chloride–induced acidosis. In the kidneys, the incidence of oncocytic tubules and nephrosis was significantly ($P < 0.05$) decreased in males treated with 2.1% ammonium chloride for 30 months. In females treated with 2.1% ammonium chloride for 30 months, there was a high incidence (not significant) of adenocarcinoma in the mammary gland; however, the authors did not ascribe this to treatment, because no preneoplastic changes were observed in the earlier studies and because the incidence was within the range of historical control data. No other effects on tumour incidence were observed, and the authors noted that treatment with ammonium chloride did not affect the type, incidence or multiplicity of tumours. They concluded that the metabolic acidosis induced by ammonium chloride was not associated with dissolution of alkaline bone salts or an increased risk of tumour formation in rats (Lina & Kuijpers, 2004).

Following on from a 13-week subchronic oral toxicity study of ammonium sulfate in rats (Takagi et al., 1999; article in Japanese and not evaluated by the Committee), in which the maximally tolerated dose for a 2-year carcinogenicity study was concluded to be 3% or more in the diet, chronic and carcinogenicity studies were performed. Ammonium sulfate was mixed into powdered basal diet and administered to male and female F344/DuCrj rats for 52 or 104 weeks. In the chronic toxicity study (52 weeks), groups of 10 males and 10 females received ammonium sulfate at 0%, 0.1%, 0.6% or 3% in the diet (equal to 0, 11, 66 and 393 mg ammonium/kg bw per day in males and 0, 12, 73 and 384 mg ammonium/kg bw per day in females, respectively). In the carcinogenicity study (104 weeks), groups of 50 males and 50 females received 0%, 1.5% or 3% ammonium sulfate in the diet (equal to 0, 145 and 332 mg ammonium/kg bw per day in males and 0, 167 and 353 mg ammonium/kg bw per day in females, respectively). Clinical signs and mortality were recorded daily throughout the treatment periods, and body weight and food consumption were recorded every 2 weeks until week 10 and then every 5 weeks thereafter. At the end of the treatment periods, all surviving animals were euthanized and exsanguinated. In the chronic toxicity study, blood was collected for haematological and serum biochemical analyses. All animals from both studies were subjected to a complete necropsy, and an extensive range of organs was collected for histological analysis.

There were no mortalities in the chronic toxicity study; however, in the carcinogenicity study, survival rates were reduced in both males and females of all groups, with no significant differences between the groups. The general condition of all of the animals was good for both studies, and there were no treatment-related effects on body weight or food intake. No effects were found on haematological or serum biochemical parameters at any dose level in the chronic study. Ammonium sulfate at 3% of the diet for 52 weeks increased ($P < 0.05$) kidney weights by 7–11.5% in both sexes and increased ($P < 0.05$) liver weights by 7% in males. Spleen weights were decreased ($P < 0.05$) by 7% in males of the 3% dose group. There were no significant histopathological changes in the chronic study. In the carcinogenicity study, the incidence of chronic nephropathy was increased at 1.5% and 3% in both sexes but was significant only in males of the 1.5% dose group (increased by 53%, $P < 0.05$). Neoplastic lesions were observed in all groups but were not attributed to treatment, because they are known to occur spontaneously and because there were no significant differences between the groups. The authors concluded that the NOAEL for ammonium sulfate was 0.6% in both sexes (equal to 66 mg ammonium/kg bw per day) (Ota et al., 2006).

2.2.4 Genotoxicity

(a) Iron

A number of in vitro and in vivo genotoxicity tests have been conducted using ferrous sulfate and ferrous fumarate (Table 2). In the Ames reverse mutation assay using the standard plate incorporation method, ferrous sulfate and ferrous fumarate were generally negative, except for infrequent non-concentration-related observations. In the mouse lymphoma assay, ferrous sulfate produced a weakly positive response in the absence of metabolic activation at the highest concentration tested (201 µg iron/ml) and a concentration-dependent (1.0–1.5 µg iron/ml) response in the presence of S9 metabolic activation. Ferrous fumarate also produced a concentration-dependent (296–316 µg iron/ml) mutagenic response in the absence of metabolic activation. Concentrations of up to 4.6 µg iron/ml as ferrous fumarate were not mutagenic in the presence of metabolic activation.

In an in vivo test for nuclear aberrations and micronucleus formation, a dose-dependent increase in the frequency of nuclear aberrations was noted in the colon cells of both feeding and fasting mice treated with ferrous sulfate, which was considered to be an indication of toxicity rather than genotoxicity.

(b) Ammonium

Ammonium sulfamate was non-mutagenic in plate and suspension tests conducted in *S. typhimurium* strains TA1535, TA1537 and TA1538 and in *Saccharomyces cerevisiae* D4 in the presence or absence of metabolic activation at a concentration of 5% (Litton Bionetics, Inc., 1975).

Ammonia (0.025–0.5%) in distilled water was tested for its ability to induce reverse mutations from streptomycin dependence to non-dependence in *Escherichia coli*. Mutagenic activity was observed only at cytotoxic concentrations

Table 2. Results of assays for mutagenicity/genotoxicity with iron

Compound	Type of assay	Test system	Concentration/dose of elemental iron	Results	Reference
In vitro					
Ferrous sulfate	Reverse mutation	*Salmonella typhimurium* TA97	16, 31, 63, 125, 250, 500, 1000 µmol/l (2.4, 4.7, 9.6, 19, 38, 76, 152 µg/ml)	Negative in sodium phosphate buffer and distilled, deionized water; positive at 250 µmol/l only in HEPES buffer	Pagano & Zeiger (1992)
	Reverse mutation	*S. typhimurium* TA97a, TA98, TA100, TA102, TA1535, TA1537, TA1538	Up to 10 000 µg/plate[a]	Negative	Dunkel et al. (1999)
	Forward mutation	L5178Y TK$^{+/-}$ mouse lymphoma cells	20, 50, 100, 150, 201 µg/ml[b]	Negative at ≤150 µg/ml; positive at 201 µg/ml	Dunkel et al. (1999)
			0.8, 1.0, 1.2, 1.5 µg/ml[c]	Negative at 0.8 µg/ml; positive at ≥1.0 µg/ml	
	Enhancement of virus-mediated cell transformation	Syrian hamster embryo cells	Range of concentrations not specified	Positive at 0.9 mmol/l and greater	Casto et al. (1979)
Ferrous fumarate	Reverse mutation	*S. typhimurium* TA97a, TA100, TA102, TA1535, TA1537, TA1538	Up to 10 000 µg/plate[a]	Negative	Dunkel et al. (1999)
	Reverse mutation	*S. typhimurium* TA98	Up to 10 000 µg/plate[a]	Negative[b]/positive[c,d]	Dunkel et al. (1999)
	Forward mutation	L5178Y TK$^{+/-}$ mouse lymphoma cells	296, 303, 309, 316 µg/ml[b]	Positive	Dunkel et al. (1999)
			1.6, 3.3, 3.9, 4.6 µg/ml[c]	Negative	

Table 2. (contd)

Compound	Type of assay	Test system	Concentration/dose of elemental iron	Results	Reference
In vivo					
Ferrous sulfate	Nuclear aberrations	C57BL/6J mouse stomach, duodenum and colon cells	2.0, 6.5 or 13.0 mg/kg bw (gavage)	Positive in colon cells only[e]	Bianchini et al. (1988)
	Micronucleus formation	C57BL/6J mouse stomach, duodenum and colon cells	2.0, 6.5 or 13.0 mg/kg bw (gavage)	Negative	Bianchini et al. (1988)

HEPES, *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.
[a] With and without metabolic activation.
[b] Without metabolic activation.
[c] With metabolic activation.
[d] Observed only at 987 µg iron/plate and 1647 µg iron/plate. At higher concentrations of iron, ferrous fumarate did not induce a mutagenic response in the TA98 strain with or without metabolic activation.
[e] Observed only at 13.0 mg/kg bw in feeding animals and 6.5 and 13.0 mg/kg bw in fasting animals.

(0.25% and 0.5%), where the proportion of survivors was less than 2% (Demerec et al., 1951).

In normal and SV40-transformed 3T3 mice fibroblasts cultured with ammonia (0, 10, 20 or 35 mg/l), dose-dependent changes in cell morphology and significant ($P < 0.001$) reductions in cell multiplications were observed (United States Environmental Protection Agency, 1989).

2.2.5 Reproductive toxicity

(a) Iron

In order to investigate zinc–iron interactions, pregnant Wistar rats were maintained on low-zinc (7 mg/kg diet) or adequate-zinc (60 mg/kg diet) diets for at least 2 weeks prior to mating. They were then fed similar diets containing normal (144 mg/kg diet) or high (576 mg/kg diet) levels of ferrous sulfate from day 1 to day 21 of gestation. The zinc and iron contents of the diets given to the four groups were calculated to be (mg/kg diet): group +Zn −Fe: Zn 59.6, Fe 36.3; group +Zn +Fe: Zn 57.6, Fe 161.5; group −Zn −Fe: Zn 6.8, Fe 35.1; group −Zn +Fe: Zn 6.9, Fe 171.5. Thus, the iron doses were equivalent to 1.8 mg/kg bw per day and 8 mg/kg bw per

day, respectively. Animals were weighed, and food intake was recorded regularly. On day 21, blood was taken from the tail for haemoglobin and packed cell volume measurements. The animals were then killed by carbon dioxide asphyxiation, and fetuses and placentas were removed and assessed for gross morphological abnormalities. The mean number of corpora lutea and the number of implantation sites and resorption sites were recorded, and the litters were homogenized for fat analysis. The mothers' left hindlimbs were removed for zinc and iron analysis. Animals fed the normal-iron, low-zinc diet ate slightly less and gained less weight than those on the high-iron, low-zinc diet in the third trimester ($P < 0.05$). There were no significant differences between the groups in maternal haemoglobin or packed cell volume values, and the only difference in the hindlimb bone analysis was a higher iron concentration in both of the supplemented groups. The mean number of resorption sites, number of fetuses in each group, fetal dry weight and fetal fat content were not significantly different among the four groups. In the adequate-zinc groups, the high-iron diet significantly ($P < 0.05$) reduced fetal wet weight compared with normal-iron diets. The low-zinc diets were associated with a higher incidence of fetal morbidity, and 57% of mothers showed uterine and fetal haemorrhages compared with only 10–18% of mothers from the adequate-zinc diets. Brown patches were observed on the uterine wall in the region of the placenta in the mothers on the low-zinc diets. The addition of iron to the diet increased fetal iron in both groups ($P < 0.01$). The authors concluded that iron supplementation did not adversely affect fetal growth from mothers given a low-zinc diet, and the results therefore did not suggest an iron–zinc interaction (Fairweather-Tait et al., 1984).

A diet supplemented with 0%, 0.5%, 1% or 2% carbonyl iron (0, 5000, 10 000 or 20 000 mg/kg diet) was fed to four Porton rat dams 2 days following parturition to initiate iron supplementation to the offspring via the breast milk. After weaning (at 3 weeks of age), the offspring continued to receive the assigned diet (equivalent to 0, 250, 500 or 1000 mg iron/kg bw per day, respectively) until 32 weeks of age. There were 20 females and 18 males in the study, arranged into groups of four or six female pups and three or five male pups. Body weight measurements were recorded weekly. At regular intervals, up to three rats from each dose group had open liver biopsies under anaesthesia for hepatic iron loading assessment. At termination, all rats had liver biopsies to assess hepatic iron content and to assess iron loading histologically. All rats receiving supplemental iron had increased hepatic iron at the first biopsy (8 weeks). Female rats had a higher hepatic iron content than males ($P < 0.001$); for those receiving higher doses, the iron levels continued to rise over the treatment period. Iron loading scores were correlated with hepatic iron content ($r = 0.95$). Growth retardation was observed in pups of both sexes supplemented with iron when compared with control pups, particularly at 1% and 2% dose levels ($P < 0.01$). At the highest iron dose level, the body weights of females and males were lower by an average of 14% and 19%, respectively, compared with controls. No evidence of fibrosis was observed in any of the livers. The authors concluded that iron supplementation was associated with a moderate retardation of growth (Plummer et al., 1997).

(b) Ammonium

Ammonium chloride was administered in the drinking-water to investigate the role of metabolic acidosis in salicylate-induced teratogenesis in pregnant Sprague-Dawley rats. Beginning on day 7 of gestation, rats received either 0.9% ammonium chloride (9000 mg/l, equivalent to 450 mg ammonium chloride/kg bw per day or 153 mg ammonium/kg bw per day), a subcutaneous injection of salicylate or both. Ammonium chloride treatment was associated with a reduction in fetal growth but did not result in any teratogenic effects. However, when administered with salicylate, ammonium chloride increased maternal and fetal mortality and increased the fetal anomaly rate, compared with that due to salicylate alone. These effects were attributed to acidosis and not to ammonia (Goldman & Yakovac, 1964).

2.2.6 Special studies

(a) Effects of iron on liver

Dietary iron overload in experimental animal models has been associated with hepatic changes, with some studies showing resultant fibrosis at very high doses of iron. The Committee noted at its twenty-seventh meeting (Annex 1, reference *62*) that iron overload in humans is a rare condition that occurs only in a few special situations and not in the general population. However, considering that a number of published animal studies have focused on the hepatic effects of dietary iron overload, these studies are summarized below. The data indicate that hepatic fibrosis and cirrhosis are observed only following repeated exposure to large doses of iron in the diet, although these effects are not observed in the majority of studies. Findings from these animal studies are in keeping with the human data previously evaluated by the Committee, which showed that only severe cases of siderosis in sub-Saharan Africans are associated with fibrosis and cirrhosis of the liver and are complicated by excessive alcohol consumption and malnutrition. Most individuals of this population group present with mild to moderate siderosis, which does not exert deleterious effects.

(i) Mice

In a series of experiments investigating the effect of excess dietary iron on liver function and hepatic iron and vitamin E status, groups of male weanling CD-1 mice (six mice per group) were assigned to one of four dietary ferrous sulfate treatments, providing 120 (control), 3000, 5000 or 8000 mg iron/kg diet, equivalent to 12, 300 (low dose), 500 (middle dose) and 800 (high dose) mg iron/kg bw per day, respectively, for 7 weeks. Body weights were measured weekly. At the end of the treatment period, all mice were killed by carbon dioxide asphyxiation, and livers were removed for analysis of iron and vitamin E content. In the high-dose group, a significant ($P < 0.05$) decrease of 36% in body weight gain was observed compared with the control group at the end of the study period. There was a significant ($P < 0.05$) increase of 39–53% in liver weight of mice of the middle- and high-dose groups. At all dose levels, an increase in hepatic iron content and a decrease in hepatic vitamin E content were observed compared with the control group ($P < 0.05$). These changes were dose dependent, and the amount of iron in

the liver was positively correlated with the amount of iron in the diet ($r = 0.980$). In a parallel experiment to determine the antidotal properties of vitamin E in acute iron poisoning, mice fed 8000 mg iron/kg diet for 7 weeks showed a 68% higher serum ALT activity compared with the control group. Microscopic examination of liver samples from these mice did not reveal any histopathological changes (Omara & Blakley, 1993).

Groups of 20 male BALB/cJ mice were fed diets supplemented with different doses of carbonyl iron and examined for potential liver effects. Four groups received diets containing carbonyl iron at levels of 0% (control), 0.5%, 1.5% or 3% (0, 5000, 15 000 or 30 000 mg iron/kg diet; equivalent to 0, 250, 750 and 1500 mg iron/kg bw per day, respectively) for 12 months. Five mice from each group were sacrificed at 2, 4, 8 and 12 months. Body weight, liver weight, hepatic iron content, electron microscopic examination of the liver, mitotic index in hepatocytes and hepatocyte nuclear size were all examined at 2, 4, 8 and 12 months. Iron supplementation was well tolerated by animals at all doses. In mice fed the highest level of dietary iron (1500 mg/kg bw per day), a significant ($P < 0.05$) decrease in body weight was observed compared with control mice at 2 and 8 months, but by 12 months, there were no significant differences in body weight. A dose-dependent and time-dependent increase in hepatic iron content was observed without any evidence of hepatic fibrosis or hepatocellular carcinoma. In the 1500 mg/kg bw per day group, liver iron concentrations were 13-fold higher ($P < 0.01$) after 12 months compared with the control group. Iron deposits were visible in all treated groups in periportal hepatocytes and sinusoidal cells from 2 months onwards. Changes of the hepatocyte nuclei, including iron-containing ferritin inclusions, enlargement of nucleus size, increase in mitotic index and the presence of abnormal mitotic figures, were observed in the mice fed the highest level of dietary iron for 12 months compared with control mice. The authors concluded that carbonyl iron supplementation produces significant iron overload in mice but does not result in liver fibrosis or hepatocellular carcinoma, although nuclear changes were produced in hepatocytes, which may represent preneoplastic changes (Pigeon et al., 1999).

(ii) Rats

Chronic experimental iron overload was induced in 30 male Sprague-Dawley rats by administering diets supplemented with 3.0% carbonyl iron for 1 month (equivalent to 1500 mg iron/kg bw per day), followed by 2.5% carbonyl iron for up to 12 months (equivalent to 1250 mg iron/kg bw per day). A similar control group was fed chow diet ad libitum, and a third group of rats was fed a chow diet in a restricted manner (to parallel the growth rate of iron-overloaded rats, which are known to have a reduced food intake and thus subsequent growth retardation). At time periods of 0.5, 1, 2, 3, 8 and 12 months, five rats from each group were killed by exsanguination via cardiac puncture under anaesthesia. A range of organs were excised for measurements of iron concentration and histological analysis. The liver was the predominant organ analysed. Compared with the two control groups, the iron-supplemented rats showed rapid increases in liver and splenic iron content. The liver iron concentration of these rats peaked at 3 months and then stabilized, whereas the splenic iron concentration increased more slowly than the liver iron

concentration but exceeded the liver concentration after 8 months of feeding. The iron-supplemented rats displayed hepatomegaly at 8 and 12 months. Within the liver, iron deposition was initially confined to periportal hepatocytes, but subsequently extended to the midzonal and centrilobular zones. Iron deposition also gradually increased in reticuloendothelial cells and became prominent after 3 months, at which time there was mild morphological evidence of hepatocellular injury. By 8 months, iron deposition was massive, and portal areas were enlarged with collections of iron-loaded macrophages and increased collagenous tissue; this periportal fibrosis was more pronounced upon examination at 12 months. Both control groups had normal hepatic histopathology. Increases in iron accumulation were also observed in the spleen, lymph nodes, intestine and kidneys. The authors concluded that the chronic administration of dietary iron resulted in hepatic fibrosis (Park et al., 1987).

To investigate the possible interaction between iron overload and alcohol metabolism, four groups of six male weanling Sprague-Dawley rats were fed one of four diets: 1) a control diet for 12 weeks; 2) a control diet for 8 weeks followed by an alcohol-containing diet (no details provided) for 4 weeks; 3) a diet supplemented with carbonyl iron at a level of 3% (equivalent to 1500 mg iron/kg bw per day) for 8 weeks followed by the control diet for 4 weeks; or 4) the iron-supplemented (3%) diet for 8 weeks followed by the alcohol-containing diet for 4 weeks. At the end of 12 weeks, animals were sacrificed, and blood was taken for serum ALT and blood alcohol analysis. The livers were removed for iron and protein analysis and microscopic investigation. No differences in food consumption were observed between the different groups. The iron-fed animals gained less weight than the controls and had higher iron concentrations in the liver (approximately 13 times higher; not statistically significant). Blood alcohol concentrations did not differ in any of the alcohol-fed animals. Serum ALT levels were increased in the rats fed iron and alcohol. Microscopically, there was no evidence of hepatic necrosis, fibrosis or any other pathological changes in the iron-fed animals, although ferritin molecules were observed in lysosomes and the cell sap of hepatocytes, which the authors concluded were signs of hepatocellular damage following iron overload (Stål & Hultcrantz, 1993).

To investigate the role of iron in ethanol-induced hepatocellular damage, groups of five male Sprague-Dawley rats received diets supplemented with carbonyl iron at a level of 2.5% or 3% (equivalent to 1250 and 1500 mg iron/kg bw per day, respectively) for 6 or 9 weeks, followed by diets not supplemented with iron, either control rat chow or an ethanol-containing diet (65 g/l), for 5 or 9 weeks. One group of animals receiving iron for 9 weeks followed by alcohol for 5 weeks received desferrioxamine subcutaneously during the final week (150–180 mg/kg bw per day; rationale for administering desferrioxamine not given). The parameters assessed included body weight, serum ALT, hepatic iron, protein, glutathione, and total and microsomal malondialdehyde levels. Microscopic examination of the liver was also performed. In rats administered 1500 mg iron/kg bw per day, significant ($P < 0.05$) increases in serum ALT activity and hepatic glutathione and microsomal malondialdehyde levels were observed (the latter two parameters were assessed only at the 1500 mg/kg bw per day dose) compared with the control group. Iron

chelation with desferrioxamine reversed the increase in serum ALT activity. At all dietary levels of iron, significant ($P < 0.05$) increases in hepatic iron content, iron deposition and malondialdehyde levels were observed. Treatment with desferrioxamine did not result in significantly decreased hepatic iron concentrations. Microscopic examination of the liver revealed no evidence of fibrosis in any of the control or treated rats. The authors concluded that the effects observed on the rat liver from the combined dietary overload of iron and ethanol are dependent on a pool of chelatable iron (Stål et al., 1996).

Conversely, a 26-week study was conducted with groups of seven newborn male and female Wistar-Furth rats to investigate the hypothesis that alcohol potentiates iron-associated liver toxicity. The pups received either a control diet or a diet supplemented with carbonyl iron at a level of 3% for 10 weeks and then a control diet, an alcohol-supplemented diet, an iron-supplemented diet (6 g iron/l, equal to 1920 and 2502 mg iron/kg bw per day in males and females, respectively) or a diet supplemented with both alcohol and iron, for an additional 16 weeks. The parameters evaluated included body weight, food consumption, hepatic iron concentration and serum aspartate aminotransferase (AST) and ALT activities. Liver biopsy was conducted at weeks 8 and 26, and livers were examined for histopathological variations. The iron-fed groups (with and without alcohol) weighed significantly ($P < 0.01$) less than the group fed alcohol or the control group, despite having consumed more diet. A significant ($P < 0.01$) increase in hepatic iron content was observed in the iron-fed rats compared with the control and alcohol-fed rats. There was a significant increase in the hepatic iron concentration in all iron-supplemented groups throughout the experimental period, compared with both the group fed an alcohol-supplemented diet and the group fed control diet. However, the co-administration of alcohol and iron resulted in a slightly lower hepatic iron concentration compared with the group fed iron only, with the value reaching statistical significance ($P < 0.02$). Serum AST and ALT activities did not differ significantly. Focal fibrosis was observed around iron-laden periportal macrophages, but was not present elsewhere in the liver of rats fed the iron-supplemented diet. The authors concluded that chronic alcohol feeding failed to potentiate hepatic fibrosis in iron-overloaded rats (Olynyk et al., 1995).

(b) Ammonium toxicity in different cell lines

The toxicity of millimole per litre concentrations of ammonium sulfate to three different cell lines was investigated. These were LLC-PK_1, an epithelial cell line of pig tubular renal origin; Jurkat cells, which are from human T-cell leukaemia; and GH_4 cells, which are from rat pituitary tumour and secrete prolactin and growth hormone. Proliferation and viability assays were performed, as well as determination of protein content and assessment of apoptosis. Intracellular calcium and cytosolic pH were measured, and cell cycle distributions were analysed. Ammonium sulfate reduced the growth of LLC-PK_1 cells in a dose-dependent manner at concentrations of $\geq$1 mmol/l, and the morphology of the cells changed, such that the treated cells were less densely packed and emitted prolongations that were not found in untreated cells. The authors suggested that this indicated that a certain degree of differentiation was occurring, despite the cell growth being

arrested. The growth of GH_4 cells was only mildly affected by treatment (at concentrations greater than 1 mmol ammonium sulfate/l), and the morphology of the cells was not affected. Concentrations from 0.1 to 6 mmol ammonium sulfate/l decreased the number of Jurkat cells in a dose-dependent manner; the cells were found to die by apoptosis after accumulating at S-phase. Ammonium chloride (15 mmol/l) treatment resulted in calcium mobilization in all cells, but predominantly in Jurkat cells, which had a median effective concentration (EC_{50}) of 8.5 ± 0.4 mmol/l. The authors concluded that the toxic effects caused by ammonium differ depending on the cell type (Mirabet et al., 1997).

(c) Effects of phosphates on kidney

Previous evaluation of phosphoric acid and phosphate salts by the Committee at its twenty-sixth meeting (Annex 1, reference *59*) focused on the development of nephrocalcinosis in the rat following oral administration of diets high in phosphorus content. Nephrocalcinosis has been defined as calcified deposits, mainly in the form of calcium phosphate, in tubules located predominantly at the corticomedullary junction of the kidney. Subsequent to the previous evaluation of phosphoric acid and phosphate salts, numerous studies have been published with regard to high phosphorus intake and nephrocalcinosis in the rat, paying special attention to the contribution of the calcium to phosphorus ratio in the diet to this effect. Female rats are particularly susceptible to phosphorus-induced nephrocalcinosis, the incidence of which is 100% and the severity moderate to severe in this sex; in males, the incidence is 0–25% and the severity mild (Clapp et al., 1982; Cockell et al., 2002). Studies on the kidney effects of phosphates that have become available subsequent to the twenty-sixth meeting of the Committee are discussed briefly below.

The incidence and severity of nephrocalcinosis were significantly greater in animals fed high-phosphorus diets compared with animals fed low to adequate dietary levels of phosphorus in the range of 2000–4000 mg/kg diet (0.2–0.4%). Animals fed phosphorus at levels of 0.2% in the diet did not develop nephrocalcinosis after 4–8 weeks of feeding, and those fed 0.3% phosphorus diets generally did not develop nephrocalcinosis or developed only very mild nephrocalcinosis after 3–16 weeks of feeding. Animals fed adequate phosphorus levels of 0.4% in the diet also developed only mild nephrocalcinosis. Diets high in phosphorus content induced nephrocalcinosis in weanling females (approximately 3–4 weeks old) of various rat strains following feeding for 2–16 weeks. High dietary phosphorus levels were in the range of 5000–12 000 mg/kg diet (0.5–1.2%) and were provided in the form of phosphate salts (calcium phosphate, monosodium phosphate, monopotassium phosphate, potassium tripolyphosphate or potassium dihydrogen phosphate) (Clapp et al., 1982; Al-Modhefer et al., 1986; Hoek et al., 1988; Ritskes-Hoitinga et al., 1989, 1992; Matsuzaki et al., 2001; Cockell et al., 2002; Cockell & Belonje, 2004).

Increasing the calcium content of the diet while maintaining high phosphorus levels (i.e. increasing the calcium to phosphorus ratio) significantly reduced the incidence and severity of nephrocalcinosis. Calcium to phosphorus ratios of less than 1 resulted in a high incidence of nephrocalcinosis. Adjusting the calcium to

phosphorus ratio to values greater than 1 and approaching values between 1.3 and 1.9 significantly reduced the incidence and severity of nephrocalcinosis (Clapp et al., 1982; Al-Modhefer et al., 1986; Hoek et al., 1988; Cockell et al., 2002).

Calcification of the kidney and nephrocalcinosis were observed to be irreversible; animals fed diets high in phosphorus for 2–8 weeks and then switched to control diets low in phosphorus (as sodium or potassium phosphate) for periods of up to 25 weeks displayed calcification of the kidney and nephrocalcinosis to a similar extent as for animals continuously fed high-phosphorus diets (Soeterboek et al., 1991; Cockell & Belonje, 2004).

A significant increase in relative kidney weights has been associated with dietary phosphorus levels (as sodium or potassium phosphate) that induce nephrocalcinosis, accompanied by a trend towards an increase, or a significant increase, in kidney mineral (calcium, phosphate and magnesium) content (Hoek et al., 1988; Ritskes-Hoitinga et al., 1989, 1992; Soeterboek et al., 1991; Cockell et al., 2002; Cockell & Belonje, 2004). Altered single-nephron function was observed in rats fed diets high in phosphorus (0.8%, form of phosphorus not stated), a diet that also produced nephrocalcinosis. Calcium deposits resulted in tubular obstruction, significantly prolonging proximal and distal fluid transit times in these rats compared with rats fed diets that did not induce nephrocalcinosis (Al-Modhefer et al., 1986).

Significant changes in urinary and faecal output and whole-body retention of minerals have been reported in rats fed diets high in phosphorus as a result of sodium phosphate dietary supplementation. In female SPF-derived Wistar rats (six per group) fed a 0.4% phosphorus diet (0.25% calcium) for 4 weeks, a significant ($P < 0.01$) increase in urinary and faecal excretion of phosphorus and a significant ($P < 0.01$) decrease in urinary calcium were observed compared with rats fed a 0.2% phosphorus diet. No significant differences in urinary, faecal or renal magnesium levels were observed that were attributable to high phosphorus intake. Whole-body retention of phosphorus was significantly ($P < 0.01$) decreased, but that of calcium and magnesium was unaffected. In the same study, rats fed a 0.8% phosphorus diet (0.5% calcium) for 4 weeks displayed a significantly ($P < 0.01$) increased urinary and faecal output of phosphorus compared with rats fed a 0.4% phosphorus diet. All other mineral parameters were unaffected (Hoek et al., 1988).

Urinary phosphorus output was also significantly ($P < 0.01$) increased in female SPF-derived Wistar rats (six per group) fed a 0.6% phosphorus diet (as sodium phosphate) for 4 weeks compared with rats fed a 0.4% phosphorus diet in a similarly designed experiment. Faecal excretion of phosphorus was unchanged, resulting in an increase in whole-body phosphorus retention. Urinary and faecal calcium and magnesium levels were unaffected. Plasma calcium and magnesium levels were significantly ($P < 0.05$) reduced, but plasma phosphorus levels were unaffected (Ritskes-Hoitinga et al., 1989).

In RP and Long-Evans female rats (six per group), urinary concentrations of calcium and magnesium were significantly ($P < 0.05$) reduced and urinary phosphorus levels significantly ($P < 0.05$) increased following 4 weeks on a 0.5% phosphorus diet as sodium phosphate (0.50% calcium), compared with rats fed a

0.2% phosphorus diet. Plasma phosphorus as well as plasma parathyroid hormone levels were also significantly ($P < 0.05$) increased in these animals, whereas plasma calcium levels were significantly ($P < 0.05$) reduced. Faecal magnesium output and whole-body magnesium retention were significantly ($P < 0.05$) increased (Ritskes-Hoitinga et al., 1992). A significant ($P < 0.05$) reduction in the urinary pH was observed (Ritskes-Hoitinga et al., 1989, 1992), as was an increase ($P < 0.05$) in urine albumin levels at higher dietary phosphorus levels as sodium phosphate, suggesting an impairment of kidney function (Ritskes-Hoitinga et al., 1989).

In a study conducted with potassium salts (potassium tripolyphosphate and potassium dihydrogen phosphate), female Wistar rats (six per group) fed a 1.2% phosphorus diet (0.54% calcium content) for 21 days exhibited significant ($P < 0.05$) decreases in phosphorus, calcium and magnesium levels in urine compared with rats fed a 0.3% phosphorus diet. Urinary creatinine, albumin, β_2-microglobulin and *N*-acetyl-β-D-glucosaminidase activity levels were significantly ($P < 0.05$) increased in rats fed the diet high in potassium tripolyphosphate, but not in rats fed high levels of potassium dihydrogen phosphate. Consumption of the high-potassium tripolyphosphate diet, but not the high-potassium dihydrogen phosphate diet, also resulted in significant ($P < 0.05$) reductions in final body weights and food intake compared with rats fed the low-potassium diets. Furthermore, rats administered the high-potassium tripolyphosphate diet displayed more severe nephrocalcinosis than did rats fed the high-potassium dihydrogen phosphate diet (Matsuzaki et al., 2001).

(d) Effects of phosphates on bone

The Committee's evaluation of phosphoric acid and phosphate salts at its twenty-ninth meeting (Annex 1, reference *70*) also raised concern regarding possible bone loss in the rat following oral administration of diets high in phosphorus content. The animal data evaluated showed that excessive dietary phosphorus resulted in a decrease in serum calcium levels, which stimulated secretion of parathyroid hormone and thus the subsequent resorption of calcium from bone to maintain plasma calcium homeostasis. The increased parathyroid hormone stimulates the synthesis of cacitriol via the renal enzyme 1-alpha-hydroxylase, which then promotes calcium absorption from the gut. Parathyroid hormone secretion is then subject to feedback regulation by calcitriol and calcium. The effects of high phosphate are difficult to distinguish from the effects of low calcium per se, as this would also trigger increased levels of parathyroid hormone. The relationship between parathyroid hormone and phosphate is complex and may differ in acute and chronic exposures. The Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (2005) considered the relationship of phosphate intake and bone health in detail and concluded that the elevated parathyroid hormone levels associated with supplemental phosphorus intakes in humans reflect a short-term adjustment to maintain plasma calcium levels and do not necessarily represent an adverse effect of phosphate on bone health. The long-term effects on bone health of elevated parathyroid hormone resulting from high phosphate intakes are, however, unknown.

Subsequent to the previous evaluation on phosphoric acid and phosphate salts, several additional animal studies have been published with regard to the effects of high phosphorus intake on bone and are summarized below.

(i) Rats

Twenty-four male weanling (4–11 weeks of age) Sprague-Dawley rats and 24 mature rats (12–19 weeks of age) were randomly assigned to each of four experimental diets with combinations of high and low levels of protein and phosphorus for 7 weeks. Diets consisted of 0.3% phosphorus with 25% casein, 0.8% phosphorus with 25% casein, 0.3% phosphorus with 45% casein or 0.8% phosphorus with 45% casein. Phosphorus was provided in the form of calcium hydrogen phosphate, and the calcium level of all diets was constant. Dietary phosphorus levels were 3000 and 8000 mg/kg, equivalent to 300 and 800 mg/kg bw per day in the weanling rats and 150 and 400 mg/kg bw per day in the mature rats. After 7 weeks, animals were killed, and blood, muscle and bone were collected for calcium and phosphorus analysis. Mechanical properties of femurs were also determined. Plasma phosphorus levels were significantly ($P < 0.01$) increased in young and mature rats fed the 0.8% phosphorus diet compared with rats fed the 0.3% phosphorus diet. Plasma calcium levels were significantly ($P < 0.05$) increased in mature rats, but not in young rats, of the 0.8% group. A significant ($P < 0.05$) increase in muscle weight was observed in young rats, but not in mature rats, fed the 0.8% phosphorus diet compared with rats fed the 0.3% phosphorus diet. Muscle phosphorus concentration remained unaffected by the dietary phosphorus level. In young rats, body weight and femur weight were significantly ($P < 0.05$) increased in the 0.8% group compared with the 0.3% group. Femur length remained unchanged. In mature rats, femur weight and length were similar in the 0.3% and 0.8% phosphorus groups. Maximum breaking load, a measure of bone strength, and compliance, a measure of bone flexibility, were unaffected by the level of dietary phosphorus in both young and mature rats. Femur calcium concentrations were not significantly different between groups. However, femur phosphorus concentrations were significantly ($P < 0.01$) elevated in young rats, but not mature rats, fed the 0.8% phosphorus diet compared with rats fed the 0.3% phosphorus diet. The authors concluded that dietary levels of protein and phosphorus can affect some parameters of bone, blood and muscle and that the effects may be dependent on the age of the rats (Howe & Beecher, 1983).

Fifty-two female weanling (34 days of age) Sprague-Dawley rats were divided into two groups, such that 27 rats were permitted free access to running wheels (treatment group), whereas the remaining rats were kept in conventional cages (control group). After 29 weeks, groups were further divided into three groups to receive one of three diets: 1) adequate calcium (0.5%) and adequate phosphorus (0.4%) levels; 2) adequate calcium (0.5%) and high phosphorus (1.2%) levels; and 3) low calcium (0.3%) and high phosphorus (1.2%) levels. Supplemental phosphorus was provided as sodium phosphate and monopotassium phosphate. Dietary phosphorus levels were equivalent to 200 (adequate) and 600 (high) mg/kg bw per day. Food consumption, body weight and voluntary wheel exercise over 24-h periods were recorded weekly. The experimental period lasted until the rats were

17 months of age, at which point they were killed by decapitation and the right and left femurs and kidneys were removed for analysis of calcium and phosphorus. The phosphorus content of the diet did not significantly affect body weight between groups. Both 1.2% phosphorus diet groups displayed significantly ($P < 0.01$) increased kidney concentrations of calcium and phosphorus compared with the 0.4% phosphorus group. However, kidney weight was significantly ($P < 0.05$) increased only when the calcium content of the diet was reduced to 0.3% calcium and the phosphorus content was increased to 1.2%. Indicators of bone quality, such as bone ash, density and specific gravity, were significantly ($P < 0.01$) decreased in vertebral bone (atlas), left tibias and right femurs of the 1.2% phosphorus groups compared with the 0.4% phosphorus group. Reducing the calcium content of the diet to 0.3% further significantly ($P < 0.0001$) decreased bone gravimetric measurements in the 1.2% phosphorus groups. The detrimental effects of a high-phosphorus diet were more pronounced in the vertebral bone (atlas) than in the long bones. Running had no effect on kidney mineralization, but was effective in improving bone gravimetric measurements (Bauer & Griminger, 1983).

Three groups of 1-month-old male Wistar rats (9 or 10 rats per group) were randomly assigned to one of three diets varying in calcium to phosphorus ratio for 8 weeks: 1) a control diet with 0.6% phosphorus (ratio 1:1); 2) an experimental diet containing 1.2% phosphorus (ratio 1:2); or 3) an experimental diet containing 1.8% phosphorus (ratio 1:3). The calcium content remained constant at 0.6% in all diets. Phosphorus was provided in the form of calcium hydrogen phosphate and potassium phosphate. Dietary phosphorus levels were equivalent to 300, 600 or 650 mg/kg bw per day, respectively. Analyses of the right and left femurs, right femoral neck and tibial shaft, and right tibias were conducted. Body weight, serum parathyroid hormone, calcium and phosphate content, bone mineral content and bone mineral density were determined. The body weights of the rats fed each experimental diet were significantly ($P < 0.001$) decreased compared with body weights of rats fed the control diet from day 28 until the end of the treatment period. Serum parathyroid hormone concentrations were significantly ($P < 0.001$) increased in the 1.8% phosphorus group compared with the control and the 1.2% phosphorus group, whereas serum calcium and phosphate levels did not differ between groups. Both high-phosphorus groups (1.2% and 1.8%) had significantly ($P < 0.001$) lower bone mineral content and bone mineral density compared with control rats. Final femur area values were unaffected by a high phosphate intake. However, femur volume was significantly ($P < 0.001$) reduced in the high-phosphate groups compared with the control group, and femur length was significantly ($P = 0.019$) shorter in the 1.8% phosphorus group compared with the 1.2% phosphorus group. In the cortical bone of the femur, high-phosphate diets resulted in significantly ($P < 0.001$) reduced bone mineral content, bone mineral density, cross-sectional area and mean thickness compared with the control diet. High-phosphate intake also resulted in significantly ($P < 0.05$) decreased tibia volume and cross-sectional area of the tibial cortical bone and negatively affected bone surface structure values in the tibial trabecular bone. In terms of the mechanical properties of bone, the high-phosphate diets significantly ($P < 0.001$) reduced femoral neck and tibial shaft ultimate strength, as well as tibia stiffness and toughness. The authors concluded that a high phosphate intake obstructed growth and reduced bone material

properties and bone strength in growing male rats, even when calcium intake was sufficient (Huttunen et al., 2007).

Groups of five female Wistar rats were fed diets containing low phosphorus (0.15%), adequate phosphorus (control diet, 0.5%) or high phosphorus (1.5%) for 42 days, equal to 74, 250 and 760 mg/kg bw per day, respectively. The calcium content remained constant at 0.5% in all diets. Supplemental phosphorus was provided as potassium phosphate. Food intake and body weights were recorded throughout the treatment period. During the last 3 days of treatment, faeces and urine of all rats were collected for calcium and phosphorus balance studies. At termination, blood samples and the fifth lumbar vertebra were collected from each rat. Measurements of bone mineral content and bone mineral density were conducted, as well as serum analyses of phosphorus, calcium parathyroid hormone and osteocalcin. Urinary deoxypyridinoline was also measured. No significant differences in the food intake, weight gain or serum calcium concentrations among the three groups were observed. The serum phosphorus and parathyroid concentrations were significantly ($P < 0.05$) decreased in the low-phosphorus group compared with the control and the high-phosphorus groups. The urinary excretion of calcium was significantly ($P < 0.05$) increased in the low-phosphorus group compared with the other two groups, and the absorption of calcium was significantly ($P < 0.05$) increased with decreasing dietary phosphorus intake. Absorption and urinary excretion of phosphorus were significantly ($P < 0.05$) increased with increasing dietary phosphorus levels. The serum osteocalcin concentration and urinary excretion of deoxypyridinoline were significantly ($P < 0.05$) higher in the high-phosphorus group than in the other two groups. Bone mineral content and compression load on the fifth lumbar vertebra were significantly ($P < 0.05$) higher in the low-phosphorus group compared with the other two groups, whereas the bone mineral density of the fifth lumbar vertebra was significantly ($P < 0.05$) increased with decreasing dietary phosphorus levels. The authors suggested that a high-phosphorus diet affects bone metabolism, although there was no discussion on the effects or mechanism. Furthermore, the authors suggested that the formation of insoluble calcium and phosphorus salts in the intestinal lumen is an important factor for the decrease in calcium absorption (Koshihara et al., 2005).

(ii) Pigs

Growing Hampshire-Yorkshire pigs were randomly assigned to receive diets containing 0.3%, 0.6% or 0.9% phosphorus, in the form of dicalcium phosphate, equivalent to 120, 240 and 360 mg/kg bw per day, respectively. For each level of phosphorus, the calcium to phosphorus ratio was adjusted by increasing the calcium content of the diet, resulting in ratios of 1:1, 2:1 or 3:1. Four pen replicates of two pigs (one barrow and one gilt) per pen were fed the diets for 28 days. At the end of the treatment period, all pigs were killed and bled. The third and fourth metatarsals and metacarpals were removed from each rear and front foot, as well as the femurs, for determination of breaking strength. Whole blood and prothrombin clotting times were determined. A fatal haemorrhagic condition (based on necropsy findings) occurred in the pigs fed the high calcium levels (1.2–2.7%), so at day 28, vitamin K (5 mg menadione/kg bw) was added to the diet in two of the four replications, and

the experiment was continued for an additional 14 days. In the absence of supplemental vitamin K, whole blood and prothrombin clotting times were related to the calcium level in the diet, with the times increasing with higher levels of dietary calcium ($P < 0.01$). However, when vitamin K was added to the diet, clotting times were constant across all dietary levels of calcium. Pigs fed the 0.3% phosphorus diet grew at a significantly ($P < 0.001$) slower rate and required significantly ($P < 0.02$) more feed per unit of weight gain (efficiency) compared with pigs fed the 0.6% and 0.9% phosphorus diets at all calcium to phosphorus ratios. In general, a trend was observed in which increasing the calcium to phosphorus ratio at all phosphorus levels also resulted in slower weight gain. Pigs fed diets with calcium to phosphorus ratios of 2:1 and 3:1 were also significantly ($P < 0.001$) less efficient than pigs fed the 1:1 calcium to phosphorus diet. Bone breaking strength of all bone types was significantly ($P < 0.001$) reduced in pigs fed the 0.3% phosphorus diet compared with pigs fed the other two phosphorus-supplemented diets. Raising the calcium to phosphorus ratio in the 0.6% and 0.9% phosphorus groups significantly ($P < 0.01$) increased bone strength, an effect that was not observed in the 0.3% phosphorus group. The authors concluded that excessive levels of dietary calcium interfered with normal blood clotting mechanisms in growing pigs, suggesting that the pigs' gut synthesis of vitamin K may not meet the pigs' requirement (Hall et al., 1991).

(iii) Non-human primates

Groups of seven young baboons (*Papio ursinus*) were fed one of four diets replete in vitamin D (1000 international units/kg diet) for 16 months. In three of the four diets, the phosphorus content was kept constant (3100 mg/kg diet), while the levels of calcium varied (400, 1400 or 4000 mg calcium/kg diet). The fourth diet was low in both calcium and phosphorus (400 mg calcium/kg diet and 900 mg phosphorus/kg diet). The form of phosphorus was not stated. At intervals of 3–4 weeks, body weight was recorded and blood was collected for haematological analyses. Radiographs of hands and forearms were recorded after 3, 8 and 16 months of treatment, and iliac crest bone biopsies were taken for histomorphometric studies after 8 months of treatment and at termination. Baboons were killed after 16 months of treatment with an overdose of sodium pentobarbitone. No clinical abnormalities or stigmata of rickets were observed in any of the baboons. Weight gains were similar in all groups. Serum calcium levels were constant except for the low-calcium group, where values fell below those of the high-calcium group between 220 and 260 days. Similarly, the low-calcium group, but not the high-calcium group, developed hyperphosphataemia between 200 and 400 days; and baboons in the 3100 mg phosphorus/kg diet group, but not the 900 mg phosphorus/kg diet group, experienced hyperphosphataemia. No significant differences were observed between the groups for serum AP, albumin or magnesium. Observations of hand and wrist radiographs showed no changes at 3 months. However, by 8 months, three of the baboons in the low-calcium group were found to have mild changes compatible with rickets. Histological features of osteomalacia were observed at 8 and 16 months and were associated with a reduction in calcium, and not phosphorus, intake (Pettifor et al., 1984).

2.3 Observations in humans

2.3.1 Clinical studies

(a) Ferrous ammonium phosphate

The human safety and tolerance of ferrous ammonium phosphate were also examined as part of the bioavailability study previously described in section 2.1.2. The study consisted of two randomized, double-blind, two-period crossover trials in which test meals containing ferrous ammonium phosphate, ferrous sulfate or ferric pyrophosphate were consumed by test subjects (38 healthy women aged 18–30 years, mean body weight 56.71 ± 4.87 kg). Test meals consisted of 250 ml of reconstituted full cream instant milk powder supplemented with radiolabelled ferrous ammonium phosphate (^{57}Fe), ferrous sulfate (^{58}Fe) or ferric pyrophosphate (^{57}Fe) at a fortification level of 2.5 mg of iron per serving. Subjects were assigned to one of two trials and, within each trial, to one of the two treatments. In the first trial, subjects consumed two servings of either ferrous ammonium phosphate or ferrous sulfate test meals on day 1 and then two servings of the other test meal on day 2. In the second trial, subjects consumed two servings of either ferric pyrophosphate or ferrous sulfate test meals on day 1 and then two servings of the other test meal on day 2. Total intake of iron from the ferrous ammonium phosphate, ferrous sulfate and ferric pyrophosphate compounds was 5.5 mg, 6.6 mg and 6.4 mg, respectively. In total, nine minor adverse events were reported by subjects, equally distributed among the three groups. These were characterized by cold- and flu-like symptoms, including runny nose (3), sore throat (2), nasal congestion (1), cough with sore throat (1) and a general account of the common cold (2). No other adverse effects were reported (Nestlé Research Center, 2007b).

(b) Iron

(i) Studies with ferrous iron supplements conducted in adults

Controlled clinical trials conducted on ferrous sulfate and other oral iron formulations subsequent to the latest evaluation on iron at the twenty-seventh meeting (Annex 1, reference *62*) are summarized below.

The tolerability of supplemental iron delivered from a wax matrix tablet of ferrous sulfate was compared with that from a conventional ferrous sulfate tablet in a randomized, single-blind, parallel group study. No control (placebo) group was included. Both tablets delivered 50 mg iron, and the 543 healthy subjects (aged 18–39 years) evaluated in the study were randomly assigned to one of the two treatments and instructed to take one tablet each morning for 56 consecutive days. Baseline haemoglobin levels were determined, and subjects were instructed to record the occurrence of any side effects. The incidence of adverse gastrointestinal effects was significantly ($P < 0.001$) greater among subjects taking the conventional tablets (50%, 1012 reports) than among those taking the wax matrix preparation (19%, 306 reports), suggesting that the wax matrix preparation was better tolerated. The most common side effects among those taking the conventional tablets were abdominal discomfort (19.5%) and constipation (17.3%). Of the 543 subjects, 44

receiving the conventional tablets and 33 receiving the wax matrix preparation did not complete the treatment course because of intolerable gastrointestinal side effects. The authors concluded that the wax matrix iron delivery system was better tolerated than the conventional iron supplement tablet (Brock et al., 1985).

In a prospective evaluation of the effects of oral ferrous sulfate therapy on gastrointestinal symptoms in healthy volunteers, 14 subjects (13 females and 1 male aged 24–48 years) received 975 mg ferrous sulfate/day (three tablets, 325 mg each, per day, equivalent to 360 mg iron/day) for 2 weeks. Before and after iron therapy, subjects completed a checklist of gastrointestinal symptoms, stool haemoccult and faecal blood loss were determined, and upper endoscopy biopsies were performed. Another group of 13 healthy volunteers (12 males and 1 female aged 23–50 years) received the same dose (equivalent to 360 mg iron/day) for 1 week. Baseline and post-treatment stool haemoccult testing was performed. All subjects developed dark brown-black stools during the study. Following 1 and 2 weeks of ferrous sulfate supplementation, symptoms of nausea and vomiting, but not abdominal pain, were significantly ($P < 0.05$) increased in severity compared with the 2 weeks prior to initiation of iron therapy. The stool samples of all 27 volunteers were haemoccult negative prior to treatment; after iron therapy, only one sample was questionably trace-positive. Faecal blood loss was not affected by iron treatment. Endoscopic analysis revealed significantly ($P = 0.003$) increased incidences of erythema and small subepithelial haemorrhages of the stomach. No endoscopic abnormalities of the duodenum were noted either before or after iron therapy. Biopsies of the stomach and duodenum did not reveal any demonstrable histological differences before or after iron administration. Endoscopy scores positively and significantly correlated with those of abdominal pain ($r = 0.64$, $P = 0.01$). The authors concluded that oral ferrous sulfate therapy may cause mild endoscopic abnormalities in the stomach, which are of uncertain clinical significance; and ferrous sulfate rarely causes haemoccult-positive stools (Laine et al., 1988).

A prospective, controlled, double-blind multicentre trial was performed to assess the tolerability of iron protein succinylate (ITF 282) compared with a ferrous sulfate controlled-release tablet. One thousand and ninety-five patients were randomized to receive either two ITF 282 tablets per day (60 mg iron/tablet, 120 mg iron/day) or one controlled-release ferrous sulfate tablet per day containing 105 mg iron; both treatments lasted 60 days. Patients (male and female, aged 15–88 years) were affected with iron deficiency or iron-deficient anaemia. Blood samples for haematology and clinical chemistry analyses were collected from patients at days 30 and 60 following initiation of iron therapy. There were no compound-related adverse effects on haematology or serum chemistry parameters, and general tolerability to both treatments was deemed favourable, but significantly ($P < 0.001$) more favourable with ITF 282. With ITF 282, 63 patients (11.5%) complained of 69 adverse reactions (25 heartburn, 19 constipation, 25 abdominal pain), compared with 141 adverse events (33 heartburn, 31 epigastric pain, 23 abdominal pain, 8 skin rash, 14 nausea) reported by 127 (26.3%) patients taking ferrous sulfate. The frequency and duration of these adverse events were significantly ($P < 0.01$) less in patients receiving ITF 282 than in those receiving ferrous sulfate. All events

experienced with both iron formulations were transient, and none could be classified as severe or potentially harmful by the author. The author concluded that the tolerability of ITF 282 is significantly more favourable than that of ferrous sulfate, and thus it may be suitable as first-line treatment for the management of iron deficiency (Liguori, 1993).

In a controlled, double-blind crossover trial, haem iron and non-haem iron were administered to 100 healthy volunteers for periods of 1 month each. The subjects (Swedish adult men and women, aged 34–52 years) were randomly assigned to one of two groups. Group 1 received two tablets per day, each containing 1.2 mg haem iron from porcine blood plus 8 mg non-haem iron as iron fumarate. Group 2 received one tablet per day containing 60 mg haem iron as iron fumarate. The study was divided into three consecutive periods of 1 month each, and all participants received a placebo for one of the last two periods. Participants served as their own placebo controls and assessed their own side effects by keeping symptom diaries. Baseline serum ferritin and haemoglobin concentrations were determined. Although no significant differences in basic iron status were detected between the two groups, the number of subjects experiencing obstipation (35%) as well as total gastrointestinal side effects (25%; nausea, epigastric pain, obstipation and diarrhoea) was significantly ($P < 0.05$) greater in the group receiving iron in the form of ferrous fumarate than in the haem/non-haem iron combination group or placebo group. The effects reported for the haem iron treatment were indistinguishable from the placebo. The authors concluded that a low-dose iron supplement containing both haem iron and non-haem iron has fewer side effects than an equipotent non-haem iron supplement (Frykman et al., 1994).

In a randomized controlled trial investigating oral and intravenous iron as an adjuvant to autologous blood donation in elective surgery, groups of 30 patients, aged 18 years and older (15 patients per sex per group), received 3 × 100 mg of iron per day from ferrous fumarate orally for 5 weeks before operation; 200 mg of iron (Fe^{3+}) as an intravenous dose given after each blood donation; or no iron supplementation (control). Blood donations were scheduled for 7-day intervals beginning on day –35 before surgery. The outcomes measured were net red blood cell production, haematological values, measures of iron metabolism and postoperative erythropoiesis. Haematological variables (haemoglobin, mean corpuscular haemoglobin, reticulocytes and mean cell haemoglobin concentration) were not significantly different among the three groups. The red blood cell distribution width was significantly ($P < 0.05$) higher in the two iron-treated groups compared with the controls, but the increases were not seen at all donations. In the intravenous-treated group, serum ferritin was increased at all donations compared with the controls, but was significant ($P < 0.01$) only at two of the four donations. Transferrin saturation fell slightly after the first donation in both iron-treated groups and then remained almost stable until surgery. Net red blood cell production was not significantly different among the three groups; however, there was a trend (non-significant) towards better erythropoiesis in iron-treated patients than in controls. Two patients from the oral iron group and two from the intravenous iron group were excluded from the study because of suspected mild adverse reactions to the iron supplementation. Of the two orally treated patients, one had abdominal pain and

the other had an itching erythema. In the intravenously treated patients, one had an itching erythema and the other experienced general discomfort and dizziness. Of the adverse effects reported, constipation and diarrhoea were reported more frequently in the orally treated group than in the intravenously treated group. The authors concluded that neither oral nor intravenous iron therapy administered to patients undergoing autologous blood donations preoperatively enhanced the success of blood donations (Weisbach et al., 1999).

The effect of weekly iron supplementation on anaemia and iron deficiency among adult non-pregnant female tea pluckers was investigated in a randomized, double-blind intervention trial. A total of 280 women received either a capsule containing 200 mg of ferrous fumarate (equivalent to 66 mg of iron) and 200 mg of folic acid or a placebo control on a weekly basis for 24 weeks. The iron equivalent dose of 66 mg/week is equivalent to approximately 10 mg/day. Baseline measurements were obtained for haemoglobin, haematocrit, ferritin and mean corpuscular haemoglobin concentration, and these were all determined again at the end of the treatment period. Mean haemoglobin concentration significantly ($P < 0.001$) increased in the iron-supplemented group compared with baseline measurements, particularly in anaemic women (those with baseline haemoglobin levels of 119 g/l or less). Both groups showed a non-significant decrease in ferritin values, and the iron-supplemented group showed a significant ($P = 0.04$) increase in haematocrit values. Supplemented anaemic women showed a greater ($P < 0.0001$) increase in haemoglobin compared with those who had normal haemoglobin values at baseline (≥120 g/l). Giddiness, dizziness, bouts of vomiting, diarrhoea and stomach pains were side effects reported in both groups: 14.4% of the supplement group and 22.7% of the control group, not statistically significantly different between the two groups (Gilgen & Mascie-Taylor, 2001).

Regular healthy blood donors (289 males and 237 females) were randomized to receive 0 (control), 20 or 40 mg iron/day for 6 months in a double-blind, placebo-controlled study. Iron supplementation was provided in the form of capsules as ferrous gluconate. The parameters assessed initially and at each follow-up visit were haemoglobin, serum ferritin and soluble transferrin receptor levels. Follow-up periods were every 2–3 months. Volunteers with haemoglobin concentrations less than 13.5 g/dl (males) or 12.5 g/dl (females) were deferred from blood donation but not excluded from the study. A total of 141 (49%) male volunteers dropped out of the study: 44%, 44% and 58% from the 40 mg, 20 mg and placebo groups, respectively. A total of 96 (41%) female volunteers dropped out of the study: 28%, 44% and 49% from the 40 mg, 20 mg and placebo groups, respectively. Volunteers withdrew from the study because of gastrointestinal complaints, poor compliance or other reasons (details not provided). Deferral from donation occurred in a small number of visits (~2%) for both males and females and was more frequently the case in the placebo group than in either of the iron-supplemented groups ($P < 0.05$). Mean serum ferritin concentrations decreased in the control groups. In the 20 mg dose group, ferritin concentrations decreased by 28% in males and increased by 18% in females; and in the 40 mg dose group, ferritin concentrations remained constant in males and increased by 63% in females. The mean concentration of soluble transferrin receptors rose in the control groups,

remained constant in the male treated groups, remained constant in females of the 20 mg dose group and decreased in females of the 40 mg dose group. Approximately 60% of the subjects did not report any side effects, and there were no significant differences in the incidence of adverse effects (such as gastrointestinal complaints) among groups. The frequency of gastrointestinal complaints specifically was low, occurring at 11%, 13% and 11% in the 40 mg, 20 mg and control groups, respectively (Radtke et al., 2004).

To determine whether low-dose iron supplementation can replace the conventional doses for treatment of iron deficiency anaemia in the elderly, patients over 80 years of age received daily iron supplementation. Ninety anaemic patients (haemoglobin levels between 80 and 119 g/dl and ferritin levels below 40 ng/ml) were randomized to receive one of three supplemental treatments (30 patients per group): 15, 50 or 150 mg (conventional dose) of elemental iron daily for 2 months. Another group of 30 non-anaemic patients from the same hospital received 15 mg of iron daily for 2 months and served as a reference group. Iron was provided as liquid ferrous gluconate mixed in simple syrup for the 15 and 50 mg dose groups, whereas 1 tablet of 500 mg ferrous calcium citrate was taken 3 times daily for 2 months for the 150 mg iron group. Iron absorption was assessed 2 h after intake of 15 mg iron in all patients. Serum iron was measured before the first ingestion of iron and then at 15, 30, 45, 60 and 120 min thereafter. Baseline haemoglobin and ferritin concentrations were measured and again on days 30 and 60 following initiation of iron therapy. Patients also completed a questionnaire on potential side effects. Serum iron concentrations increased in all patients after ingestion of 15 mg iron, but were significant ($P < 0.001$) only in the anaemic patients. In the first 2 h, iron was absorbed more quickly in the anaemic patients than in the non-anaemic group ($P = 0.029$). At the end of the 2-month treatment period, serum haemoglobin and ferritin concentrations had increased significantly ($P < 0.001$) in all anaemic patients compared with baseline measurements, with no statistically significant differences among the three dose groups. There was no significant change in serum haemoglobin or ferritin in the reference group. The lower the baseline haemoglobin level, the more it increased with iron treatment ($P < 0.01$). Black stools were reported in a dose-dependent manner in the 50 mg and 150 mg iron groups, but not in the 15 mg group ($P < 0.05$). Nausea and vomiting occurred in a dose-dependent manner in all three dose groups ($P < 0.05$). Other reported adverse events included abdominal discomfort, diarrhoea, constipation and darkened stools, all of which occurred significantly ($P < 0.05$) in the 50 and 150 mg dose groups compared with the 15 mg dose group (Rimon et al., 2005).

Female blood donors (less than 45 years of age, mean age 29 years) were recruited in a study to investigate the effects of iron supplementation on improving iron stores for safe blood donation in women. A total of 412 non-pregnant women received either 150 mg of ferrous sulfate ($n = 207$) or a placebo ($n = 205$) 3 times daily (total iron intake of 166 mg/day) for 1 week. Each volunteer was scheduled for a total of four visits: an initial visit and three subsequent predonation visits at 4-month intervals. Treatment or placebo was taken for 1 week after each blood donation. The haemoglobin concentration, haematocrit, serum ferritin, total iron-binding capacity and per cent saturation of total iron-binding capacity were

measured throughout the study period. Subjects were also asked about any side effects. Of the 412 subjects, only 252 completed all four visits. Prior to the initiation of treatment, there were no significant differences between the two groups for all haematological parameters assessed, and iron deficiency was found in 53 (12.9%) donors. The prevalence of iron deficiency decreased in the iron-supplemented group, whereas it increased in the placebo group. Of the ferrous sulfate–treated subjects, there was a significant ($P \leq 0.001$) decrease in haemoglobin concentration at the end of the study period compared with the beginning of the study period. There were no other significant differences in haematological parameters of subjects taking ferrous sulfate throughout the study period. In the control group (placebo treatment), there was a significant decline in haemoglobin ($P \leq 0.001$), haematocrit ($P \leq 0.001$), serum iron ($P \leq 0.001$), serum ferritin ($P = 0.02$) and per cent saturation ($P \leq 0.001$) between initial and final results. Side effects observed in both the iron-supplemented group and placebo group included nausea and vomiting, abdominal cramps, headaches and constipation. Side effects were recorded in approximately 15%, 18% and 18% of the iron-supplemented group compared with approximately 7%, 6% and 8% of the placebo group at visits 1, 2 and 3, respectively. There was a significant ($P < 0.05$) increase in the overall incidence of side effects and in the occurrence ($P < 0.05$) of nausea and vomiting in the iron-supplemented group compared with the placebo group (Maghsudlu et al., 2008).

(ii) Studies with ferrous iron supplements or fortified foods conducted during gestation and in infants and children

Iron supplementation during pregnancy is commonly recommended in order to prevent and treat iron deficiency anaemia. Several randomized, placebo-controlled, double-blind studies have evaluated the safety of iron supplementation during pregnancy on birth outcome measures, as summarized below. Additionally, studies conducted in infants and children have also been summarized.

In a prospective study conducted in a poor community of Bangladesh, 309 infants and young children of both sexes aged 2–48 months were randomly assigned to receive either 15 mg iron/day in the form of ferrous gluconate plus vitamins or vitamins alone (control). House-to-house visits were made on alternate days to monitor intake and to record incidences and duration of diarrhoea, dysentery and respiratory infections for a period of 15 months. The majority of the children were between 1 and 4 years of age and mildly to moderately malnourished at the end of the supplementation period. The median number of episodes of watery diarrhoea and dysentery was three per child per year for both iron-treated and control groups. The average duration of each episode of watery diarrhoea and dysentery was 3 and 4 days, respectively. The total number of days of illness due to watery diarrhoea was 8 in iron-treated infants and 6 in control infants. The total number of days of illness due to dysentery was 11 in both iron-treated and control infants. When the data were stratified according to age, the results revealed that infants aged 2–11 months receiving iron therapy experienced a significantly higher number of dysentery episodes ($P = 0.03$) and total number of days of illness due to dysentery ($P = 0.02$) compared with control infants. Such an effect of iron therapy

was not found in children aged 12–48 months. Iron therapy did not affect the incidence of acute respiratory tract infections in either age group (Mitra et al., 1997).

A single-blind, placebo-controlled study was conducted in 150 stunted and anaemic children aged 18–30 months (35–39 children per group). Children were randomly assigned to receive one of four supplemental treatments daily for 6 weeks: multivitamin–multimineral plus iron, multivitamin–multimineral plus placebo, placebo plus placebo, or placebo plus iron. The multivitamin–multimineral tablets each contained 11 vitamins and 10 minerals, and the iron supplements were in the form of ferrous fumarate tablets, each containing 66 mg of iron. Appetite, knee–heel length, dietary intakes, morbidity, body length, weight, arm circumference and haemoglobin concentrations were assessed before and after supplementation. No significant differences in appetite or food intake were observed among the groups. Likewise, anthropometric measurements and haemoglobin levels did not differ among the four groups 4 months after the intervention, although haemoglobin concentrations were significantly ($P < 0.05$) higher in the iron-treated children than in the children on placebo 1 week after treatment. Occurrences of fever, diarrhoea, runny nose and coughing were comparable in all groups before and after supplementation. The authors concluded that iron supplementation failed to improve the appetite and growth of young stunted and anaemic children (Dossa et al., 2001).

Two hundred and seventy-five iron-replete women (serum haemoglobin concentration ≥110 g/l and serum ferritin concentration ≥20 µg/l) were randomly assigned to receive either 30 mg iron/day as ferrous sulfate or placebo control from enrolment (<20 weeks of gestation, average 11 weeks) to 28 weeks of gestation. At 28 weeks, iron status was re-evaluated; women with ferritin concentrations of 12–<20 µg/l were given 30 mg iron/day, and those with ferritin concentrations of <2 µg/l were given 60 mg iron/day, regardless of initial assignment. This protocol was repeated at 38 weeks of gestation. At 28 and 38 weeks of pregnancy, blood was collected for iron and haematological analyses. Iron supplementation from the time of enrolment to 28 weeks of gestation resulted in infants with significantly ($P = 0.01$) increased birth weight compared with infants of the placebo group. Infants from the iron supplementation group also displayed a significant ($P = 0.017$) decrease in the incidences of preterm low birth weight and small-for-gestational-age birth weight (6.8%) compared with placebo infants (17.7%, $P = 0.014$). Iron supplementation did not significantly affect the prevalence of anaemia, but led to a significantly higher mean birth weight ($P = 0.01$) (Cogswell et al., 2003).

To determine the safety of an iron-fortified powdered human milk, a 28-day prospective, randomized, double-blind study was conducted in which preterm infants were randomized to receive either 5.76 mg iron/day from an iron-fortified human milk fortifier or a control human milk fortifier. Ninety-six infants received the milk fortified with iron, and 85 received the control fortifier. On days 0, 14 and 28, blood was taken for haematological analyses. Infant characteristics did not differ significantly between the fortified groups throughout the course of the study. Such characteristics included daily and weekly weight gain, body length gain and head circumference gain. Both fortifiers were well tolerated by infants, and no differences in morbidity or adverse events were observed between the two groups. Specifically,

haematology and clinical chemistry parameters were not significantly affected by iron fortification on days 14 and 28 of the study. Incidences of septic infections and necrotizing enterocolitis were also similar between the groups and were reported as very low. The authors concluded that iron-fortified human milk is safe and well tolerated and facilitates good growth and development (Berseth et al., 2004).

A randomized, double-blind, placebo-controlled trial was conducted in healthy children (3–4 years old) with the aim of evaluating the effects of iron supplementation on vigilance, attention and conceptual learning in preschool children. The study population was divided into two groups: children with iron deficiency anaemia (haemoglobin $\leq$115 g/l) and children with good iron status (haemoglobin $\geq$120 g/l). Subjects within each group were randomized to receive iron supplementation or a placebo. In total, 48 children received either 15 mg of iron in the form of ferrous fumarate with multivitamins or multivitamins alone (placebo) for 2 months. Blood samples were obtained at baseline (2 months prior to supplementation) and after supplementation to determine levels of haemoglobin, haematocrit, mean corpuscular volume, red and white blood cell counts, serum iron and iron binding capacity, serum ferritin and whole blood lead. Similarly, computerized tests of attention and learning were administered to all subjects at baseline and after supplementation. A significant ($P < 0.05$) increase in haemoglobin level was observed among anaemic children treated with iron compared with anaemic children in the placebo group. Similarly, serum iron was significantly ($P < 0.05$) increased in anaemic children treated with iron compared with those on placebo. Ferritin was significantly ($P < 0.05$) increased following iron supplementation in anaemic children, but no significant changes were observed in the placebo group. In the anaemic children, no significant change over time was observed in transferrin saturation in either the treatment or placebo group. In the good iron status children, transferrin saturation significantly ($P < 0.05$) decreased in the placebo-treated group, but no significant changes were observed in the iron-treated group. In the cognitive tests, the anaemic children with iron supplementation made significantly ($P < 0.05$) fewer errors of commission, exhibited higher accuracy ($P < 0.05$) and were significantly ($P < 0.05$) more efficient than anaemic children given placebo. These effects were not observed in the children with good iron status. The authors concluded that iron supplementation in iron-deficient anaemic preschool children resulted in an improvement in discrimination—specifically, selective attention (Metallinos-Katsaras et al., 2004).

Four hundred and twenty-nine iron-replete, non-anaemic pregnant women at <20 weeks of gestation (average 11 weeks) were randomly assigned to receive either 30 mg iron/day as ferrous sulfate provided in a multivitamin or a multivitamin without iron from 26 to 29 weeks of gestation. At birth, infants from the iron supplement group weighed, on average, 108 g more than infants from the control group ($P = 0.03$). The incidence of preterm delivery was approximately 50% less in the iron group compared with the control group (7.5% versus 13.9%), but this was not statistically significant. Iron supplementation did not significantly affect the incidences of low birth weight or small-for-gestational-age birth weight or the gestational age at delivery (Siega-Riz et al., 2006).

To determine whether iron supplementation during pregnancy influences childhood intelligence quotient (IQ), pregnant women who participated in a double-blind, randomized, controlled trial of iron supplementation in pregnancy were subsequently followed. Women were randomly allocated to receive either iron (20 mg/day) or placebo from 20 weeks of pregnancy until birth. Women in the iron group had higher concentrations of haemoglobin and serum ferritin ($P < 0.001$) and lower incidences of iron deficiency ($P < 0.001$) and iron deficiency anaemia ($P = 0.001$). The children were followed up at 4 years to assess development. A standardized IQ assessment revealed that children from the iron (153 infants) and placebo (149 infants) groups were similar in their mean composite IQ scores. The iron and placebo groups did not differ significantly in any of the IQ subscales, such as verbal reasoning, visual reasoning, quantitative reasoning and short-term memory. The proportion of children that fell 1 or 2 standard deviations below the mean IQ was similar between the iron and placebo groups. The mean scores for behavioural difficulties (emotion, conduct, hyperactivity, peer relation and social behaviour, assessed by means of a parental questionnaire) did not differ significantly between the groups; however, the total behaviour score was significantly ($P = 0.037$) elevated in children of the iron group compared with children of the placebo group, indicating abnormal behaviour. The authors noted that this result needs to be interpreted with caution, because it may have been due to chance. In the mothers, there were no significant differences in any of the health concepts assessed (physical, emotional, social functioning; bodily pain; mental health; vitality) between the two groups. The authors concluded that prenatal iron supplementation had no effect on the IQ of the offspring at 4 years of age (Zhou et al., 2006).

Breastfed infants (4–6 months of age) attending a healthy baby clinic were recruited to determine the long-term effects of oral iron supplementation on growth parameters and general health. The nutritional status of the infants was assessed, such that there were two treatment groups receiving 1 mg iron/kg bw per day in the form of ferrous gluconate (a well-nourished group, $n = 86$; and a malnourished group, $n = 112$). The placebo control group was composed of 50 normal healthy infants. The two treatment groups (well-nourished and malnourished) were in turn stratified into anaemic (56 well-nourished and 72 malnourished) and non-anaemic (30 well-nourished and 40 malnourished) subgroups. Basal blood samples were taken from all infants to determine iron status parameters. Treatment continued for 1 year, during which mothers recorded stool frequency and consistency and any symptoms of illness. Additional iron status assessment was performed at 6 and 12 months. After 6 and 12 months of treatment, body weight gain and body length gain were significantly ($P < 0.05$) higher in iron-supplemented infants compared with infants receiving the placebo. Head circumference gain was unaffected by iron therapy. Within the iron-treated groups, weight and length gains were significantly ($P < 0.05$) greater in malnourished infants compared with well-nourished infants as assessed at 6 and 12 months. Within the malnourished group, anaemic infants displayed significantly ($P < 0.01$) greater weight and length gains compared with non-anaemic infants after 6 months of iron therapy. Morbidity risk in this study was measured by incidences of diarrhoea and fever and was found to be significantly ($P < 0.05$) higher in malnourished infants compared with well-nourished infants, both receiving iron treatment. Because the iron-treated groups consisted of a large

portion of malnourished infants compared with the placebo group, which did not consist of any malnourished infants, iron supplementation appeared to significantly increase morbidity risk. The anaemic status of the iron-treated infants did not affect morbidity risk. The authors noted that the apparent effect of iron therapy on morbidity was linked to the immunological background of the infants rather than to the iron status or iron supplementation itself. The authors concluded that oral iron supplementation resulted in better effects on growth, especially in those who were initially malnourished and anaemic (Abdelrazik et al., 2007).

Twenty-one healthy and very low birth weight infants were given 18 mg iron/day in the form of ferrous fumarate to evaluate the effects of iron supplementation on oxidative stress and antioxidant status. At 6 weeks of age, infants received 1 ml of ferrous fumarate mixed with milk (9 mg iron/ml) twice daily, for 1 week. Blood and urine samples were obtained twice before initiation of iron supplementation (at weeks 5 and 6) and 1 week after iron supplementation (week 7). Urinary concentration of 8-iso-prostaglandin $F_{2\alpha}$ (a marker of oxidative stress), plasma total hydroperoxides, antioxidant status and haematological parameters were measured. Antioxidant status was assessed by measuring plasma glutathione and vitamin C and E levels and the iron-reducing ability of plasma. Results were compared with baseline measurements taken at week 6. After 1 week of iron supplementation, a significant ($P < 0.05$) increase was observed in serum iron concentration and transferrin saturation, compared with baseline values. Concentrations of urinary 8-iso-prostaglandin $F_{2\alpha}$ and its endogenous beta-oxidation metabolite, 2,3-dinor-8-isoprostaglandin $F_{2\alpha}$, and plasma total hydroperoxides remained unchanged compared with baseline concentrations. Plasma concentrations of most antioxidants remained largely unaltered following iron supplementation, with the exception of small, significant ($P < 0.05$) decreases observed in plasma vitamin E, total glutathione concentrations and the iron-reducing ability of plasma, compared with baseline concentrations. The authors concluded that markers of oxidative stress in urine and plasma antioxidant status in healthy low birth weight infants fed human milk remained unchanged after a high dose (18 mg/day) of oral iron supplementation (Braekke et al., 2007).

In a study assessing the effect of iron supplementation on neurocognitive development, low birth weight infants were randomly assigned to early- or late-iron supplementation groups. In the early-iron group ($n = 100$), iron supplementation was initiated at a median age of 14 days, whereas in the late-iron group ($n = 94$), iron supplementation was initiated at a median age of 61 days. Iron was administered via infant formula at a dose of 2 mg/kg bw per day and increased to 4 mg/kg bw per day if iron deficiency was detected during the study. Children completed a standardized follow-up assessment at a median age of 5.3 years. Mildly or severely abnormal results on the standardized neurology examination were significantly ($P = 0.02$) higher in the late-iron group compared with the early-iron group. Standardized assessments of mobility, motor coordination, cognitive function, visual impairment and childhood behaviour were similar between the two groups. There were, however, non-significant suggestive trends towards improved cognitive development in infants receiving early iron supplementation compared with infants in the late-iron group. The authors concluded that early iron supplementation

showed trends towards improved neurocognitive development and showed no evidence for any adverse effect (Steinmacher et al., 2007).

A double-blind, randomized, community-based trial was conducted in India with 432 children (aged 6–18 months) to determine the haematological response to different doses and forms of iron. The subjects were randomly assigned to one of five groups: micronutrient sachets containing 12.5, 20 or 30 mg iron as ferrous fumarate, micronutrient sachets containing 20 mg iron as ferric pyrophosphate or liquid drops containing 20 mg of iron in the form of ferrous glycine sulfate, once daily for 8 weeks. The micronutrient sachet is a novel form of iron, additionally containing ascorbic acid, vitamin A acetate, folic acid and zinc gluconate, that is added to infants' weaning foods at home. The different forms and doses of iron were added to the children's food. Haemoglobin levels were estimated at baseline, week 3 and week 8. Ferritin levels were assessed at baseline and week 8. Investigators visited the children weekly to record consumption, side effects and any illness. After 8 weeks, no significant differences in haemoglobin or ferritin levels were observed among the groups; however, there was a significant ($P < 0.0001$) increase in haemoglobin at both 3 and 8 weeks in all groups. There were two deaths reported in the study: one child in the 30 mg/day (ferrous fumarate) group died as a result of infective gastroenteritis and dehydration; and the other child was from the 20 mg/day (ferric pyrophosphate) group and died as a result of meningitis or aspiration following a febrile convulsion. The authors reported that both deaths were unrelated directly or indirectly to the consumption of iron. Median compliance across all groups was between 46% and 62% and was significantly lower in the 30 mg/day sachet (ferrous fumarate) and the 20 mg/day drop (ferrous glycine) groups, compared with the other groups (statistical data not provided). Side effects included diarrhoea, vomiting, discoloration of stools and staining of teeth and occurred in all groups. Incidences of diarrhoea and vomiting per child and the proportion of children that reported staining of teeth were significantly ($P < 0.05$) higher in the group receiving 20 mg/day drops (ferrous glycine) compared with the other groups. Common cold, cough and fever were significantly ($P < 0.01$) more common among the 20 mg/day drop (ferrous glycine) group, compared with all the sachet groups. A dose-dependent relationship was not seen with any of the reported side effects (Hirve et al., 2007).

(c) Ammonium chloride

The World Health Organization (WHO) noted that ingestion of ammonium chloride induces metabolic acidosis and diuresis, and it is administered for these effects (International Programme on Chemical Safety, 1986). Ammonium chloride has been used in the study of metabolic acidosis, and WHO noted that the metabolic acidosis induced by ammonium chloride is caused by the chloride ion. Therefore, such studies are of little relevance when evaluating the toxicity of ammonia (International Programme on Chemical Safety, 1986).

(d) Phosphate

There is a concern that high dietary intakes of phosphates and phosphorus may result in bone loss through parathyroid hormone–driven demineralization of the

bone matrix. This concern was raised by the Committee at its twenty-sixth meeting (Annex 1, reference *59*) following evaluation of animal studies conducted on phosphoric acid and phosphate salts. To address this concern, clinical studies have been conducted in healthy human subjects given diets high in phosphorus with low or normal levels of calcium (approximately 400 and 1000–2000 mg calcium/day, respectively). The results of these studies are briefly summarized below.

Daily supplementation with high levels of phosphates, providing 1500–3000 mg phosphorus/day, for periods of 1 week to 4 months generally resulted in significantly increased serum phosphate and/or urinary phosphate levels, accompanied by decreased urinary calcium levels, compared with low-phosphate diets that provided approximately 1000 mg phosphorus/day (Portale et al., 1986; Silverberg et al., 1986; Calvo & Heath, 1988; Calvo et al., 1988, 1990; Bizik et al., 1996; Whybro et al., 1998). In certain studies, high-phosphorus diets have also been shown to result in decreased serum calcium levels (Silverberg et al., 1986; Calvo et al., 1988, 1990; Bizik et al., 1996), whereas other studies have shown no change in serum calcium levels (Portale et al., 1986; Heaney & Recker, 1987; Calvo & Heath, 1988; Brixen et al., 1992; Whybro et al., 1998).

Some studies have shown that high-phosphorus diets increased serum parathyroid hormone levels (Silverberg et al., 1986; Calvo & Heath, 1988; Calvo et al., 1988, 1990; Brixen et al., 1992); however, changes in parathyroid hormone levels were not observed in other studies (Bizik et al., 1996; Whybro et al*.,* 1998), and increases may in fact be a result of low calcium intake. Furthermore, 1,25-dihydroxyvitamin D and osteocalcin levels and markers of kidney function and bone resorption were typically unaffected by high-phosphorus diets (Calvo et al., 1990; Brixen et al., 1992; Whybro et al*.,* 1998; Grimm et al., 2001).

Reported side effects related to high phosphorus intakes (750 mg phosphorus/day or greater) were gastrointestinal in nature and included nausea, loose stools, diarrhoea, vomiting and abdominal discomfort (Broadus et al., 1983; Brixen et al., 1992; Whybro et al., 1998; Grimm et al., 2001).

2.3.2 Case-studies

(a) Ammonium

Several reports of accidental ingestion of ammonia solutions resulting in adverse effects in humans were reviewed by WHO as part of its assessment of ammonia (International Programme on Chemical Safety, 1986). In such cases of accidental exposure, tissue-destructive effects such as oesophageal burns have been reported. Solutions of ammonia have a caustic effect as a result of their high pH. In one case, the ingestion of a solution containing 24 g ammonia/l resulted in death. Upon autopsy, it was revealed that the solution induced haemorrhagic inflammatory changes in the oesophagus, stomach and small intestine (Klendshoj & Rejent, 1966).

Ammonia dose levels resulting from the accidental ingestion of ammonia solutions are considerably greater than those that occur from the consumption of ammonia from food sources. WHO noted that no data on the acute oral toxicity of

ammonium compounds in humans, other than ammonium chloride, are available (International Programme on Chemical Safety, 1986).

3. DIETARY EXPOSURE

3.1 Use in foods

Ferrous ammonium phosphate is proposed for use as a replacement for ferrous iron–containing substances currently being used for iron fortification throughout the world. Specifically, it finds application in those foods in which other fortificants create undesired organoleptic properties, primarily foods containing high fat and high polyphenol levels. The salt will be used in a wide range of general food categories at levels that typically provide between 15% and 30% of the recommended daily allowance for iron. The exact products and use levels will depend on the region and population group but will be consistent with current iron fortification programmes.

3.2 Dietary exposure estimates

The Committee received a submission on the potential dietary exposure to ferrous ammonium phosphate. Food consumption data based on individual dietary records were combined with expected ferrous ammonium phosphate concentrations in various foods to produce assessments based on dietary patterns in the United Kingdom and the USA. Because ferrous ammonium phosphate dissociates into its component ions at low pH (physiological conditions), the assessments considered each ion, iron, ammonium and phosphate, separately. The Committee concluded that this approach was appropriate.

For the United Kingdom population, on a consumer basis, the highest mean and 97.5th-percentile dietary exposures to ferrous ammonium phosphate from all proposed food uses were estimated to be 3.4 mg/person per day (0.04 mg/kg bw per day for a 60-kg individual)[1] and 16.9 mg/person per day (0.30 mg/kg bw per day). Children consumed the greatest amount of ferrous ammonium phosphate on a body weight basis, with the highest mean and 97.5th-percentile consumer dietary exposures of 0.10 and 0.71 mg/kg bw per day, respectively.

Ferrous ammonium phosphate has a maximum iron content load of 30%. Thus, the highest mean consumer dietary exposure to iron from consumption of ferrous ammonium phosphate was estimated to be 1.0 mg/person per day (15 µg/kg bw per day), and the highest 97.5th-percentile dietary exposure of iron was 5.1 mg/person per day (90 µg/kg bw per day). For children, these estimates were 30 and 213 µg/kg bw per day, respectively. These exposure estimates are representative of the use of targeted fortification levels for ferrous ammonium phosphate and are well within the estimated dietary exposure of 44 mg iron/person per day from food, water and supplements, as reported by the United Kingdom's

[1] All of the dietary estimates shown in parentheses were derived using the actual body weights of the participants of the surveys and are not estimates based on standard body weight assumptions.

Expert Group on Vitamins and Minerals (2003). In addition, these levels comply with European Union regulations and scientific opinions.

The highest mean and 97.5th-percentile dietary exposures to ammonia for the United Kingdom population from ferrous ammonium phosphate were estimated to be 0.3 mg/person per day (4 µg/kg bw per day) and 1.5 mg/person per day (27 µg/kg bw per day), respectively. Again, children would consume the greatest amount of ammonia (from ferrous ammonium phosphate) on a body weight basis, with the highest mean and 97.5th-percentile consumer dietary exposures of 9 and 64 µg/kg bw per day, respectively. These exposure estimates are negligible compared with the estimated 4000 mg/day produced endogenously in the human intestines (International Programme on Chemical Safety, 1986).

The highest mean consumer dietary exposure to phosphorus (as phosphate) from ferrous ammonium phosphate for the United Kingdom population was estimated to be 0.6 mg/person per day (9 µg/kg bw per day), and the highest 97.5th-percentile exposure was 3.0 mg/person per day (54 µg/kg bw per day). Children would consume the highest amount of phosphorus on a body weight basis, with the highest mean and 97.5th-percentile consumer dietary exposures of 18 and 127 µg/kg bw per day, respectively. These exposure estimates are well below the mean and 97.5th-percentile dietary exposures to phosphorus from foods, ranging from 1112 to 1570 mg/person per day and from 1763 to 2601 mg/person per day, respectively, for populations in five European countries (European Food Safety Authority, 2005). In addition, the 97.5th-percentile estimates for phosphorus dietary exposure in each population group resulting from the consumption of ferrous ammonium phosphate are roughly 1/1000 of the tolerable upper intake levels for phosphorus in each population group established by the Institute of Medicine (1997).

For the USA population, on a consumer basis, the mean dietary exposure to ferrous ammonium phosphate from all proposed food uses was estimated to be 13 mg/person per day or 0.2 mg/kg bw per day. The 90th-percentile exposure was estimated to be 29 mg/person per day or 0.6 mg/kg bw per day. Ferrous ammonium phosphate has a maximum iron content load of 30%. Thus, on a consumer basis, the mean dietary exposure to iron was estimated to be 3.9 mg/person per day or 74 µg/kg bw per day. The 90th-percentile exposure was estimated to be 8.7 mg/person per day or 181 µg/kg bw per day.

These consumption estimates are also representative of the use of targeted fortification levels and fall well below the tolerable upper limit of 45 mg/person per day for iron established by the Institute of Medicine (2001). Furthermore, these levels are consistent with current fortification guidelines and labelling requirements in the USA.

The estimated mean and 90th-percentile consumer dietary exposures to ammonia are 1.1 mg/person per day (22 µg/kg bw per day) and 2.6 mg/person per day (54 µg/kg bw per day), respectively. Infants were determined to have the highest consumer mean and 90th-percentile dietary exposures to ammonia on a body weight basis, 63 and 151 µg/kg bw per day, respectively. All of these estimates are

negligible when compared with the estimated 4000 mg/day produced endogenously by humans (International Programme on Chemical Safety, 1986).

The estimated mean and 90th-percentile consumer dietary exposures to phosphorus are 2.3 mg/person per day (44 µg/kg bw per day) and 5.2 mg/person per day (108 µg/kg bw per day), respectively. On a body weight basis, mean and 90th-percentile consumer dietary exposures to phosphorus were highest in infants, with estimated dietary exposures of 126 and 302 µg/kg bw per day, respectively. The 90th-percentile consumer dietary exposures to phosphorus for the different population groups are approximately 1/1000 of the tolerable upper intake levels for phosphorus established by the Institute of Medicine (1997).

Overall, the dietary exposure calculations for both the United Kingdom and the USA, covering all the proposed food uses of ferrous ammonium phosphate and using use levels that reflect current fortification programmes in the respective communities, indicate levels of consumption of each of the individual components (iron, ammonium and phosphate) that fall well below acceptable amounts previously established by the Committee.

As ferrous ammonium phosphate is intended to replace current sources of ferrous iron in national fortification programmes, the Committee concluded that its introduction into the food supply will result in no increase in estimates of dietary exposure to iron. Dietary exposures to ammonium and phosphate ions would not be significantly impacted by the introduction of ferrous ammonium phosphate as a ferrous iron fortificant in food.

4. COMMENTS

4.1 Toxicological data

The toxicological data related specifically to ferrous ammonium phosphate are limited. The Committee received a submission containing unpublished studies on the bioavailability of iron from ferrous ammonium phosphate and studies on the constituent ions—iron, ammonium and phosphate. The submission included new toxicological data on iron, phosphate and ammonium salts that have become available subsequent to the latest evaluations conducted by the Committee, as well as older studies not previously reviewed by the Committee. The latest toxicological information on ammonium and phosphate salts was discussed in order to identify whether the new information would indicate a need to revise the Committee's previous opinions on these components. It was concluded that the safety of ferrous ammonium phosphate depended primarily on the iron component.

In one of the few studies available on ferrous ammonium phosphate, the bioavailability of iron from ferrous ammonium phosphate in humans was shown to be similar to that of other iron salts used for fortification purposes. In this study, no gastrointestinal complaints or other adverse effects were reported following consumption of a milk product fortified with ferrous ammonium phosphate, providing a total iron dose of 5 mg.

Results of acute toxicity studies in rodents indicate that large doses of iron (as ferrous sulfate) produce adverse effects that consist largely of gastrointestinal disturbances, such as diarrhoea. The oral LD_{50} values for iron derived from ferrous sulfate were reported to be approximately 250 mg/kg bw in mice, to range from 300 to 1100 mg/kg bw in rats and to be greater than 200 mg/kg bw in dogs.

Results of short-term studies of toxicity in rodents, including studies specifically designed to produce iron overload, indicate that repeated administration of large doses of iron in the diet (mainly in the form of ferrous sulfate or carbonyl iron), in the range of 37.5 mg iron/kg bw per day or greater, was associated with hepatic changes. Consistent observations in these studies included a reduction in body weight gain, increases in serum indicators of hepatic toxicity and increased hepatic microsomal lipid peroxidation, coupled with increases in hepatic iron content. However, these findings in the liver were not accompanied by histopathological abnormalities.

In a rat carcinogenicity study, dietary administration of iron lactate at 167 mg iron/kg bw per day, but not at 82 mg/kg bw per day, resulted in an increased incidence of hyperplasia in the pancreatic acinar cells and endometrium. There was no increase in tumour incidence at either dose. A number of in vitro and in vivo genotoxicity studies have been conducted using ferrous sulfate and ferrous fumarate. No genotoxic effects were seen, except for infrequent non-concentration-dependent effects that were observed in the presence of cytotoxicity. The Committee concluded that ferrous sulfate and ferrous fumarate are not genotoxic.

In reproductive toxicity studies, iron supplementation of the diets of pregnant rats did not affect fetal growth. Pups supplemented via breast milk and then the diet at doses of 500 mg iron/kg bw per day and greater showed significant retardation of growth.

The additional toxicological studies on iron reviewed in this evaluation support the safety of iron in the diet, which was evaluated by the Committee at its twenty-seventh meeting (Annex 1, reference *62*).

The Committee noted previously that healthy individuals have taken supplements of 50 mg iron/day as ferrous sulfate for long periods of time without any adverse effects. Repeated oral iron supplementation at dosages greater than 50 mg iron/day may lead to adverse gastrointestinal effects. Gastrointestinal symptoms such as abdominal discomfort, diarrhoea, constipation and darkened stools were observed in studies where iron was provided at dose levels equal to or greater than 50 mg/day, with the frequency of effects increasing with the dose. These observations are consistent with the previous evaluation of iron and derivation of the PMTDI (Annex 1, references *62* and *63*). Studies with ferrous iron supplements in pregnant women (up to 60 mg/day) and infants (up to 66 mg/day) did not result in adverse birth outcomes or adverse effects on growth or development.

Individuals with iron storage disorders such as haemochromatosis are particularly at risk from exposure to iron, primarily as a result of an increased rate of iron absorption, even under conditions of normal iron stores.

The Committee concluded that the latest toxicological information on ammonium and phosphate salts did not indicate a need to revise the Committee's previous evaluations on these ions.

4.2 Assessment of dietary exposure

The Committee received a submission detailing the potential dietary exposure to ferrous ammonium phosphate. Food consumption data based on individual dietary records were combined with expected ferrous ammonium phosphate concentrations in various foods to produce assessments based on dietary patterns in the United Kingdom and the USA.

For the population in the United Kingdom, the highest mean dietary exposure to iron from consumption of ferrous ammonium phosphate for consumers only was estimated to be 1.0 mg/person per day (15 µg/kg bw per day), and the highest 97.5th-percentile dietary exposure to iron was 5.1 mg/person per day (90 µg/kg bw per day). For the population in the USA, for consumers only, the mean dietary exposure to iron from ferrous ammonium phosphate was estimated to be 3.9 mg/person per day or 74 µg/kg bw per day, and the 90th-percentile exposure was 8.7 mg/person per day or 181 µg/kg bw per day. For both population groups, the intakes of phosphate and ammonium ions were insignificant when compared with background dietary exposures.

Overall, the dietary exposure calculations for both the United Kingdom and the USA, covering all the proposed food uses of ferrous ammonium phosphate and using use levels that reflect current fortification programmes in the respective communities, indicate levels of consumption of each of the individual components (iron, ammonium and phosphate) that fall well below acceptable amounts previously established by the Committee.

As ferrous ammonium phosphate is intended to replace current sources of ferrous iron in national fortification programmes, the Committee concluded that its introduction into the food supply will result in no increase in estimates of dietary exposure to iron.

5. EVALUATION

The newly available information on the toxicity of iron did not identify any toxicological effects additional to those previously identified by the Committee and did not indicate a need to revise the PMTDI. On the basis of the available data on the bioavailability of iron from ferrous ammonium phosphate and consideration of the toxicity of its constituent ions, the Committee concluded that ferrous ammonium phosphate is acceptable for use as a source of iron for dietary fortification, provided that the total intake of iron does not exceed the PMTDI for iron of 0.8 mg/kg bw.

Products, including ferrous ammonium phosphate, that are intended to provide a source of additional iron should not be consumed by individuals with any type of iron storage disease, except under medical supervision.

Consideration of the toxicity of ammonium or phosphate did not indicate a need to revise the Committee's previous evaluations on these ions.

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GLYCEROL ESTER OF GUM ROSIN

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1. EXPLANATION

Glycerol ester of gum rosin (GEGR) was placed on the agenda of the current meeting at the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008). The Committee had not previously evaluated GEGR. However, the Committee previously considered, at its eighteenth, twentieth, thirty-third, thirty-seventh, forty-fourth and forty-sixth meetings (Annex 1, references *35*, *41*, *83*, *94*, *116* and *122*), a related substance, glycerol ester of wood rosin (GEWR). At its forty-sixth meeting, the Committee allocated an acceptable daily intake (ADI) for GEWR of 0–25 mg/kg body weight (bw).

GEGR is intended to be used as an emulsifier/density adjustment agent for flavouring substances in non-alcoholic beverages and cloudy spirit drinks.

1.1 Chemical and technical considerations

GEGR is a complex mixture of triglycerol and diglycerol esters of resin acids from gum rosin (GR), with a residual fraction of monoglycerol esters. It is obtained by the esterification of refined GR under a nitrogen atmosphere with food-grade glycerol and purified by direct countercurrent steam distillation. Refined GR is obtained by extracting oleoresin gum from living pine trees and refining it through washing, filtration and distillation.

The refined rosin contains approximately 90% resin acids and 10% neutrals. The resin acid fractions are a complex mixture of isomeric diterpenoid monocarboxylic acids having the empirical formula $C_{20}H_{30}O_2$ and grouped into three main classes: abietane, pimarane and isopimarane. The resin acids of these classes are similar in structure, differing only in the number and arrangement of double bonds or in the stereochemistry of the C-13 position. The neutral fraction is composed of esters of resin acids, esters of fatty acids and various unsaponifiable materials. The composition of the resin acid portion of the esters in the neutral fraction is similar to that of the resin acids in the acid fraction; the fatty acid portion of the esters in the neutral fraction is predominantly C_{18} or higher straight-chain acids with varying degrees of unsaturation.

The chemical composition of GEGR varies depending on the pine species, environmental factors, geographical differences and the techniques used in the processes of rosin purification and refinement. During the esterification procedure, because of the high temperature and severe conditions employed, some isomerization and dehydration reactions of the resin acids also occur, so that the resin acid distribution in the final ester is different from that in the original rosin. Limited data were available on the variability of the resin acid composition of GEGR in commerce. According to submitted data, the major resin acids found in GEGR are abietic, dehydroabietic, communic, pimaric and isopimaric acids.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Chemical similarity of the glycerol esters of gum and wood rosin

At its present meeting, the Committee reviewed and evaluated the available data pertaining to the chemical composition of both GEGR and GEWR and the various analytical techniques employed to determine their chemical similarity. The three types of rosins—gum, tall oil and wood—are differentiated primarily by the part of the pine tree from which they are obtained, the variety of pine tree species used and the manufacturing process. The Committee concluded that the chemical components of GEGR and GEWR are qualitatively similar and that the data from GEWR could be used in the evaluation of GEGR.

2.1.2 Absorption, distribution, metabolism and excretion

There are no data available on the absorption, distribution, metabolism or excretion of GR or GEGR.

The Committee evaluated the results of several unpublished pharmacokinetic studies on GEWR at its forty-fourth and forty-sixth meetings and concluded that food-grade GEWR is metabolically stable in the gastrointestinal tract and that only a minor fraction, most probably the monoglycerol ester fraction, undergoes partial hydrolysis. These studies are described in detail in the previous evaluations by the Committee (Annex 1, references *117* and *123*). However, a brief summary of each study is given below.

In the first study, Fischer 344 rats (six per sex per group) were fed diets containing ester gum 8BG (a commercial food-grade preparation of GEWR) under two treatment regimens: 1) for 24 h at a concentration of 7000 or 28 000 mg/kg diet, and 2) for 10 days at a concentration of 14 000 or 28 000 mg/kg diet. Feed intake was measured, and ester gum 8BG intake was calculated for each treatment group. Faeces were collected during each 24-h treatment period and for subsequent 24-h periods until ester gum 8BG was no longer detected. After a 24-h dietary intake, most of the ingested ester gum 8BG was excreted in the faeces within 48–72 h. The faecal recovery was 75% of the amount ingested at the low dose and 95% at the higher dose. The low faecal recovery at the low dose was attributed to the lack of sensitivity of the analytical method. After 10 days of repeated dietary intake, faecal recoveries of the ester gum 8BG were essentially equal to the amount ingested. The author concluded that neither enterohepatic cycling nor measurable hydrolysis of ester gum 8BG took place in the rat intestine (Blair, 1995).

In another pharmacokinetic study, Fischer 344 rats received a single dose of about 200 mg [1,3-^{14}C]glycerol ester gum 8BG/kg bw by gavage after 1 day (five male and five female rats) or 10 days (five male rats) of dietary administration of unlabelled compound. In both male and female rats fed ester gum 8BG for 1 day, more than 95% of the administered dose was recovered in the faeces or cage rinses, 1% or less was excreted either as carbon dioxide in the expired air or in urine within 120 h and only traces ($\leq$0.2%) of the total dose of radiolabel were detected in 8 of 15 carcasses 120 h after treatment. Similar results were obtained in male rats given the radiolabelled ester gum after a 10-day dietary administration of unlabelled compound. According to the author, analysis of the faecal extracts using reversed-phase high-performance liquid chromatography (HPLC) indicated that a very small percentage of the administered ^{14}C-labelled ester gum 8BG, probably monoglycerol esters, was hydrolysed (Noker, 1996).

In a separate study employing five male rats with jugular vein and biliary duct cannulae, radioactivity was measured in bile and blood at 4-h and 12-h intervals for 24 h following oral administration of ^{14}C-labelled ester gum 8BG. During the 24-h period, 1.6–2.9% of the administered dose was excreted in the bile, 0.1% or less was accounted for in the blood and 0.1–0.2% was found in livers collected from the rats. HPLC analyses of two bile samples indicated the presence of hydrolysed components. The results of this and the previous study indicate that ^{14}C-labelled

ester gum 8BG is absorbed at very low levels and undergoes minimal hydrolysis or degradation in the gastrointestinal tract (Noker, 1996).

In order to determine the metabolic fate of ester gum 8BG in an in vitro system, [1,3-^{14}C]glycerol ester gum 8BG was incubated with human faecal extract, simulated gastric fluid or sterile water (as a negative control) at a concentration of 0.5 or 4.4 mg/ml for 24 h. Samples were collected at 0, 6 and 24 h and analysed in an HPLC–radiochemical detector system. The elution patterns on the radiochromatogram from the three incubation systems were similar. As ester gum is a mixture, further analysis was done based on changes in radioactivity in seven regions related to the peaks of radioactivity on the radiochromatograms. With the negative control, no significant changes were observed over time at either concentration. With human faecal extract, occasional minor differences were reported in regions containing small peaks. With simulated gastric fluid, there were no significant changes in peak regions up to 24 h at the low concentration. At the higher concentration, however, a slight decrease was seen in one peak region associated with the triglycerides of rosins in the 24-h sample compared with the 6-h sample; there was no significant difference in that same peak between the 0-h and 6-h samples. As the emptying time of the stomach is normally about 4 h, changes observed after that time would have no effect on the stability of ester gum in the stomach in vivo. These results demonstrate the metabolic stability of ester gum 8BG under conditions resembling those present in the human gastrointestinal tract (Lin, 1996).

The Committee also considered the previous evaluation of the absorption studies in rats with tritiated resin acids—namely, dehydroabietic, tetrahydroabietic and isopimaric acids—which indicated that these resin acids were recovered primarily from the faeces within 2 weeks (most within 4 days) after oral administration. The small amount of dehydroabietic acid absorbed appeared to have been metabolized in the liver to three or four uncharacterized metabolites, which were then excreted in the bile and urine. There was limited evidence to show that tetrahydroabietic and isopimaric acids were metabolized (Radomski, 1965).

2.2 Toxicological studies

At the present meeting, the Committee evaluated the toxicological studies with GR, which included acute toxicity, 90-day toxicity and 2-year toxicity/carcinogenicity studies, and a summary statement by the sponsor regarding the results of two unpublished 90-day toxicity studies with GEGR. These studies are described below, together with brief summaries of previously evaluated studies on wood rosin (WR) and GEWR.

2.2.1 Acute toxicity

The Committee, at its present meeting, considered the summary of acute oral toxicity studies with GR in several rodent species. Acute oral median lethal doses (LD_{50} values) for GR in mice, rats and guinea-pigs were reported to be 4600, 7600 and 4100 mg/kg bw, respectively.

2.2.2 Short-term studies of toxicity

(a) Rats

(i) Gum rosin

At its present meeting, the Committee evaluated a 90-day oral toxicity study in rats administered GR. The sponsor provided only the summary of the study.

In this 90-day oral toxicity study, Sprague-Dawley rats (10 per sex per group) were given diets containing 0%, 0.01%, 0.05%, 0.2%, 1.0% or 5.0% GR (equivalent to 0, 5, 25, 100, 500 and 2500 mg/kg bw per day). The group of rats administered the highest concentration (5.0% GR) was discontinued early in the study because all animals experienced drastic reductions in body weight and died during the first 7 days of the treatment period. The mortality was associated with feed refusal. At dietary concentrations up to 1.0%, there was no mortality, and there were no treatment-related effects on haematology, urinalysis, or gross and microscopic histopathology. At the 1.0% dietary concentration, body weight gain and feed consumption were reduced, particularly during the first few weeks of the study. Differences in organ weights in the 1.0% dietary concentration group were not accompanied by histopathological changes (Kay, 1960a).

(ii) Wood rosin

In the 90-day oral toxicity study that was previously evaluated by the Committee, Sprague-Dawley rats (10 per sex per dose) were fed a diet containing 0%, 0.01%, 0.05%, 0.2% or 1.0% *N*-wood rosin (equal to 0, 6.4, 36, 119 or 674 mg/kg bw per day). A 5.0% dietary group was discontinued early in the study because all animals in this group died during the first week of the treatment period. This mortality was associated with feed refusal. At dietary concentrations up to 1.0%, there was no mortality. However, body weights and feed consumption were significantly reduced in male and female rats throughout the study. Liver to body weight and brain to body weight ratios for male and female rats were significantly increased at the 1.0% dietary concentration. There were no significant differences in haematology, urinalysis, or gross and microscopic histopathology observed in any of the treated animals (Kay, 1960b; Annex 1, reference *117*).

(iii) Glycerol ester of gum rosin

The Committee was informed by the sponsor of the results of two unpublished 90-day toxicity studies with GEGR in rats given dietary concentrations of 0%, 0.2%, 0.5% and 1.0%. In each study, the no-observed-effect level (NOEL) was claimed to be 1.0% in the diet. However, the full reports were not available for evaluation by the Committee. These studies,[1] conducted in 1989, were from a "white paper" issued by the National Association of Chewing Gum Manufacturers and European Association of the Chewing Gum Industry (1996).

[1] The primary study reports were considered to be confidential business information and therefore were not available for review.

(iv) Glycerol ester of wood rosin

The Committee previously evaluated two 90-day oral toxicity studies in rats with ester gum 8BG, a commercial food-grade GEWR (Annex 1, reference *117*). These studies are briefly summarized below.

In a 13-week oral toxicity study, groups of Fischer 344 rats (20 per sex) were fed a diet containing ester gum 8BG at a dose level of 0, 625, 1250 or 2500 mg/kg bw per day. There were no deaths among treated or control rats during the course of the study and no changes in appearance, behaviour, ophthalmoscopic examinations, haematology, clinical chemistry, or gross or microscopic histopathology that were attributed to treatment. Slight, but significant, decreases in body weight gain were noted in female rats at 1250 and 2500 mg/kg bw per day during the last weeks of the study. However, these minor effects were considered negligible and attributed to dietary dilution. Dietary dilution was also thought to account for a slight dose-related increase in feed consumption at all dose levels in both sexes. Minor differences between controls and high-dose groups in organ to body weight ratios and organ to brain weight ratios were small and not associated with any histological abnormalities, and they were not considered to be an important effect of treatment. The NOEL in this study was 2500 mg/kg bw per day, the highest dose tested. On the basis of these results, the Committee allocated an ADI for GEWR of 0–25 mg/kg bw (Blair, 1991, 1992).

In another 90-day oral toxicity study, Sprague-Dawley rats were fed ester gum 8D (GEWR) at a dietary level of 0%, 0.01%, 0.05%, 0.2%, 1.0% or 5.0% (equal to 0, 6, 31, 120, 630 and 2660 mg/kg bw per day). No mortality occurred among treated or control animals. At the 5.0% dietary concentration, feed consumption was slightly lower than in controls. At dietary concentrations up to 1.0%, there were no significant effects on body weight, feed intake, haematology, urinalysis, or gross and microscopic histopathology. The NOEL was considered to be the 1.0% dietary concentration (equal to 630 mg/kg bw per day) (Kay, 1960c; Annex 1, reference *117*).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rats

(i) Gum rosin

At its present meeting, the Committee evaluated the 2-year oral toxicity study in rats administered GR. The sponsor provided only the summary of the study.

In a 2-year toxicity/carcinogenicity study with GR, Sprague-Dawley rats (30 per sex per group) were fed diets containing 0%, 0.05% or 1.0% GR (equivalent to 0, 25 and 500 mg/kg bw per day). Five animals from each group were sacrificed at the end of 12 months for gross and microscopic pathology studies. All surviving animals were killed at 24 months; organ weights were recorded, and pathological examinations were conducted.

At the 1.0% dietary concentration, body weights were significantly lower than those of control rats throughout the study, which was attributed to lower feed consumption. Some sporadic differences in ratios of organ to body weight were noted; in the absence of any accompanying pathology, these were not considered to be of toxicological significance. There were no significant differences between GR-treated groups and control rats with respect to survival, haematology, urinalysis, or gross and microscopic histopathology. There was no evidence of carcinogenicity at dietary concentrations of 0.05% or 1.0% (Kohn, 1962a).

(ii) Wood rosin

In a 2-year toxicity/carcinogenicity study previously evaluated by the Committee, Sprague-Dawley rats (30 per sex per group) were fed dietary concentrations of 0%, 0.05%, 0.2% or 1.0% WR for 24 months (equal to 0, 24, 88 and 434 mg/kg bw per day). Five animals from each group were sacrificed at 12 months for gross and histopathological examination. At the 1.0% dietary concentration, body weights were significantly lower than in controls at both 12 and 24 months. Decreased body weights were again attributed to reduced feed consumption. Some sporadic differences in organ to body weight ratios were noted, but were not considered to be of toxicological significance. There were no significant differences between WR-treated and control rats with respect to mortality, haematology, urinalysis, gross and microscopic histopathology, or tumour incidence (Kohn, 1962c; Annex 1, reference *117*).

(b) Dogs

(i) Gum rosin

At its present meeting, the Committee evaluated a 2-year oral toxicity study in dogs administered GR. The sponsor provided only the summary of the study.

Pure-bred Beagle dogs (three per sex per group) were fed dietary concentrations of 0%, 0.05% or 1.0% (equivalent to 0, 12.5 and 250 mg/kg bw per day) GR in corn oil for 24 months. The test article had no effect on growth, food consumption, survival, haematology, urinalysis, liver or kidney function, organ weights, or gross or microscopic histopathology. The author concluded that the NOEL in this study was 1.0% in the diet (Kohn, 1962b).

(ii) Wood rosin

In a 2-year oral toxicity study previously evaluated by the Committee, Beagle dogs (three per sex per group) were fed diets containing 0%, 0.05% or 1.0% *N*-wood rosin (equal to 0, 14 or 260 mg/kg bw per day). Body weight appeared to be somewhat decreased in the high-dose males, consistent with reduced feed consumption; however, a clear treatment-related effect on body weight gain was not discernible. The author concluded that the NOEL in this study was 1.0% in the diet (Kohn, 1962d; Annex 1, reference *117*).

2.2.4 Genotoxicity

In its previous evaluation of GEWR, the Committee concluded that GEWR is not genotoxic in several in vitro test systems (Annex 1, reference *123*).

2.3 Observations in humans

2.3.1 Contact sensitization potential

Rosin (commonly known as colophony) has long been recognized as a dermal sensitizer. Rosin was first used in routine dermatological patch test series in 1939. The European Union currently classifies all types of rosin (GR, WR and tall oil rosin [TOR]) as skin sensitizers (Karlberg et al., 1999).

The Committee, at its forty-fourth meeting, noted the sensitization potential of rosins. At the present meeting, further review of several case reports in humans illustrated that dermal contact sensitization is seen with all types of rosins and rosin components, as well as with glycerol esters of gum, tall oil and wood rosins. Moreover, there is no evidence in humans to suggest that glycerol esters of gum, tall oil and wood rosins differ in their potential to produce sensitization reactions when applied to the skin.

Similarly, dermal studies in guinea-pigs have also shown that there is no difference in the contact sensitization potential of GR, TOR and modified rosins (Hausen et al., 1989; Shao et al., 1993; Gäfvert et al., 1994; Färm, 1997). Thus, both GEGR and GEWR appear to be substantially equivalent in their ability to cause dermal sensitization in humans and experimental animals following application to the skin.

According to the American Conference of Governmental Industrial Hygienists (1996), abietic acid, the primary resin acid found in rosin, is not believed to be the sensitizing agent; it is only when humans and experimental animals are patch tested with unpurified commercial rosin that sensitization occurs. However, Hausen & Mohnert (1989) found that of 44 dermatology patients, 17 (39%) reacted to abietic acid (as well as to standard rosin).

Abietic acid may be a weak contact allergen, and it is important to note that the sensitization potential for the various rosins and their respective glycerol esters was noted exclusively following dermal application. So far, no reports of allergic responses from ingestion of GEGR or GEWR in beverages have been found.

3. DIETARY EXPOSURE

GEGR is intended to be used as an emulsifier/density adjustment agent in oil-based flavourings that are added to non-alcoholic beverages and cloudy spirit drinks to increase the density of flavouring oils (e.g. lemon oil, orange oil). When dispersed in the finished beverage, GEGR acts as an emulsifier, resulting in a desirable degree of cloudiness, and also improves emulsion stability (Bailey, 1998). The maximum use level proposed by the petitioners is 100 mg/kg, and this amount is expected to be present in the final product.

In the 1970s, the United States Food and Drug Administration conducted an analysis to estimate the dietary exposure to GEWR associated with its proposed use as a density adjuster. This resulted in a dietary exposure estimate of 97.5 mg/day, based on a soft drink consumption estimate of 1.5 litres/day and an average GEWR use level of 65 mg/kg.

For the purpose of the current assessment, the dietary exposure was based on a maximum use level of 100 mg/kg combined with the total consumption of soft drinks in Australia (1995 Australian National Nutrition Survey; Food Standards Australia New Zealand, 2009), Europe (European Food Safety Authority [EFSA] Concise European Food Consumption Database; European Food Safety Authority, 2009) and the USA (2003–2004 National Health and Nutrition Examination Survey [NHANES]; Foods Analysis and Residue Evaluation Program, 2009) for adults, consumers only (Table 1). The median values were calculated for both the mean and the 95th percentile of the consumption distribution across the 19 countries considered and were, respectively, 340 and 990 g/day. Based on these figures, the estimated mean dietary exposure to GEGR would be 34 mg/day, corresponding to 0.57 mg/kg bw per day for a 60-kg adult. For high consumers, the estimated dietary exposure to GEGR would be 99 mg/day, corresponding to 1.65 mg/kg bw per day for a 60-kg adult.

Table 1. Mean and high-percentile soft drink consumption for adult consumers only

				Consumption (g/day)	
Country	Method	Number of days	Category	Mean	95th percentile
Australia	24-h recall	1	Soft drinks, flavoured mineral waters and electrolyte drinks	569.0	1500.0
Austria	24-h recall	1	Soft drinks	323.2	1000.0
Belgium	24-h recall	2	Soft drinks	476.0	1340.0
Bulgaria	24-h recall	1	Soft drinks	346.2	800.0
Czech Republic	24-h recall	2	Soft drinks	282.3	950.0
Denmark	Precoded food diary with open fields	7	Soft drinks	259.3	850.0
France	Dietary record	7	Soft drinks	127.9	426.3
Germany	Dietary history	28	Soft drinks	369.5	1442.9
Hungary	Dietary record	3	Soft drinks	223.2	600.0
Iceland	24-h recall	1	Soft drinks	674.0	1700.0

Table 1 (contd)

Country	Method	Number of days	Category	Consumption (g/day) Mean	95th percentile
Ireland	Dietary record	7	Soft drinks	203.6	635.9
Italy	Dietary record	7	Soft drinks	114.9	347.1
The Netherlands	Dietary record	2	Soft drinks	338.8	990.0
Norway	Quantitative food frequency questionnaire	0	Soft drinks	375.6	1300.0
Poland	24-h recall	1	Soft drinks	370.1	1000.0
Slovakia	24-h recall	1	Soft drinks	613.6	2000.0
Sweden	Dietary record	7	Soft drinks	243.4	685.7
United Kingdom	Dietary record	7	Soft drinks	292.5	891.6
USA	24-h recall	2	Soft drinks	510.0	1060.0[a]
Median dietary exposure across all countries				**340**	**990**

[a] 90th percentile.

4. COMMENTS

4.1 Toxicological data

At the present meeting, the Committee evaluated the information available on the chemical composition of GR, the toxicological studies with GR, which included acute toxicity, 90-day toxicity and 2-year toxicity/carcinogenicity studies, and a summary statement from the sponsor about the results of two unpublished 90-day toxicity studies with GEGR. There are no data available on the absorption, distribution, metabolism or excretion of GR or GEGR.

Acute oral LD_{50} values for GR in mice, rats and guinea-pigs were reported to be 4600, 7600 and 4100 mg/kg bw, respectively.

Results of the 90-day oral toxicity study with GR demonstrated that Sprague-Dawley rats given diets with the highest concentration of 5.0% GR experienced drastic reductions in body weights and died during the first 7 days of the treatment period. The mortality was associated with feed refusal. At dietary concentrations up to 1.0% (equivalent to 500 mg/kg bw per day), there was no mortality, and there were no treatment-related effects on haematology, urinalysis, or gross or microscopic histopathology. Body weight gain and feed consumption were reduced, particularly during the first few weeks of the study. Decreases in organ weights in the 1.0% dietary concentration group were not accompanied by histopathological changes and were not considered to be of toxicological significance.

Results of the 2-year toxicity/carcinogenicity study in rats at dietary concentrations of 0.05% or 1.0% GR indicate that at the 1.0% dietary concentration (equivalent to 500 mg/kg bw per day), body weights were significantly lower than those of controls throughout the study, which again was attributed to lower feed consumption. Some sporadic differences in the ratios of organ weight to body weight were noted but, in the absence of any accompanying pathology, were not considered to be of toxicological significance. No significant dose-related systemic toxicity was noted in rats. In the same study, there was no evidence of carcinogenicity in rats fed GR at dietary concentrations of either 0.05% or 1.0%.

In the 2-year toxicity study, GR fed to Beagle dogs at a dietary concentration of 0.05% or 1.0% had no effect on growth, feed consumption, survival, organ weights, haematology, urinalysis, liver or kidney function, or gross or microscopic histopathology. No significant dose-related systemic toxicity was noted in dogs. The NOEL in this study was 1.0% in the diet.

The results of the studies with GR were compared with those of the related substance WR. The Committee concluded that the results of the studies with GR were consistent with those of the 90-day toxicity and 2-year toxicity/carcinogenicity studies with WR in rats that were previously evaluated (Annex 1, reference *116*). Furthermore, the results from the 90-day toxicity studies with GEWR indicate that the feed acceptance was improved. This effect is reflected by the absence of deaths even in the highest GEWR dose group (2500 mg/kg bw per day).

The Committee was informed by the sponsor of the results of two 90-day toxicity studies with GEGR in rats for which the NOEL was claimed to be 1.0% in the diet. However, the full reports were not available for evaluation by the Committee.

The variations in the amounts of both the individual resin acids and the components of the neutral fraction were considered to be of no toxicological consequence.

In its previous evaluation of GEWR at the forty-sixth meeting, the Committee concluded that GEWR is metabolically stable in the gastrointestinal tract, with more than 95% being recovered unchanged in the faeces. Only a minor fraction, most probably the monoglycerol ester fraction, undergoes partial hydrolysis (Annex 1, reference *116*). Although the proportion of the monoglycerol esters is dependent upon the ratio of the GR to the glycerol used in the esterification process, the variations observed in the monoglycerol esters of GEGR are comparable with those observed in the monoglycerol esters of GEWR.

The Committee also considered the previous evaluation of the absorption studies in rats with tritiated resin acids—namely, dehydroabietic, tetrahydroabietic and isopimaric acids—which indicated that these resin acids were primarily recovered from the faeces within 2 weeks (most within 4 days) after oral administration. The small amount of dehydroabietic acid absorbed appeared to have been metabolized in the liver to three or four uncharacterized metabolites, which were then excreted in the bile and urine. There was limited evidence to show that tetrahydroabietic and isopimaric acids were metabolized.

In its previous evaluation of GEWR, the Committee concluded that GEWR is not genotoxic in several in vitro test systems.

At the forty-sixth meeting, the Committee allocated an ADI for GEWR of 0–25 mg/kg bw based on the 13-week toxicity study in rats. The NOEL was 2500 mg/kg bw per day, the highest dose tested.

4.2 Assessment of dietary exposure

GEGR is intended to be used as an emulsifier/density adjustment agent for flavouring substances in non-alcoholic beverages and cloudy spirit drinks at a maximum use level of 100 mg/kg. This amount is expected to be present in the final product.

For the purpose of the current assessment, the maximum use level was combined with the total consumption of soft drinks in Australia (1995 Australian National Nutrition Survey), Europe (EFSA Concise European Food Consumption Database) and the USA (2003–2004 NHANES) for adults, consumers only. The median values were calculated for both the mean and the 95th percentile of the consumption distribution across the 19 considered countries and were 340 and 990 g/day, respectively.

Based on these figures, the mean and the high percentile for dietary exposure to GEGR would be 34 and 99 mg/day, respectively, corresponding to 0.57 and 1.65 mg/kg bw per day, respectively, for a 60-kg adult.

5. EVALUATION

The Committee concluded that the data from GEWR could be used in the evaluation of GEGR because of the absence of toxicological effects of their respective rosins and the qualitative similarity of the chemical components of GEGR and GEWR. In addition, these esters undergo very limited hydrolysis in the gastro-intestinal tract.

The Committee decided to include GEGR in the ADI for GEWR of 0–25 mg/kg bw, thereby establishing a group ADI of 0–25 mg/kg bw for GEWR and GEGR.

The Committee requested that it be provided with full reports of the two 90-day toxicity studies with GEGR in rats fed dietary concentrations of up to 1.0% to confirm the validity of the comparison of GEWR with GEGR.

The Committee considered that although GEWR and GEGR are chemically similar, they are produced from different sources, processed using different procedures and conditions, and not identical in composition. The Committee therefore developed separate specifications for GEGR. The specifications were made tentative pending the submission of infrared spectra that correspond to the commercially available products, data on the resin acid composition obtained with updated chromatographic techniques and additional information on methods that enable the identification of the individual rosin esters and their differentiation. This information should be submitted by the end of 2010.

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GLYCEROL ESTER OF TALL OIL ROSIN

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1. EXPLANATION

Glycerol ester of tall oil rosin (GETOR) was placed on the agenda of the current meeting at the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008). The Committee had not previously evaluated GETOR. However, the Committee previously considered, at its eighteenth, twentieth, thirty-third, thirty-seventh, forty-fourth and forty-sixth meetings, a related substance, glycerol ester of wood rosin (GEWR) (Annex 1, references *35*, *41*, *83*, *94*, *116* and *122*). At its forty-sixth meeting, the Committee allocated an acceptable daily intake (ADI) for GEWR of 0–25 mg/kg body weight (bw).

GETOR is intended to be used as an emulsifier/density adjustment agent for flavouring substances in non-alcoholic beverages.

1.1 Chemical and technical considerations

GETOR is a complex mixture of triglycerol and diglycerol esters of resin acids from tall oil rosin (TOR), with a residual fraction of monoglycerol esters. It is obtained by the esterification of TOR under a nitrogen atmosphere with food-grade glycerol and purified by steam stripping. TOR is obtained as a by-product of the kraft (paper) sulfate pulping process.

TOR contains approximately 90% resin acids and 10% neutrals. The resin acid fractions are a complex mixture of isomeric diterpenoid monocarboxylic acids having the empirical formula $C_{20}H_{30}O_2$ and grouped into three main classes: abietane, pimarane and isopimarane. Resin acids of these classes are similar in structure, differing only in the number and arrangement of double bonds or in the stereochemistry of the C-13 position. The neutral fraction is composed of esters of resin acids, esters of fatty acids and various unsaponifiable materials. The composition of the resin acid portion of the esters in the neutral fraction is similar to that of the resin acids in the acid fraction; the fatty acid portion of the esters in the neutral fraction is predominantly C_{18} or higher straight-chain acids with varying degrees of unsaturation.

The chemical composition of GETOR varies depending on the pine species, environmental factors, geographical differences and the techniques used in the processes of rosin purification and refinement. During the esterification procedure, because of the high temperature and severe conditions employed, some isomerization and dehydration reactions of the resin acids also occur, so that the resin acid distribution in the final ester is different from that in the original rosin. Additionally, the quality and consistency of TOR will depend upon the quality of the crude tall oil by-product from which it is distilled. Characteristic impurities in GETOR are sulfur compounds, which are derived from the use of sulfate in the kraft paper-making process. No data were available on the resin acid composition of GETOR in commerce.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Chemical similarity of the glycerol esters of tall oil and wood rosin

At its present meeting, the Committee reviewed and evaluated the available data pertaining to the chemical composition of both GETOR and GEWR and the various analytical techniques employed to determine their chemical similarity. The three types of rosins—gum, tall oil and wood—are differentiated primarily by the part of the pine tree from which they are obtained, the variety of pine tree species used and the manufacturing process. The Committee also noted the compositional differences that are unique to TOR. The Committee noted that several sulfur

compounds have been detected in crude tall oil. These include sodium sulfate, hydrogen sulfide, methyl mercaptan, dimethyl sulfide and dimethyl disulfide. However, it is unlikely that the four latter compounds, which are volatile, would be retained during the harsh conditions of subsequent refining and purification. The most likely residual sulfur compounds in TOR would be sodium sulfate and possibly traces of dimethyl sulfide and dimethyl disulfide. However, there are no data to confirm the presence of these specific compounds and whether their presence in trace amounts would pose any toxicological concern. The Committee did not have adequate information on the composition of GETOR, considering that the source material and production processes are different, which may result in different by-products.

2.1.2 Absorption, distribution, metabolism and excretion

There are no data available on the absorption, distribution, metabolism or excretion of TOR or GETOR.

The Committee evaluated the results of several unpublished pharmacokinetic studies on GEWR at its forty-fourth and forty-sixth meetings and concluded that food-grade GEWR is metabolically stable in the gastrointestinal tract and that only a minor fraction, most probably the monoglycerol ester fraction, undergoes partial hydrolysis. These studies are described in detail in the previous evaluations by the Committee (Annex 1, references *117* and *123*). However, a brief summary of each study is given below.

In the first study, Fischer 344 rats (six per sex per group) were fed diets containing ester gum 8BG (a commercial food-grade preparation of GEWR) under two treatment regimens: 1) for 24 h at a concentration of 7000 or 28 000 mg/kg diet, and 2) for 10 days at a concentration of 14 000 or 28 000 mg/kg diet. Feed intake was measured, and ester gum 8BG intake was calculated for each treatment group. Faeces were collected during each 24-h treatment period and for subsequent 24-h periods until ester gum 8BG was no longer detected. After a 24-h dietary intake, most of the ingested ester gum 8BG was excreted in the faeces within 48–72 h. The faecal recovery was 75% of the amount ingested at the low dose and 95% at the higher dose. The low faecal recovery at the low dose was attributed to the lack of sensitivity of the analytical method. After 10 days of repeated dietary intake, faecal recoveries of the ester gum 8BG were essentially equal to the amount ingested. The author concluded that neither enterohepatic cycling nor measurable hydrolysis of ester gum 8BG took place in the rat intestine (Blair, 1995).

In another pharmacokinetic study, Fischer 344 rats received a single dose of about 200 mg [1,3-^{14}C]glycerol ester gum 8BG/kg bw by gavage after 1 day (five male and five female rats) or 10 days (five male rats) of dietary administration of unlabelled compound. In both male and female rats fed ester gum 8BG for 1 day, more than 95% of the administered dose was recovered in the faeces or cage rinses, 1% or less was excreted either as carbon dioxide in the expired air or in urine within 120 h and only traces ($\leq$0.2%) of the total dose of radiolabel were detected in 8 of 15 carcasses 120 h after treatment. Similar results were obtained in male rats given the radiolabelled ester gum after a 10-day dietary administration of unlabelled

compound. According to the author, analysis of the faecal extracts using reversed-phase high-performance liquid chromatography (HPLC) indicated that a very small percentage of the administered ^{14}C-labelled ester gum 8BG, probably monoglycerol esters, was hydrolysed (Noker, 1996).

In a separate study employing five male rats with jugular vein and biliary duct cannulae, radioactivity was measured in bile and blood at 4-h and 12-h intervals for 24 h following oral administration of ^{14}C-labelled ester gum 8BG. During the 24-h period, 1.6–2.9% of the administered dose was excreted in the bile, 0.1% or less was accounted for in the blood and 0.1–0.2% was found in livers collected from the rats. HPLC analyses of two bile samples indicated the presence of hydrolysed components. The results of this and the previous study indicate that ^{14}C-labelled ester gum 8BG is absorbed at very low levels and undergoes minimal hydrolysis or degradation in the gastrointestinal tract (Noker, 1996).

In order to determine the metabolic fate of ester gum 8BG in an in vitro system, [1,3-^{14}C]glycerol ester gum 8BG was incubated with human faecal extract, simulated gastric fluid or sterile water (as a negative control) at a concentration of 0.5 or 4.4 mg/ml for 24 h. Samples were collected at 0, 6 and 24 h and analysed in an HPLC–radiochemical detector system. The elution patterns on the radiochromatogram from the three incubation systems were similar. As ester gum is a mixture, further analysis was done based on changes in radioactivity in seven regions related to the peaks of radioactivity on the radiochromatograms. With the negative control, no significant changes were observed over time at either concentration. With human faecal extract, occasional minor differences were reported in regions containing small peaks. With simulated gastric fluid, there were no significant changes in peak regions up to 24 h at the low concentration. At the higher concentration, however, a slight decrease was seen in one peak region associated with the triglycerides of rosins in the 24-h sample compared with the 6-h sample; there was no significant difference in that same peak between the 0-h and 6-h samples. As the emptying time of the stomach is normally about 4 h, changes observed after that time would have no effect on the stability of ester gum in the stomach in vivo. These results demonstrate the metabolic stability of ester gum 8BG under conditions resembling those present in the human gastrointestinal tract (Lin, 1996).

The Committee also considered the previous evaluation of the absorption studies in rats with tritiated resin acids—namely, dehydroabietic, tetrahydroabietic and isopimaric acids—which indicated that these resin acids were recovered primarily from the faeces within 2 weeks (most within 4 days) after oral administration. The small amount of dehydroabietic acid absorbed appeared to have been metabolized in the liver to three or four uncharacterized metabolites, which were then excreted in the bile and urine. There was limited evidence to show that tetrahydroabietic and isopimaric acids were metabolized (Radomski, 1965).

2.2 Toxicological studies

At the present meeting, the Committee evaluated the toxicological studies with TOR, which included acute toxicity, 90-day toxicity and 2-year toxicity/

carcinogenicity studies, acute oral toxicity studies with GETOR and two new 90-day toxicity studies with GEWR. These studies are described below, together with brief summaries of previously evaluated short-term toxicity studies on wood rosin (WR) and GEWR.

2.2.1 Acute toxicity

(a) Tall oil rosin

The Committee, at its present meeting, considered the acute oral toxicity studies with TOR in several rodent species.

Male mice, rats and guinea-pigs (10 of each species) received graded oral gavage doses of TOR in corn oil, and the animals were observed for 14 days. The acute oral median lethal doses (LD_{50} values) for TOR in mice, rats and guinea-pigs were reported to be 4600, 7600 and 4600 mg/kg, respectively (Kay, 1961).

(b) Glycerol ester of tall oil rosin

The Committee, at its present meeting, considered the acute oral toxicity studies with GETOR in rats.

In two separate studies, GETOR was tested for acute oral toxicity in Sprague-Dawley rats (five males and five females). A single dose (5.0 g/kg bw) of the test compound as a 10% suspension in corn oil was administered by gavage. Animals were observed daily for 14 days. Parameters evaluated included clinical signs, mortality and gross necropsy. Physical signs included urinary and faecal staining, soft stool and decreased motor activity within 48 h after dosing. With the exception of piloerection and decreased motor activity in some animals, all signs diminished during the second week. As no animals died during the period of observation, the median lethal doses (LD_{50} values) for GETOR were considered to be greater than 5.0 g/kg bw (Auletta, 1979a,b).

2.2.2 Short-term studies of toxicity

(a) Rats

(i) Tall oil rosin

At its present meeting, the Committee evaluated the new 90-day oral toxicity study in rats administered TOR. In this study, Sprague-Dawley rats were given diets containing TOR (prepared as a 40% suspension in corn oil and blended into the diet) at a dietary concentration of 0%, 0.01%, 0.05%, 0.2%, 1.0% or 5.0%, equivalent to 0, 5, 25, 100, 500 and 2500 mg/kg bw per day. Two identical control groups were utilized. The diets of controls contained 2.3% corn oil, except for the diet of the 5.0% group, which contained 11.7% corn oil. Test parameters included clinical observations, mortality, body weights and body weight gain, feed consumption, feed utilization, haematology, urinalysis, organ weights, and gross and microscopic pathology. The group of rats administered the highest concentration of

5.0% TOR was discontinued early in the study because all animals experienced drastic reductions in body weight and died during the first 7 days of the treatment period. The mortality was associated with feed refusal. At dietary concentrations up to 1.0%, there was no mortality, and there were no treatment-related effects on haematology, urinalysis, or gross and microscopic histopathology. Body weight gain and feed consumption were reduced, particularly during the first few weeks of the study. Decreases in organ weights in the 1.0% dietary group were not accompanied by histopathological changes and were not considered to be of toxicological significance (Kay & Calandra, 1960).

(ii) Glycerol ester of tall oil rosin

No 90-day oral toxicity data are available for GETOR.

(iii) Wood rosin

In the 90-day oral toxicity study that was previously evaluated by the Committee, Sprague-Dawley rats (10 per sex per dose) were fed a diet containing 0%, 0.01%, 0.05%, 0.2% or 1.0% *N*-wood rosin (equal to 0, 6.4, 36, 119 or 674 mg/kg bw per day, respectively). A 5.0% dietary group was discontinued early in the study because all animals in this group died during the first week of the treatment period. This mortality was associated with feed refusal. At dietary concentrations up to 1.0%, there was no mortality. However, body weights and feed consumption were significantly reduced in male and female rats throughout the study. Liver to body weight and brain to body weight ratios for male and female rats were significantly increased at the 1.0% dietary concentration. There were no significant differences in haematology, urinalysis, or gross and microscopic histopathology observed in any of the treated animals (Kay, 1960; Annex 1, reference *117*).

(iv) Glycerol ester of wood rosin

The Committee previously evaluated two 90-day oral toxicity studies in rats with ester gum 8BG, a commercial food-grade GEWR (Annex 1, reference *117*). It also evaluated two new studies with GEWR at the current meeting (Mann et al., 1982; Tompkins, 1989). All these studies are briefly summarized below.

In a 13-week oral toxicity study, groups of Fischer 344 rats (20 per sex) were fed a diet containing ester gum 8BG at a dose level of 0, 625, 1250 or 2500 mg/kg bw per day. There were no deaths among treated or control rats during the course of the study and no changes in appearance, behaviour, ophthalmoscopic examination, haematology, clinical chemistry, or gross and microscopic histopathology that were attributed to treatment. Slight, but significant, decreases in body weight gain were noted in female rats at 1250 and 2500 mg/kg bw per day during the last weeks of the study. However, these minor effects were considered negligible and attributed to dietary dilution. Dietary dilution was also thought to account for a slight dose-related increase in food consumption at all dose levels in both sexes. Minor differences between controls and high-dose groups in organ to body weight ratios and organ to brain weight ratios were small and not associated with any histological abnormalities, and they were not considered to be an important effect of treatment.

The no-observed-effect level (NOEL) in this study was 2500 mg/kg bw per day, the highest dose tested. On the basis of these results, the Committee allocated an ADI for GEWR of 0–25 mg/kg bw (Blair, 1991, 1992).

In another 90-day oral toxicity study in Sprague-Dawley rats (10 per sex per group), animals were treated with GEWR at a dietary concentration of 0%, 0.01%, 0.05%, 0.2%, 1.0% or 5.0%. The approximate doses were 0, 5, 25, 100, 500 or 2500 mg/kg bw per day. Body weight and body weight gain were not affected by treatment. In the high-dose group, feed consumption was slightly decreased, but feed utilization was increased. The higher utilization values were related to the higher caloric content of the 5.0% dietary concentration group. No treatment-related effects on haematology, urinalysis, gross pathology, histopathology or organ weights were reported. No deaths occurred, and no adverse clinical signs were noted. Based on these data, the NOEL was approximately 500 mg/kg bw per day (Calandra, 1960a).

In another study, groups of Crl:CD BR rats (n = 25 per sex, except for the low dose, where n = 20 per sex) were fed GEWR at a dietary concentration of 0, 2000, 5000 or 10 000 mg/kg for 90 days. Mean GEWR consumption was calculated to be approximately 136–139 mg/kg bw per day for males and 156–171 mg/kg bw per day for females ingesting 2000 mg/kg; 339–340 mg/kg bw per day for males and 402–403 mg/kg bw per day for females ingesting 5000 mg/kg; and 714 mg/kg bw per day for males and 815–831 mg/kg bw per day for females ingesting 10 000 mg/kg. Parameters evaluated included mortality, clinical signs, body weight, feed consumption, haematology, clinical chemistry, urinalysis, ophthalmology, faecal examination, organ weights, and gross and microscopic pathology. An interim sacrifice occurred on day 30, at which rats (five per sex per group) from the control, mid-dose and high-dose groups were necropsied. GEWR did not affect mortality (100% survival), clinical signs, body weight, body weight gain, feed consumption, organ weights, haematology, clinical chemistry, urinalysis, ophthalmology, faecal observations, gross pathology or microscopic pathology at any dose level. Based on these data, the NOEL was considered to be 10 000 mg/kg (equivalent to 714 and 815–831 mg/kg bw per day for males and females, respectively), the highest dose tested (Tompkins, 1989).

In another study, Sprague-Dawley rats (15 per sex per group) were fed GEWR at a dietary concentration of 0%, 0.2%, 1.0% or 5.0% for 90 days. The approximate doses were 0, 200, 1000 and 5000 mg/kg bw per day, based on standard conversion factors (International Programme on Chemical Safety, 1987). Parameters evaluated included mortality, clinical signs, body weight, feed consumption, haematology, clinical chemistry, urinalysis, faecal examination, organ weights, and gross and microscopic pathology. No clinical signs were observed in any dose group except for one death of a male rat in the high-dose group. GEWR administration did not affect body weight, haematology, clinical chemistry, urinalysis or faecal examination. Statistically significant decreases in feed consumption were noted in the high-dose males during the first 5 weeks and also in the 13th week and in the high-dose females during the first 3 weeks and later during the 5th and 9th weeks. Dose-dependent, statistically significant increases were reported in absolute and relative liver weights in the high-dose females and in relative liver weight in the

mid-dose females and in the mid-dose and high-dose males. Histopathology revealed slight periportal hepatocytic vacuolation in the high-dose females only. No other histopathological changes were noted. Based on these data, the NOEL was approximately 1000 mg/kg bw per day (Mann et al., 1982).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rats

(i) Tall oil rosin

At its present meeting, the Committee evaluated the 2-year oral toxicity study in rats administered TOR.

Groups of weanling Sprague-Dawley rats (30 per sex, individually housed) were fed a dietary concentration of 0%, 0.05%, 0.2% or 1.0% TOR, prepared as a 30% suspension in corn oil (weight per weight [w/w]), for 2 years, equivalent to 0, 25, 100 and 500 mg/kg bw per day. The final corn oil content was 2.3% in all test and control diets. At the end of 12 months, five animals of each sex were sacrificed for gross and microscopic pathology studies. All surviving animals were killed at 24 months; organ weights were recorded, and pathological examinations were conducted.

At both 12 and 24 months, body weights were significantly lower than those of controls in both males and females (statistically significant) at the 1.0% dietary concentration. The decreased body weight was attributed to the reduced feed consumption level. There were no significant differences between TOR-treated groups and controls with respect to survival, tumour rate, haematology, urinalysis, or gross or microscopic pathology (Kohn & Kay, 1962a).

(ii) Wood rosin

In a 2-year toxicity/carcinogenicity study previously evaluated by the Committee, Sprague-Dawley rats (30 per sex per group) were fed dietary concentrations of 0%, 0.05%, 0.2% or 1.0% WR for 24 months (equal to 0, 24, 88 and 434 mg/kg bw per day). Five animals from each group were sacrificed at 12 months for gross and histopathological examination. At the 1.0% dietary concentration, body weights were significantly lower than in controls at both 12 and 24 months. Decreased body weights were again attributed to reduced feed consumption. Some sporadic differences in organ to body weight ratios were noted, but were not considered to be of toxicological significance. There were no significant differences between WR-treated and control rats with respect to mortality, haematology, urinalysis, gross and microscopic histopathology, or tumour incidence (Kohn, 1962a; Annex 1, reference *117*).

(b) Dogs

(i) Tall oil rosin

At its present meeting, the Committee evaluated the 2-year oral toxicity study in dogs administered tall oil rosin.

Groups of Beagle dogs (three per sex) were fed TOR, prepared as a 30% suspension in corn oil (w/w), at a dietary concentration of 0.05% or 1.0% (equivalent to 12.5 and 250 mg/kg bw per day) for 2 years. A control group consisting of six animals of each sex received the basal diet. The final corn oil content was 2.3% in all test and control diets. Test parameters included body weight, food consumption, mortality and behavioural changes, haematology, urine analysis, liver and kidney function tests, and gross and microscopic examinations. The feed intake was slightly lower in the high-dose group than in the control dogs. No significant effects were observed in body weight, organ weights, mortality, behavioural changes, haematology, urine analysis, liver and kidney function tests, or gross or microscopic pathological examinations (Kohn & Kay, 1962b).

(ii) Wood rosin

In a 2-year oral toxicity study previously evaluated by the Committee, Beagle dogs (three per sex per group) were fed diets containing 0%, 0.05% or 1.0% *N*-wood rosin (equal to 0, 14 or 260 mg/kg bw per day). Body weight appeared to be somewhat decreased in the high-dose males, consistent with reduced feed consumption; however, a clear treatment-related effect on body weight gain was not discernible. The author concluded that the NOEL in this study was 1.0% in the diet (Kohn, 1962b; Annex 1, reference *117*).

2.2.4 Genotoxicity

In its previous evaluation of GEWR, the Committee concluded that GEWR is not genotoxic in several in vitro test systems (Annex 1, reference *123*).

2.3 Observations in humans

2.3.1 Contact sensitization potential

Rosin (commonly known as colophony) has long been recognized as a dermal sensitizer. Rosin was first used in routine dermatological patch test series in 1939. The European Union currently classifies all types of rosin (gum rosin, wood rosin [WR] and TOR) as skin sensitizers (Karlberg et al., 1999).

The Committee, at its forty-fourth meeting, noted the sensitization potential of rosins. At the present meeting, further review of several case reports in humans illustrated that dermal contact sensitization is seen with all types of rosins and rosin components, as well as for glycerol esters of gum, tall oil and wood rosins. Moreover, there is no evidence in humans to suggest that glycerol esters of gum, tall oil and wood rosins differ in their potential to produce sensitization reactions when applied to the skin.

Similarly, dermal studies in guinea-pigs have also shown that there is no difference in the contact sensitization potential of gum rosin, TOR and modified rosins (Hausen & Mohnert, 1989; Hausen et al., 1989; Shao et al., 1993; Gäfvert et al., 1996; Färm, 1998). Thus, both GETOR and GEWR appear to be substantially equivalent in their ability to cause dermal sensitization in humans and experimental animals following application to the skin.

According to the American Conference of Governmental Industrial Hygienists (1996), abietic acid, the primary resin acid found in rosin, is not believed to be the sensitizing agent; it is only when humans and experimental animals are patch tested with unpurified commercial rosin that sensitization occurs. However, Hausen & Mohnert (1989) found that of 44 dermatology patients, 17 (39%) reacted to abietic acid (as well as to standard rosin).

Abietic acid may be a weak contact allergen, and it is important to note that the sensitization potential for the various rosins and their respective glycerol esters was noted exclusively following dermal application. So far, no reports of allergic responses from ingestion of GETOR or GEWR in beverages have been found.

3. DIETARY EXPOSURE

GETOR is intended to be used as an emulsifier/density adjustment agent in oil-based flavourings that are added to non-alcoholic beverages to increase the density of flavouring oils (e.g. lemon oil, orange oil). When dispersed in the finished beverage, GETOR acts as an emulsifier, resulting in a desirable degree of cloudiness, and also improves emulsion stability. The maximum use level proposed by the petitioners is 100 mg/kg, and this amount is expected to be present in the final product.

In the 1970s, the United States Food and Drug Administration conducted an analysis to estimate the dietary exposure to GEWR associated with its proposed use as a density adjuster. This resulted in a dietary exposure estimate of 97.5 mg/day, based on a soft drink consumption estimate of 1.5 litres/day and an average GEWR use level of 65 mg/kg.

For the purpose of the current assessment, the dietary exposure was based on a maximum use level of 100 mg/kg combined with the total consumption of soft drinks in Australia (1995 Australian National Nutrition Survey; Food Standards Australia New Zealand, 2009), Europe (European Food Safety Authority [EFSA] Concise European Food Consumption Database; European Food Safety Authority, 2009) and the USA (2003–2004 National Health and Nutrition Examination Survey [NHANES]; Foods Analysis and Residue Evaluation Program, 2009) for adults, consumers only (Table 1). The median values were calculated for both the mean and the 95th percentile of the consumption distribution across the 19 considered countries and were, respectively, 340 and 990 g/day. Based on these figures, the estimated mean dietary exposure to GETOR would be 34 mg/day, corresponding to 0.57 mg/kg bw per day for a 60-kg adult. For high consumers, the estimated dietary exposure to GETOR would be 99 mg/day, corresponding to 1.65 mg/kg bw per day for a 60-kg adult.

Table 1. Mean and high-percentile soft drink consumption for adult consumers only

				Consumption (g/day)	
Country	Method	Number of days	Category	Mean	95th percentile
Austria	24-h recall	1	Soft drinks	323.2	1000.0
Australia	24-h recall	1	Soft drinks, flavoured mineral waters and electrolyte drinks	569.0	1500.0
Belgium	24-h recall	2	Soft drinks	476.0	1340.0
Bulgaria	24-h recall	1	Soft drinks	346.2	800.0
Czech Republic	24-h recall	2	Soft drinks	282.3	950.0
Denmark	Precoded food diary with open fields	7	Soft drinks	259.3	850.0
France	Dietary record	7	Soft drinks	127.9	426.3
Germany	Dietary history	28	Soft drinks	369.5	1442.9
Hungary	Dietary record	3	Soft drinks	223.2	600.0
Iceland	24-h recall	1	Soft drinks	674.0	1700.0
Ireland	Dietary record	7	Soft drinks	203.6	635.9
Italy	Dietary record	7	Soft drinks	114.9	347.1
The Netherlands	Dietary record	2	Soft drinks	338.8	990.0
Norway	Quantitative food frequency questionnaire	0	Soft drinks	375.6	1300.0
Poland	24-h recall	1	Soft drinks	370.1	1000.0
Slovakia	24-h recall	1	Soft drinks	613.6	2000.0
Sweden	Dietary record	7	Soft drinks	243.4	685.7
United Kingdom	Dietary record	7	Soft drinks	292.5	891.6
USA	24-h recall	2	Soft drinks	510.0	1060.0[a]
Median dietary exposure across all countries				**340**	**990**

[a] 90th percentile.

4. COMMENTS

4.1 Toxicological data

At the present meeting, the Committee evaluated the information available on the chemical composition of TOR, the toxicological studies with TOR, which included acute toxicity, 90-day toxicity and 2-year toxicity/carcinogenicity studies, and two acute toxicity studies with GETOR. There are no data available on the absorption, distribution, metabolism or excretion of TOR or GETOR.

Acute oral LD_{50} values for TOR in mice, rats and guinea-pigs were reported to be 4600, 7600 and 4600 mg/kg bw, respectively.

Results of the 90-day oral toxicity study with TOR demonstrate that Sprague-Dawley rats given diets with the highest concentration of 5.0% TOR experienced drastic reductions in body weight and died during the first 7 days of the treatment period. The mortality was associated with feed refusal. At dietary concentrations up to 1.0% (equivalent to 500 mg/kg bw per day), there was no mortality, and there were no treatment-related effects on haematology, urinalysis, or gross and microscopic histopathology. Body weight gain and feed consumption were reduced, particularly during the first few weeks of the study. Decreases in organ weights in the 1.0% dietary level group were not accompanied by histopathological changes and were not considered to be of toxicological significance.

Results of the 2-year toxicity/carcinogenicity study in rats at dietary concentrations of 0.05–1.0% with TOR indicate that at the 1.0% dietary concentration (equivalent to 500 mg/kg bw per day), body weights were significantly lower than those of controls throughout the study, which again was attributed to lower feed consumption. No significant dose-related systemic toxicity was noted in rats. In the same study, there was no evidence of carcinogenicity in rats fed TOR at dietary concentrations up to 1.0%.

In the 2-year toxicity study, TOR fed to Beagle dogs at a dietary concentration of 0.05% or 1.0% had no significant effect on growth, survival, organ weights, haematology, urinalysis, liver or kidney function, or gross or microscopic histopathology. The feed intake was slightly lower in the high-dose group than in the control dogs. No significant dose-related systemic toxicity was noted in dogs. The NOEL in this study was 1.0% in the diet (equivalent to 250 mg/kg bw per day).

The results of the studies with TOR were compared with those of the related substance WR. The Committee concluded that the results of the studies with TOR were consistent with those of both the 90-day toxicity and 2-year toxicity/carcinogenicity studies with WR, which were previously evaluated (Annex 1, reference *117*). Furthermore, the results from the 90-day toxicity studies with GEWR indicate that the feed acceptance was improved. This effect is reflected by the absence of deaths even in the highest GEWR dose group (2500 mg/kg bw per day).

In its previous evaluation of GEWR at the forty-sixth meeting, the Committee concluded that GEWR is metabolically stable in the gastrointestinal tract, with more than 95% recovered unchanged in the faeces. Only a minor fraction, most probably the monoglycerol ester fraction, undergoes partial hydrolysis (Annex 1, reference

117). Although the proportion of the monoglycerol esters is dependent upon the ratio of TOR to glycerol used in the esterification process, the variations observed in the monoglycerol esters of GETOR are comparable with those observed in the monoglycerol esters of GEWR.

The Committee also considered the previous evaluation of the absorption studies with tritiated resin acids in rats—namely, dehydroabietic, tetrahydroabietic and isopimaric acids—which indicated that these resin acids were primarily recovered in faeces within 2 weeks (most within 4 days) after oral administration. The small amount of dehydroabietic acid absorbed appeared to have been metabolized in the liver to three or four uncharacterized metabolites, which were then excreted in the bile and urine. There was limited evidence to show that tetrahydroabietic and isopimaric acids were metabolized.

The Committee also noted the compositional differences that are unique to TOR. The Committee noted that several sulfur compounds have been detected in crude tall oil. These include sodium sulfate, hydrogen sulfide, methyl mercaptan, dimethyl sulfide and dimethyl disulfide. However, it is unlikely that the four latter compounds, which are volatile, would be retained during the harsh conditions of subsequent refining and purification. The most likely residual sulfur compounds in TOR would be sodium sulfate with possible traces of dimethyl sulfide and dimethyl disulfide. However, there are no data to confirm the identity of the sulfur compounds and whether their presence in trace amounts would pose any toxicological concern.

In its previous evaluation of GEWR, the Committee concluded that GEWR is not genotoxic in several in vitro test systems.

The Committee at its forty-sixth meeting allocated an ADI for GEWR of 0–25 mg/kg bw based on the 13-week toxicity study in rats. The NOEL was 2500 mg/kg bw per day, the highest dose tested.

4.2 Assessment of dietary exposure

GETOR is intended to be used as an emulsifier/density adjustment agent for flavouring substances in non-alcoholic beverages at a maximum use level of 100 mg/kg. This amount is expected to be present in the final product.

For the purpose of the current assessment, the maximum use level was combined with the total consumption of soft drinks in Australia (1995 Australian National Nutrition Survey), Europe (EFSA Concise European Food Consumption Database) and the USA (2003–2004 NHANES) for adults, consumers only. The median values were calculated for both the mean and the 95th percentile of the consumption distribution across the 19 countries considered and were 340 and 990 g/day, respectively.

Based on these figures, the mean and the high percentile for dietary exposure to GETOR would be 34 and 99 mg/day, respectively, corresponding to 0.57 and 1.65 mg/kg bw per day, respectively, for a 60-kg adult.

5. EVALUATION

The Committee concluded in principle that the data from GEWR could be used in the evaluation of GETOR because of the absence of any toxicological effects of their respective rosins and because these esters undergo very limited hydrolysis in the gastrointestinal tract. However, the Committee did not have adequate information on the composition of GETOR, considering that the source material and production processes are different, which may result in different by-products. Therefore, the Committee decided that it could not evaluate GETOR without additional information on its composition in order to clarify the extent and significance of any differences relative to other glycerol esters of rosins.

The Committee considered that although GEWR and GETOR are chemically similar, they are produced from different sources, processed using different procedures and conditions, and not identical in composition. The Committee therefore developed separate specifications for GETOR. The specifications were made tentative pending the submission of infrared spectra that correspond to the commercially available products, data on the resin acid composition obtained with updated chromatographic techniques and additional information on methods that enable the identification of the individual rosin esters and their differentiation. The Committee also requested information on the identity of the sulfur compounds in the commercial product. This information should be submitted by the end of 2010.

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LYCOPENE FROM ALL SOURCES

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1. EXPLANATION

At the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008), the Committee evaluated lycopene extract from tomato for safety and specifications for its intended use as a food colour. The substance was originally placed on the agenda under the name "Lycopene oleoresin extract from tomato"; however, the Committee decided that "Lycopene extract from tomato" should be the name under which it would be evaluated, because the compound is not a lycopene oleoresin, but an extract that contains lycopene and other constituents dissolved and suspended in the tomato's lipids. Lycopene is the functional component of the extract intended for use as a food colour. Lycopene extract from tomato is obtained by ethyl acetate extraction of the pulp of a non-genetically modified variety of ripe tomatoes (*Lycopersicon esculentum* L.) that has a high lycopene content (6%).

The Committee previously evaluated lycopene for use as a food colour at its eighth, eighteenth and twenty-first meetings (Annex 1, references *8*, *35* and *44*). A lack of adequate information precluded the Committee from developing specifications and establishing an acceptable daily intake (ADI) for lycopene as a food colour. At its sixty-seventh meeting (Annex 1, reference *184*), the Committee evaluated both synthetic lycopene and lycopene derived from *Blakeslea trispora* and developed specifications and established a group ADI of 0–0.5 mg/kg body weight (bw).

During the Committee's evaluation of lycopene extract from tomato, it became apparent that the assessment of this extract depends on the evaluation of lycopene from other sources. The Committee therefore reconsidered all the available toxicological studies on lycopene, including a new 28-day toxicity study completed after the sixty-seventh meeting.

1.1 Chemical and technical considerations

Lycopene extract is obtained from ripe tomatoes with a high lycopene content by first crushing the tomatoes and then extracting the pulp with ethyl acetate. The solvent is removed, and the remaining extract is a dark red viscous liquid containing 5–15% lycopene, of which at least 86% is all-*trans*-lycopene; the balance of the extract is made up primarily of other naturally occurring fatty acids (72%), waxes (6%) and flavour components. Minor amounts of *cis* isomers of lycopene and other carotenoids and related substances, including β-carotene, phytofluene, phytoene and tocopherols, are also present. Because lycopene is susceptible to chemical changes such as isomerization and degradation when exposed to light, heat or oxygen, lycopene extract from tomato is packed under nitrogen and stored at low temperatures.

Lycopene extract from tomato is intended for use as a food colour in dairy products, non-alcoholic flavoured drinks, cereal and cereal products, bread and baked goods, and spreads, providing colour shades from yellow to red.

2. BIOLOGICAL DATA

All of the information in this monograph is taken from reference *186* in Annex 1, unless otherwise stated.

2.1 Biochemical aspects

Lycopene belongs to the family of carotenoids and is a biochemical precursor of higher carotenoids (e.g. β-carotene). However, unlike β-carotene, lycopene lacks a β-ionone ring structure and is therefore devoid of provitamin A activity.

In analogy with naturally occurring lycopene, the predominant isomer in synthetic lycopene is all-*trans*-lycopene (Chemical Abstracts Service No. 502-65-8). The chemical name for lycopene is 2,6,10,14,19,23,27,31-octamethyl-2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene, and synonyms are Ψ,Ψ-carotene, all-*trans*-lycopene and (all-*E*)-lycopene. Lycopene is an unsaturated acyclic hydrocarbon with the chemical structure shown in Figure 1.

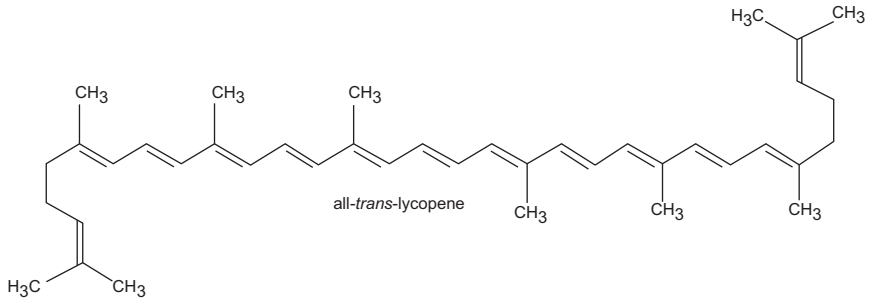

Figure 1. Chemical structure of lycopene

2.1.1 Stereochemical isomerism

All carotenoids contain an extended conjugated polyene backbone. Although theoretically each of these carbon–carbon double bonds can exist in either *trans* or *cis* configurations (also referred to as *E* and *Z* configurations, respectively), the vast majority of carotenoids, including lycopene, exhibit predominantly the all-*trans* configuration. Lycopene, however, can undergo a conversion of one or more of its *trans*-configured carbon–carbon double bonds to the corresponding *cis* forms, a conversion that occurs more readily than with most other carotenoids. The *cis* isomers of lycopene have physical and chemical characteristics that are different from those of all-*trans*-lycopene, such as lower melting points and decreased colour intensity (Nguyen & Schwartz, 1999).

In most natural matrices, all-*trans*-lycopene is thus quantitatively the most important isomer.[1] Among the naturally occurring *cis* isomers, 5-*cis*-, 9-*cis*- and 13-*cis*-lycopene usually predominate. For illustrative purposes, the isomer composition of lycopene typically found in unprocessed, raw (red) tomatoes and in tomato-based foodstuffs is provided in Table 1 (Schierle et al., 1997). It is to be noted that in other fruits and vegetables, including tangerine-type tomatoes, other *cis* isomers and/or *cis*/*trans* isomer ratios may be prevalent. Processing and storage generally do not affect the lycopene content (Nguyen & Schwartz, 1999; Agarwal et al., 2001), but heat treatment has sometimes been reported to result in *trans* to *cis* isomerization, leading to a higher content of *cis* isomers in processed foods. Table 1 also contains data on lycopene in human plasma, in which it is one of the most abundant carotenoids. A significant proportion of lycopene in human plasma is present as *cis* isomers (Khachik et al., 1997a; Schierle et al., 1997).

Table 1. Isomer composition of lycopene in different samples[a]

	Isomer (as a percentage of total lycopene)				
Sample	All-*trans*-lycopene	5-*cis*-lycopene	9-*cis*-lycopene	13-*cis*- and 15-*cis*-lycopene	Other *cis* isomers of lycopene
Raw tomatoes	94–96	3–5	0–1	1	<1
Cooked tomato-based foodstuffs	35–96	4–27	<1–14	<1–7	<1–22
Human blood plasma	32–46	20–31	1–4	8–19	11–28

[a] From Schierle et al. (1997).

2.1.2 Specifications

The Committee previously received two applications for synthetic lycopene. Synthetic lycopene is a high-purity crystalline product containing at least 96% total lycopene. Synthetic lycopene consists predominantly of all-*trans*-lycopene (not less than 70%) and 5-*cis*-lycopene, with minor quantities of other isomers. It may contain low concentrations of reaction by-products, such as triphenyl phosphine oxide (TPPO), apo-12′-lycopenal (also referred to as lycopene C25-aldehyde, lycopyl C25-aldehyde or C25-aldehyde of lycopene) and/or other lycopene-related substances, such as 1,2-dihydro-1-hydroxylycopene (rhodopin) or 1,2-dihydro-1-acetyllycopene (acetylrhodopin). Apo-12′-lycopenal and TPPO may be present in synthetic lycopene at concentrations of up to 0.15% and 0.01%, respectively. Synthetic lycopene may also contain residual solvents at low concentrations. The

[1] Before 1992, it was not possible to separate 5-*cis*-lycopene analytically from all-*trans*-lycopene. As the separation of the two stereoisomers became a standard only after 1998, earlier reported all-*trans*-lycopene contents may have been overestimated.

purity of synthetic lycopene is several times greater than that of lycopene extracted from tomatoes, which contains approximately 5% or more of total lycopene. Compared with natural lycopene in raw tomatoes, synthetic lycopene contains more 5-*cis*-lycopene. This difference is not considered to be toxicologically relevant, in view of the variation in *cis*/*trans* ratios in food sources and the fact that in processed foods, the content of 5-*cis*-lycopene is also increased. Moreover, *cis* isomers of lycopene (in particular 5-*cis*-lycopene) are present in human blood plasma at relatively high concentrations. The Committee noted that most of the available toxicological studies had been performed with synthetic lycopene formulations complying with the specifications.

The Committee previously received one application for lycopene from *Blakeslea trispora* from another sponsor. Lycopene crystals from *B. trispora* contain at least 95% total lycopene (of which at least 90% is all-*trans*-lycopene) and up to 5% other carotenoids (α- and β-carotene). The extraction solvents isopropanol and isobutyl acetate may be present in the final product at concentrations of less than 0.1% and 1%, respectively. Imidazole used during fermentation may be found in lycopene at a concentration of less than 0.0001%. The purity of lycopene from *B. trispora* is several times greater than that of lycopene extracted from tomatoes, which contains approximately 5% or more of total lycopene, whereas the *cis*/*trans* isomer ratio is comparable in lycopene from both sources.

Synthetic lycopene and lycopene from *B. trispora* are red crystalline powders. They are freely soluble in chloroform and tetrahydrofuran, sparingly soluble in vegetable oils, ether and hexane and insoluble in water. These lycopenes are sensitive to light, heat and oxygen, but are stable when stored under inert gas in light-proof containers in a cool place.

As it is not possible to get crystalline lycopene into an aqueous solution, and because of its susceptibility to oxidative degradation in the presence of light and oxygen, lycopene crystals are not suitable for commercial use. Only formulated material is marketed for use in food. Synthetic lycopene crystals and lycopene crystals from *B. trispora* are formulated as suspensions in edible oils or as water-dispersible powders and are stabilized with antioxidants. The other substances present in the marketed formulations (e.g. sucrose, corn starch, gelatine, corn oil, sunflower seed oil, ascorbyl palmitate, α-tocopherol) are common food ingredients and do not raise safety concerns.

At the current meeting, the Committee received one application for lycopene extract from tomato. The tomato extract described in this application is the ethyl acetate extract of ripe tomato fruits with high lycopene content (150–250 mg/kg). Lycopene extracted from tomatoes and tomato products contains carotenoid (5–15% by weight) and non-carotenoid components. The carotenoid fraction of the tomato extract consists mainly of lycopenes, of which ~86% is all-*trans*-lycopene, ~6% is 5-*cis*-lycopene, ~2% is 9-*cis*-lycopene and ~2% is 13-*cis*-lycopene, and other carotenoids (~4%). The major non-carotenoid components of tomato extract include fatty acids and acylglycerols (69–74%), phospholipids (8.9–14%) and waxes (5–8.4%). Lycopene extract from tomato is a dark red viscous liquid. It is freely soluble in ethyl acetate and hexane, partially soluble in ethanol and acetone and insoluble in water.

2.1.3 Absorption, distribution and excretion

(a) Rats

Absorption, distribution and excretion of lycopene were determined in male and female Wistar rats given a 10% beadlet formulation of 6,7,6′,7′-[^{14}C]lycopene (specific activity, 3.61 MBq/mg; radiochemical purity, 98%) as a single dose by gavage. Except for the radiolabel, the beadlet formulation was comparable to the commercial formulation, Lycopene 10% CWS, used in the pretreatment experiment. A series of experiments was performed:

- a balance and blood level study in groups of five male and five female rats that were given a lowest dose of 0.2 mg/kg bw or highest dose of 2.0 mg/kg bw and sacrificed at 96 h after dosing;
- a bioretention study in 16 male and 16 female rats given a dose of 2.0 mg/kg bw and sacrificed at 2, 4, 8 or 12 h after dosing;
- a balance study in five male and five female rats that were given a dose of 2.0 mg/kg bw and pretreated for 14 days by feed admix with non-radiolabelled test article at a concentration of 25 mg lycopene/kg feed; sacrifice was at 96 h after the last (radiolabelled test article) dosing; and
- a balance study in four male and four female bile duct–cannulated rats given a dose of 2.0 mg/kg bw and sacrificed at 48 h after dosing.

Depending on the study, radioactivity was analysed using liquid scintillation counting (LSC) in samples of expired air, urine, faeces, blood, plasma and/or bile collected at several time points during the study and in organs and tissues sampled at sacrifice. The study was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). Overall recovery of radiolabel in the studies was more than 92.5%. Blood and plasma concentrations peaked at 2 h after administration of both the lowest dose and the highest dose, with maximum blood concentrations being approximately 60% of maximum plasma concentrations. Average half-lives in blood and plasma were 5.2 and 3.9 h, respectively. Plasma concentrations were not linear with dose, given that the area under the curve values differed by a factor of only approximately 5 instead of 10. During the 96 h after administration, radioactivity (as a percentage of administered dose) was excreted mainly via the faeces (88–97%). Only small amounts of radioactivity were recovered from the urine (3.1–4%), expired air (1.1%) and organs and tissues (1.6–2.8%). In the study with bile duct–cannulated rats, of which only two males completed the experimental period of 48 h, radioactivity excreted in the bile in 48 h amounted to 1.3–2% of the administered dose. This low rate of biliary excretion indicates that the large amount of radioactivity found in the faeces represents mainly non-absorbed material. On average, only 8.7% of the administered dose was absorbed, of which 44% was excreted in urine, 20% in bile and 13% in expired air; 24% remained in organs and tissues at 96 h after administration. Owing to poor absorption, low absolute amounts of radioactivity (<1 µg lycopene equivalents/g) were measured in organs and tissues, blood and plasma at any time. The highest levels of radioactivity in organs and tissues were found in the liver (with a maximum level reached at 8 h after administration, and with somewhat higher levels in females than in males), followed by spleen, ovaries/uterus, fat, adrenals and intestinal tract; levels were

generally linear with dose. Pretreatment for 14 days did not affect the excretion pattern, but reduced the amount of residual tissue radioactivity: only 0.9–1% of the administered dose was recovered from organs and tissues at 96 h after administration. No obvious sex differences were observed (Wendt & Bausch, 1995).

Analysis of plasma samples demonstrated systemic absorption of lycopene in toxicological studies in rats receiving Lycopene 10% CWS for 4 weeks at a lycopene dose of 1000 mg/kg bw per day or for 14 weeks at a dose of 50, 150 or 500 mg/kg bw per day (Buser & Urwyler, 1996; Niederhauser et al., 1996; see section 2.2.2).

Systemic absorption was also demonstrated in toxicological studies in which lycopene was administered at lower doses than described above but over a longer period. When rats were given lycopene (as Lycopene 10% WS beadlets) at a dose of 10, 50 or 250 mg/kg bw per day for 52 weeks, followed by a 13-week treatment-free period, lycopene was found in plasma and liver samples of all groups during the treatment phase of the study. Mean plasma concentrations of lycopene at weeks 13, 26 and 52 showed an approximately dose-related (but not dose-proportional) increase, with the highest values occurring at 250 mg/kg bw per day. They tended to be higher for females than for the corresponding males at 50 and 250 mg/kg bw per day and did not seem to increase after week 26. At week 6 of the recovery period, lycopene was detectable (at a very low level) only in plasma of 1 out of 10 animals at the highest dose. Liver samples of male rats showed a dose-related (but not dose-proportional) increase in lycopene concentration at week 52; in liver samples of females, the lycopene concentration reached a maximum in rats given 50 mg/kg bw per day. The concentrations in liver were 3–4 times higher in females than in males (47, 176 and 200 mg/kg in males and 172, 709 and 657 mg/kg in females at lycopene doses of 10, 50 and 250 mg/kg bw per day, respectively). At the end of the recovery period, lycopene concentrations in the liver of animals at the highest dose had fallen to approximately 10% of the week 52 values (Smith et al., 2005).

When rats were given lycopene (as Lycopene 10% WS beadlets) at a dose of 2, 10 or 50 mg/kg bw per day for 104 weeks, mean lycopene plasma concentrations at weeks 52 and 104 showed a dose-related (but not dose-proportional) increase, with higher values occurring at week 104 than at week 52, and tended to be higher for males than for the corresponding females. Liver samples showed a dose-related (but not dose-proportional) increase in lycopene concentration at week 104, with concentrations being approximately 2 times higher in females than in males (33, 80 and 193 mg/kg in males and 54, 154 and 442 mg/kg in females at lycopene doses of 2, 10 and 50 mg/kg bw per day, respectively) (Edwards et al., 2006).

The distribution of 6,7,6′,7′-[^{14}C]lycopene (specific radioactivity, 0.07 MBq/mg) was examined in male F344 rats prefed a lycopene-enriched diet for 30 days and subsequently given 0.81 MBq of [^{14}C]lycopene (in 0.5 ml cottonseed oil) via gavage. Lycopene was added to the diet in the form of water-dispersible beadlets containing 10% lycopene, at a target concentration of 0.25 g/kg. The dose of lycopene administered via gavage (0.246 mg) was approximately 5% of the daily dose of lycopene received via the diet and contained 98% all-*trans*-lycopene. After

gavage, each rat was placed in a metabolism cage, and faeces and urine were collected every 24 h until sacrifice. At 0, 3, 6, 24, 72 and 168 h, eight rats per time point were sacrificed, and blood, tissues and organs were collected. Radioactivity in excreta, serum and tissues was analysed using LSC. Over 168 h, 68% of the administered radioactivity was excreted in the faeces, the majority of this within the first 48 h. The amount of radioactivity retained in the body (tissues and serum) at 168 h was 3%. Total recovery at 168 h (including urine and gastrointestinal contents) was 74%. Serum concentrations of radioactivity were highest between 3 and 24 h and declined thereafter. In liver, peak concentrations were reached at 24 h. At all time points, approximately 72% of tissue radioactivity was found in the liver. Hepatic radioactivity was made up of approximately 80% all-*trans*-lycopene and major *cis* isomers of lycopene (including 5-*cis*-lycopene) and approximately 20% polar metabolites. In extrahepatic tissue, radioactivity was greatest in adipose tissue, spleen and adrenals (Zaripheh et al., 2003).

In a follow-up study, 48 male F344 rats were divided into four groups prefed either a control diet or a lycopene-enriched diet for 30 days and killed at 5 or 24 h after receiving a single dose of 421.8 kBq of 6,7,6′,7′-[^{14}C]lycopene (specific radioactivity, 6771 kBq/mg) by gavage in 0.5 ml cottonseed oil. Lycopene was added to the diet in the form of 5% water-dispersible lycopene beadlets, at a target concentration of 0.25 g lycopene/kg. The lycopene dose via gavage (0.152 mg) was approximately 2% of the daily lycopene dose via the diet. Radioactivity in excreta collected at 3, 6 and 24 h and in serum and tissues collected at sacrifice was analysed using LSC. Non-radioactive lycopene was detectable in serum and liver at both time points. Irrespective of time, non-radioactive lycopene concentrations in serum and liver were higher in rats prefed lycopene than in rats fed the control diet. Absorption of radioactivity at 24 h was less for rats prefed lycopene (5.5%) than for control rats (6.9%). Irrespective of pretreatment diet, elimination of radioactivity was primarily in faeces (10–12% in 24 h), with only minor elimination in urine (0.088–0.091% in 24 h). Total recovery at 24 h from all tissues, gastrointestinal contents and excreta was 75% and 57% for rats prefed lycopene and control rats, respectively. In tissues and organs, most radioactivity was found in the contents and tissues of the gastrointestinal tract, followed by liver and spleen. Other extrahepatic tissues contained only small amounts of radioactivity. In liver and spleen, radioactivity increased with time and was affected by the diet, such that rats prefed the control diet had higher concentrations of radioactivity compared with the rats prefed lycopene, particularly at 24 h. Hepatic radioactivity was made up of 71–76% all-*trans*-lycopene and major *cis* isomers of lycopene (including 5-*cis*-lycopene) and 24–29% polar metabolites, irrespective of pretreatment diet and time (Zaripheh & Erdman, 2005).

Crystalline 6,7,6′,7′-[^{14}C]lycopene (specific activity, 3.74 MBq/mg) was administered by gavage to three male and three female Sprague-Dawley rats at a single dose of 0.74 MBq (in 1 ml olive oil containing α-tocopherol at 1 mg/ml). Blood was drawn before dosing and at 4, 8, 24, 48 and 72 h after dosing, after which the rats were killed and organs were removed for radioactivity analysis. Maximal absorption of radioactive lycopene into the blood occurred between 4 and 8 h after administration. All organs examined contained trace amounts of radioactivity except for the liver, which contained by far the highest amount (Mathews-Roth et al., 1990).

Using mesenteric lymph duct–cannulated male albino rats as an animal model, the absorption of purified lycopene (from tomato paste) was investigated after continuous infusion with a lipid emulsion (olive oil) containing 20 µmol lycopene/l (2.5 ml/h) via a feeding tube placed into the duodenum. Lymph samples were collected for analysis from three animals at 2-h intervals up to 12 h after the start of infusion. In a second experiment, emulsions with four different concentrations of lycopene (5, 10, 15, 20 µmol/l) were intraduodenally infused into three rats per treatment, and samples of lymph collected between 6 and 12 h of infusion were analysed (representing absorption under steady-state conditions). In a third experiment, the possible interactions of lycopene (20 µmol/l) with canthaxanthin (20 µmol/l) were studied in four animals per treatment group. Absorption, calculated by dividing the concentration of lycopene recovered in the lymph per hour by the concentration of lycopene infused into the duodenum per hour, ranged from 2% to 8%, with an average recovery of 6%. Lycopene was absorbed intact in a dose-dependent manner, with a steady state reached in the lymph after 6 h of continuous intraduodenal infusion. Lycopene and canthaxanthin did not significantly affect each other's absorption (Clark et al., 1998). In an additional study, similar to the second experiment, the effect of different oils (olive oil and corn oil) on the absorption of lycopene was investigated. Lycopene was less efficiently absorbed from corn oil emulsions (average recovery, 2.5%) than from olive oil emulsions (average recovery, 6%) (Clark et al., 2000).

The colonic absorption and distribution of lycopene were investigated in female Sprague-Dawley rats. Groups of six rats, with or without a single-barrelled colostomy at the mid-colon, received a single intragastric or intracolonic dose of 12 mg lycopene (56 mg/kg bw; source not specified) in corn oil or plain corn oil (controls) and were euthanized 24 h after exposure for determination of lycopene content in the faeces and tissues (jejunum, colon, liver). Lycopene was detected in the blood in trace amounts within 4–8 h after the single dose and was subsequently deposited in the liver, suggesting that lycopene is absorbed from the colon as well as from the small intestine of rats. A large amount of lycopene was recovered in the faeces. After intragastric lycopene treatment, lycopene was detected in the mucosae of the proximal and distal colon of the colostomized rats (Oshima et al., 1999).

Groups of F344 rats received diets containing lycopene at a concentration of 0%, 0.005%, 0.012%, 0.024%, 0.05% or 0.124% for 10 weeks (equivalent to 0, 2.5, 6, 12, 25 and 60 mg lycopene/kg bw per day, respectively) in a study investigating the uptake and tissue disposition of lycopene. There were 10 and 20 animals of each sex in the treated and control groups, respectively, and lycopene was administered as a tomato oleoresin (Betatene) containing 5.7% carotenoids (3.7% lycopene, 0.04% 2,6-cyclolycopene-1,5-diol and almost 2% others) in medium-chain triglyceride. Approximately 55% of the administered lycopene was recovered in the faeces. In males and females, concentrations of lycopene were highest in the liver (42–120 µg/g tissue), followed by mammary gland (174–309 ng/g tissue), serum (145–313 ng/g tissue), lung (134–227 ng/g tissue) and prostate (47–97 ng/g tissue). Tissue concentrations were not related to the dose administered (Zhao et al., 1998).

(b) Rabbits

In order to establish the lycopene absorption and tolerance for rabbits, four non-pregnant female New Zealand White rabbits were given Lycopene 10% WS beadlets (batch no. UT00120002; 10.5% synthetic lycopene, of which 74% was all-*trans*-lycopene) at a maximum practical daily dose of lycopene of 131 mg/kg bw by gavage for 21 days. After a washout period of 20 days, this was followed by a single gavage dose of a 10% beadlet formulation of 6,7,6′,7′-[^{14}C]lycopene (specific radioactivity, 0.11 MBq/mg; radiochemical purity, 98%) at a dose of 6 mg/kg bw. Blood samples were taken up to 24 h after dosing on days 1, 6 and 21 and up to 72 h after dosing the radiolabel. One rabbit died after the first dose as a result of a dosing error. The remaining three animals tolerated administration well, with no signs of ill health or effects on body weight. After the first dose, lycopene appeared in plasma after 1 h, and a maximum concentration (56 µg/l) was reached after 2 h. Plasma lycopene concentrations remained at this level thereafter, with the proportion of the all-*trans* isomer increasing from 19% at 1 h to 43% at 24 h. Plasma lycopene concentrations after 6 and 21 doses showed mean plasma lycopene concentrations of between 96 and 163 µg/l at all time points (27–29% all-*trans*-lycopene), with a slight rise occurring at the 2-h time point. Steady state was reached by day 6. After the radiolabelled dose, lycopene appeared in plasma after 1–2 h, and a maximum concentration (18 µg/l) was reached after 10 h, with the proportion of the all-*trans* isomer increasing from 36% after 2 h to 93% after 48 h. Radioactivity, however, appeared in plasma after 0.5 h, with maximum concentrations (approximately 50 µg equivalents/l) reached between 2 and 6 h. Lycopene as a percentage of radioactivity increased from 0% at 0.5 h to 36% at 10 h and then declined to 33% at 24 h and 5% at 72 h (Edwards et al., 2002).

In a further study, three female New Zealand White rabbits received lycopene at a dose of 400 mg/kg bw per day, achieved by dosing twice rather than once daily. Lycopene was administered as Lycopene 10% WS beadlets (batch no. UT02070001; 13.2% synthetic lycopene, with 74% being all-*trans*-lycopene) for 21 consecutive days. One animal died after the first dose as a result of a dosing error. The remaining two animals showed good tolerance for treatment with lycopene, but displayed dark red discoloured faeces, stomach and intestinal contents. Lycopene was observed in the plasma 2–4 h after the first dose, with a maximum concentration of 69 µg/l reached at 8 h. On days 6 and 21 of dosing, maximum plasma lycopene concentrations were 171 µg/l (at 4 and 8 h) and 339 µg/l (at 4 h), respectively. Samples of liver taken at termination showed lycopene concentrations of 1 and 2.3 mg/kg (Edwards et al., 2004a).

(c) Dogs

The pharmacokinetics and tissue distribution of lycopene were studied in male Beagle dogs. The dogs were fed a lycopene-free dog food with a high fat content to aid lycopene absorption. The test material (a 5% lycopene-containing granular reddish powder) was administered in gelatine capsules 2 h after initiation of feeding as single doses containing lycopene at 10, 30 or 50 mg/kg bw to two dogs per dose or at a dose of 30 mg/kg bw per day for 28 days to six dogs. In the single-dose and repeated-dose studies, blood was collected for plasma lycopene

analysis at several time points. In the repeated-dose study, three dogs per time point were killed at 1 day and 5 days after the last dose, and tissues and organs were collected for lycopene analysis. Peak plasma concentrations (56–129, 112–507 and 364–452 nmol/l from lowest to highest dose, respectively) were observed between 11 and 21 h in the single-dose studies, where half-lives ranged from 23 to 59 h. In the repeated-dose study, the plasma half-life of lycopene was approximately 30 h, and a steady-state plasma concentration of approximately 800 nmol/l was reached approximately 4 days after the first dose. In tissues and organs, lycopene concentrations were highest in the liver, followed by adrenals, spleen, lymph nodes and intestinal tissues. Tissue concentrations were generally higher at 5 days than at 1 day after the last dose. Although the test material contained 70% all-*trans*-lycopene, most of the lycopene identified in plasma and tissues was *cis*-lycopene, with only 23–41% all-*trans*-lycopene. Moreover, the percentage of all-*trans*-lycopene in many tissues, including liver, was significantly lower than that in plasma (Korytko et al., 2003).

(d) Monkeys

Five female Rhesus monkeys were given crystalline 6,7,6′,7′-[^{14}C]lycopene (specific activity, 3.74 MBq/mg) by gavage as a single dose at 1.85 MBq (in 2.5 ml olive oil containing α-tocopherol at 1 mg/ml). Blood was drawn before dosing and at 2, 4, 8, 24 and 48 h after dosing, after which the monkeys were killed and organs were removed for radioactivity analysis. The monkeys absorbed lycopene with considerable individual variation, both in blood, where peak concentrations were reached between 8 and 48 h, and in organs. Liver contained the largest amount of radioactivity, but considerable amounts were also found in the spleen and organs of the digestive tract (Mathews-Roth et al., 1990).

(e) Calves

In a 2-week feeding study in preruminant calves, differences in lycopene absorption from a synthetic lycopene preparation (LycoVit 10%; 10% lycopene) and absorption of lycopene from a natural tomato resin (Lyc-O-Mato Beads 5%; 5% lycopene) were evaluated. Groups of eight calves received lycopene as daily doses at 15 mg for 14 days in milk replacer. Plasma was analysed for carotenoids before, directly after and at several time points during a 10-day post-treatment period. In plasma, all-*trans*-lycopene and 5-*cis*-lycopene were present, as well as three lycopene metabolites not previously found. These metabolites (probably hydrogenation products of all-*trans*-lycopene and 5-*cis*-lycopene) contributed 52% to the total lycopene content measured at the end of the intervention period. On days 14–21, the concentration of total lycopene in the plasma in calves receiving lycopene from the synthetic preparation (216–286 nmol/l) was more than 3-fold that in calves fed lycopene from the tomato resin (46–72 nmol/l); however, no differences were observed in the distribution of the isomers and metabolites of lycopene (Sicilia et al., 2005).

(f) Humans

Lycopene, like all carotenoids, is fat soluble and follows the same digestion and intestinal absorption pathways followed by dietary fat (Rao & Agarwal, 1999).

Absorption of dietary carotenoids begins with their release from the food matrix and dissolution in the lipid phase, followed by incorporation into lipid micelles in the small intestine, which is required for mucosal uptake, and, finally, transport to the lymphatic and/or portal circulation (Erdman et al., 1993; Parker, 1996; Holloway et al., 2000).

Movement of carotenoids from the mixed lipid micelle into the mucosal cells of the duodenum appears to occur via passive diffusion, and subsequent transport from the enterocytes to the bloodstream involves incorporation into chylomicrons (with lycopene in the hydrophobic core) and secretion into the lymphatics. There is no evidence for significant portal absorption of carotenoids in humans (Parker, 1996). Carotenoids are transported in plasma exclusively by lipoprotein, with lycopene being transported primarily by low-density lipoprotein (LDL) (Erdman et al., 1993; Parker, 1996; Holloway et al., 2000). Lycopene accumulates in tissues rich in LDL receptors, such as liver, adrenals and testes (Holloway et al., 2000). Other human tissues containing detectable concentrations of lycopene include blood plasma, adipose tissue, prostate and lung (Schmitz et al., 1991; Redlich et al., 1996; Rao & Agarwal, 1999). Reported tissue concentrations of lycopene vary by about 100-fold between individuals (Rao & Agarwal, 1999).

Lycopene is the most predominant carotenoid in human plasma. It reaches its maximum concentration in the plasma 24–48 h after dosing (Stahl & Sies, 1992); with repeated dosing, the blood concentration continues to rise until a steady state is reached. Rao & Agarwal (1999) reported a half-life of lycopene in plasma in the order of 2–3 days, whereas Cohn et al. (2004) reported estimated half-lives of 5 days and 9 days for all-*trans*-lycopene and 5-*cis*-lycopene, respectively. When subjects were given a diet low in or without lycopene, their plasma lycopene concentrations were significantly lower after 1–2 weeks (Böhm & Bitsch, 1999; Porrini & Riso, 2000; Cohn et al., 2004); half-lives were estimated to be between 12 and 33 days (Rock et al., 1992; Burri et al., 2001). Besides the effect of dietary fat (and dietary fibre content) on lycopene bioavailability, a number of conditions have been suggested to affect the absorption, metabolism or clearance of carotenoids, such as smoking and alcohol consumption (Bowen et al., 1993; Gärtner et al., 1997). Smokers have been reported to have lower plasma concentrations of most carotenoids compared with non-smokers. This was also demonstrated for lycopene by Pamuk et al. (1994). However, other studies in smokers (Peng et al., 1995; Brady et al., 1996; Driskell et al., 1996; Mayne et al., 1999) showed no effect of smoking on plasma lycopene concentrations. Chronic alcohol consumption has been reported to decrease dietary absorption of carotenoids (Leo & Lieber, 1999). However, Brady et al. (1996) found no interaction between alcohol consumption and plasma lycopene.

Plasma lycopene concentrations reported for controls in several studies ranged from 0.2 to 1.9 µmol/l (Schierle et al., 1997; Paetau et al., 1998; Mayne et al., 1999; Porrini & Riso, 2000; Hoppe et al., 2003; Cohn et al., 2004). The most abundant geometrical isomers in human plasma are all-*trans*-lycopene and 5-*cis*-lycopene, with all the *cis* isomers contributing to more than 50% of total lycopene (Schierle et al., 1997; Rao & Agarwal, 1999). The most prominent geometric isomer that occurs in plant sources is all-*trans*-lycopene. Although heat treatment and

processing may result in *trans* to *cis* isomerization, leading to increases (<10%) in the *cis*-lycopene content of foods (Schierle et al., 1997; Boileau et al., 2002), this cannot fully explain the higher concentrations of *cis* isomers found in human blood (and tissues) compared with concentrations in the foods consumed. Hence, biological conversions may take place in humans after consumption (Holloway et al., 2000). Indeed, exposure to low pH in the stomach has been shown to result in a small increase in *cis* isomers of lycopene (Boileau et al., 2002). It is not known whether the proportion of *cis* isomers is increased in plasma because of their greater intrinsic bioavailability or because of a faster catabolism of the all-*trans* isomer in the body, or both. It was speculated that *cis* isomers are probably more readily bioavailable because of the shorter length of the molecule, their greater solubility in mixed micelles and their lower tendency to aggregate (Boileau et al., 1999, 2002).

The bioavailability of carotenoids is affected by food preparation in the presence of lipids and the functional status of the intestine. In humans, depending on the presence of fat in the meal, appreciable quantities of carotenoids are absorbed and can be found in circulating plasma and later in adipose tissue (Su et al., 1998). Studies have demonstrated that the absorption of lycopene is increased when it is ingested with a high-fat diet. The addition of oil to tomato juice before heating also improves the bioavailability of lycopene (Stahl & Sies, 1992; Fielding et al., 2005). Heat treatment in the processing of raw tomatoes results in the release of lycopene from the cellular matrix, making it more bioavailable (Gärtner et al., 1997).

Studies on the bioavailability of synthetic lycopene and lycopene from natural sources have reported conflicting results. Some studies did not find a difference in bioavailability between supplements containing natural lycopene extracts and lycopene from tomato products (Paetau et al., 1998; Rao & Agarwal, 1998), whereas others reported that lycopene from tomato juice or lycopene in the form of a tomato oleoresin was clearly better absorbed than lycopene from fresh tomatoes (Böhm & Bitsch, 1999). Tang et al. (2005) reported that lycopene from cooked tomatoes was about 3 times less bioavailable than synthetic lycopene dissolved in corn oil. However, no oil was added to the lycopene extracted from tomatoes, which might have lowered the bioavailability, as was reported by Stahl & Sies (1992) and Fielding et al. (2005). Other studies reported that the bioavailability of synthetic lycopene was comparable with that of lycopene extracted from tomatoes in the form of an oleoresin (Hoppe et al., 2003) or from tomato juice (Paetau et al., 1998). Whereas the absorption of lycopene can be affected by other carotenoids (Wahlqvist et al., 1994; Gaziano et al., 1995), lycopene did not affect the absorption of other carotenoids, such as α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin and lutein (Hoppe et al., 2003).

Diwadkar-Navsariwala et al. (2003) presented a physiologically based pharmacokinetic model, comprising seven compartments: gastrointestinal tract, enterocytes, chylomicrons, plasma lipoproteins, fast-turnover liver, slow-turnover tissues and a delay compartment before the enterocytes, with the slow-turnover tissues serving as a slow-depleting reservoir for lycopene. The model was validated by a phase I study in groups of five healthy male subjects, describing the disposition of lycopene delivered as a tomato beverage formulation (i.e. tomato paste mixed with olive oil and distilled water) as single lycopene doses of 10, 30, 60, 90 or

120 mg. The per cent absorption decreased with increasing dose; the absolute amount of lycopene absorbed was not statistically different between the doses and amounted to a mean value of 4.7 mg. Independent of the dose, 80% of the subjects absorbed less than 6 mg lycopene, suggesting that lycopene absorption is saturated at doses above 10 mg/person.

In a randomized, parallel group study design involving six male volunteers per group, the relative plasma response to lycopene intake from different sources was investigated, with restriction of intake of lycopene-rich food products during the study. After a 2-week lycopene depletion phase, the subjects were given 20 mg lycopene daily for 8 days as tomato juice, soup prepared from tomato paste or lycopene tablets (Lycopene 5% TG; batch no. UT990059720, containing a nominal content of 5 mg of synthetic lycopene per tablet) with dinner. For reasons of comparability, subjects received dinners with similar fat contents. Plasma concentrations of lycopene were monitored throughout the depletion and dosing phases and for 22 days after dosing, and kinetics were evaluated. The three preparations of lycopene differed with respect to their isomer composition, although the all-*trans* isomer was the predominant form (92%, 86% and 73% for tomato juice, tomato soup and tablets, respectively) and the 5-*cis* isomer was the predominant *cis* isomer (4%, 6% and 19%, respectively) for all preparations. The plasma response was qualitatively the same for the three preparations, with a decline in lycopene concentrations during the depletion phase, a rise during treatment, with peak concentrations being reached after 8–9 days, and a decline in the after-dosing phase. Quantitatively, the plasma response of total and all-*trans*-lycopene was comparable for tablets and tomato soup, but was much lower for tomato juice. The plasma response of 5-*cis*-lycopene was relatively high for tablets when compared with tomato soup and tomato juice, but this difference disappeared upon normalization to cholesterol concentrations. The latter was done to reduce the considerable interindividual variation observed in plasma concentrations of lycopene. The estimated half-lives were approximately 5 days and 9 days for all-*trans*-lycopene and 5-*cis*-lycopene, respectively, and did not differ between groups. The systemic availability of synthetic lycopene from a tablet formulation was comparable with that from processed tomatoes (soup from tomato paste) and superior to that from tomato juice (Cohn et al., 2000, 2004).

2.1.4 Metabolism

(a) Rats

Three male bile duct–cannulated Wistar rats received 6,7,6′,7′-[^{14}C]lycopene (specific activity, 4.26 MBq/mg; radiochemical purity, 98%) as a single dose of 2 mg/kg bw administered by gavage. The [^{14}C]lycopene was formulated as simulated beadlets with fish gelatine, similar to commercial lycopene formulations (radiochemical purity in formulation, 75%). Urine and bile were collected at 0–6, 6–12 and 12–24 h after dosing. The rats were sacrificed 24 h after dosing, and radioactivity in blood plasma, urine, bile and liver was determined by LSC. The metabolite profile was determined in urine and bile extracts by reversed-phase radio–high-performance liquid chromatography (HPLC). Twenty-four hours after

dosing, 1.4% of the administered dose on average was excreted via urine, and 0.84% and 0.78% were found in bile and liver, respectively. The chromatograms of urine and bile were comparable, and both exhibited three broad peak regions, all showing more polarity than lycopene. The isolation and characterization of individual metabolites were not achieved. Lycopene itself was not detected in urine or bile samples (Mair et al., 2005).

(b) Humans

The metabolic pathway of lycopene has not been fully described. A number of oxidative metabolites of lycopene have been identified in tomato paste, tomato juice and human serum (Khachik et al., 1998a). The major metabolites were identified as lycopene 1,2-epoxide and lycopene 5,6-epoxide. Other minor metabolites were also identified, including 2,6-cyclolycopene-1,5-diol I and 2,6-cyclolycopene-1,5-diol II. A few of the metabolites are naturally found in tomato products at low concentrations. In human serum and human milk, only 2,6-cyclolycopene-1,5-diol I and 2,6-cyclolycopene-1,5-diol II were found (Khachik et al., 1997a, 1998a). These two epimeric isomers are dihydroxylycopenes with a five-member ring end group. Lycopene apparently undergoes oxidation to yield lycopene 5,6-oxide followed by cyclization and enzymatic reduction to form the two epimeric isomers of 2,6-cyclolycopene-1,5-diol (Khachik et al., 1997b, 1998b). None of the products formed by cleavage in the respective 11 conjugated double bonds of lycopene (as described by Kim et al., 2001) were detected in the blood of humans.

(c) In vitro studies

A post-mitochondrial preparation of rat intestinal mucosa with or without added soya bean lipoxygenase was used to investigate the metabolism of deuterated all-*trans*-lycopene (12,12′,14,14′,19,19,19,19′,19′,19′-$^{2}H_{10}$-lycopene or $^{2}H_{10}$-lycopene). Metabolites were identified by atmospheric pressure chemical ionization mass spectrometry. The addition of lipoxygenase significantly increased the production of lycopene metabolites. All metabolites were formed after 15 min of incubation, and the amount was not significantly changed after 60–90 min of incubation. Both central and excentric cleavage of lycopene occurred, and cleavage products as well as oxidation products of the original all-*trans*-lycopene were observed, including *cis* isomers (Ferreira et al., 2004).

2.1.5 Effects on enzymes and other biochemical parameters

The effect of the administration of lycopene (purity, 98.7%) in corn oil on drug-metabolizing enzyme capacity—pentoxyresorufin *O*-dealkylase (PROD), ethoxyresorufin *O*-deethylase (EROD), benzyloxyresorufin *O*-dealkylase (BROD), methoxyresorufin *O*-demethylase (MROD), quinone reductase (QR), uridine 5′-diphosphate (UDP) glucuronosyltransferase (UDPGT) and glutathione transferase (GST)—antioxidant enzyme activities and the ability to modulate 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP)-induced deoxyribonucleic acid (DNA) adducts in liver and colon and oxidative stress in blood was investigated in rats. Groups of four female Wistar rats received lycopene at a dose of 0 (corn oil

only), 0.001, 0.005, 0.05 or 0.1 g/kg bw per day for 14 days. Blood was collected on day 14 before administration of the last dose in order to determine plasma lycopene concentration. The last dose was given in conjunction with tritium-labelled PhIP; 24 h later, blood was taken, animals were killed and liver was removed for preparation of subcellular fractions. Plasma lycopene concentrations in the lycopene-supplemented groups were low (16–71 nmol/l), indicating poor absorption. BROD activity in the liver was induced in a dose-dependent fashion (up to and including 0.05 g/kg bw per day) at all doses investigated. EROD activity was induced only at the two highest concentrations tested. The extent of induction, however, was relatively minor for both enzymes (2-fold or less). Neither PROD nor MROD was affected at any dose. An investigation of selected phase II detoxification enzymes showed that lycopene is capable of inducing hepatic QR, approximately a 2-fold increase over control levels, at doses between 0.001 and 0.05 g/kg bw per day, whereas at the highest dose of 0.1 g/kg bw per day, the activity was comparable with the control level. UDPGT and GST activity were only slightly increased (less than 2-fold) at 0.005 and 0.1 g/kg bw per day, respectively. Investigation of antioxidant enzymes showed slight induction of superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase at the lower doses; at the higher doses, the (still slightly increased) activities returned to control levels. PhIP-induced oxidative stress in plasma was not affected by lycopene at any dose, nor was the level of PhIP–DNA adducts in liver or colon (Breinholt et al., 2000). The minor inducing effects of lycopene on drug-metabolizing enzymes as observed in the above study were not observed in other studies. Gradelet et al. (1996) investigated phase I (PROD, EROD, BROD, MROD, *N*-nitrosodimethylamine *N*-demethylase [NDMAD], erythromycin *N*-demethylase) and phase II (QR, GST, *p*-nitrophenol UDPGT, 4-hydroxybiphenyl UDPGT, aldehyde dehydrogenase [ALDH1, ALDH3]) xenobiotic-metabolizing enzyme activities in liver microsomes and cytosol of five male Wistar rats given diets containing lycopene at 300 mg/kg (as 5% lycopene extract from tomato, mixed with corn oil) for 15 days. In this study, lycopene affected only the activity of NDMAD, which was reduced to 60% of the control value. In another study, eight male Wistar rats were given 15 g/day of a diet containing 300 mg lycopene/kg (as 3.6% lycopene extract from tomato, mixed with maize oil) for 16 days, after which the effect on xenobiotic-metabolizing enzyme activities in the liver, lung, kidney and small intestine was investigated. BROD activity in the lung was reduced to 41% of the control value, whereas other enzyme activities (EROD, PROD, MROD, GST) were not affected by lycopene treatment (Jewell & O'Brien, 1999). With the exception of hepatic QR, Zaripheh et al. (2005) did not observe changes in the activity of phase I (EROD, BROD, MROD) or phase II (QR, GST) enzymes in liver and selected extrahepatic tissues of F344 rats fed lycopene at a concentration of 0.25 g/kg diet for periods of 0, 3, 7, 30 or 37 days or for 30 days followed by a basal diet period for 7 days. Hepatic QR was increased in rats receiving the lycopene-supplemented diet for 3 and 7 days, but not in rats treated for 0, 30 or 37 days. Withdrawal of lycopene from the diet significantly reduced hepatic QR activity compared with the enzyme activity observed at 30 days of treatment.

2.1.6 Special studies on the accumulation of lycopene in rat liver

As the liver was shown to be the main target organ after lycopene administration, a series of studies was performed to investigate possible differences between lycopene administered as beadlets and lycopene administered as tomato concentrate, and the reversibility of changes was observed. Most studies were conducted with female rats, as female rats showed higher accumulation of lycopene in liver than did male rats. In a pair-feeding experiment, groups of eight male Wistar rats were given daily 20 g of feed enriched with lycopene, using either beadlets or tomato concentrate, at a target concentration of 25 mg lycopene/kg diet for 5 weeks or for 5 weeks followed by control diet for 3 weeks. This dose was equivalent to approximately 2 mg/kg bw per day. The beadlet formulation (Lycopene 10% CWS; batch no. 11/12 7/95-1) contained lycopene as 75% all-*trans* and 23% 5-*cis* configuration at 104 mg/g; the tomato concentrate contained lycopene as a 91% all-*trans* and 5% 5-*cis* configuration at 435 µg/g. Control groups of four male rats received control diet for 5 or 8 weeks. Accumulation of lycopene was monitored in rat tissues and plasma, and liver was examined histopathologically. After 5 weeks, the plasma concentration of lycopene was lower in rats fed beadlets (2.2 ng/ml, 36% all-*trans*-lycopene) than in rats fed tomato concentrate (3.8 ng/ml, 57% all-*trans*-lycopene). In contrast, lycopene concentrations in tissues were higher in rats fed beadlets than in rats fed tomato concentrate. In tissues, the highest concentration of lycopene was found in the liver, followed by spleen and small intestine. Total mean concentrations of lycopene in liver were 2.5 times higher in beadlet-fed rats (18 µg/g, 62% all-*trans*-lycopene) than in rats fed tomato concentrate (7.1 µg/g, 82% all-*trans*-lycopene). In the spleen, total mean concentrations of lycopene were 5 times higher in the group receiving beadlets (6.3 µg/g, 78% all-*trans*-lycopene) than in the group receiving tomato concentrate (1.2 µg/g, 80% all-*trans*-lycopene). In the small intestine, total mean concentrations of lycopene were almost 3 times higher in the group receiving beadlets (2.8 µg/g, 61% all-*trans*-lycopene) than in the group receiving tomato concentrate (1.0 µg/g, 68% all-*trans*-lycopene). For all other tissues investigated (adrenal, heart, lung, kidney, brain, fat, eye, muscle, skin, testes, thyroid gland), the concentrations of lycopene found were less than 1 µg/g or not detectable. Compared with the feed given, supplementation resulted in lower relative amounts of the all-*trans* isomer in all tissues with detectable lycopene, except for spleen, where feeding with beadlets resulted in a somewhat higher proportion of the all-*trans* isomer. Depletion for 3 weeks led to significantly lower concentrations of lycopene in tissues in both the group receiving beadlets and the group receiving tomato concentrate, with a significant increase in the relative amount of the all-*trans* isomer in liver, spleen and small intestine. Histological examination of the liver did not reveal carotenoid inclusions in the treated or control animals (Glatzle et al., 1997).

The time course of lycopene levels in liver, spleen and plasma was investigated in a pair-feeding experiment in which female Wistar rats were given 20 g/day of feed enriched with lycopene beadlets (Lycopene 10% CWS; batch no. 511821, containing lycopene of a 72% all-*trans* and 22% 5-*cis* configuration at 113 mg/g) at a target concentration of 110–120 mg lycopene/kg diet for up to 64 weeks (equivalent to approximately 10 mg/kg bw per day). The concentration of

lycopene was monitored in liver, spleen and plasma of rats sacrificed after 2, 4, 6, 8, 10, 12, 14, 18, 22, 26 and 64 weeks of treatment (3–10 rats per time point). Control animals (3–6 per time point) were sacrificed at the start of the study and after 20, 26 and 64 weeks. Histopathological and morphological examinations of liver and spleen were also performed. Lycopene was found in the liver and spleen of all animals receiving supplementation and in the plasma in most animals. Liver showed the highest concentration of lycopene, with mean concentrations increasing from 80 µg/g after 2 weeks of treatment to 248 µg/g after 64 weeks; no steady state was reached. The spleen showed mean concentrations of lycopene of 33 µg/g after 2 weeks, 87 µg/g after 14 weeks, 104 µg/g after 22 weeks and 78 µg/g after 64 weeks; a steady state was found after approximately 14 weeks of treatment. There was a large variability in plasma concentrations, with mean concentrations of lycopene of 29–54 ng/ml in weeks 2–10 and 57–120 ng/ml in weeks 12–64. Compared with the feed given, 5-*cis*-lycopene was significantly increased in tissues and plasma. The proportion of all-*trans*-lycopene was 52–62% in the liver, 61–71% in spleen and 40–47% in plasma. At necropsy, brown-orange staining of the abdominal adipose tissue and red-orange to dark red discoloration of the liver were increasingly noted after 26 and 6 weeks of treatment, respectively. Upon histopathology, deposits were found in the liver and, to a lesser extent, spleen of all treated animals. To further investigate these deposits, morphological examinations were performed by semiquantitative light microscopy and electron microscopy. Light microscopy revealed a time-related increase in pigment deposition in hepatocytes between week 2 (average grading 1.2) and week 18 (average grading 3.8), but no apparent further increase thereafter (average grading 3.8–4 between weeks 18 and 64). Pigments appeared as brown-orange gritty deposits in the cytoplasm in normal light and as birefringent material in polarized light. There was a significant correlation between total liver lycopene concentration and birefringent deposits. Despite significant accumulation of pigment in liver cells, there was no evidence of lycopene-related liver damage after 64 weeks of continuous exposure. The deposits in the spleen were also birefringent pigments (average grading, 1–1.3). Upon ultrastructural localization of lycopene, the intracellular inclusions, varying in size from submicrometre sizes up to several micrometres, were found to be present in a compartment with a clearly discernible membrane bilayer, indicating a pathway that involves the endocytotic system of the hepatocytes (Glatzle et al., 1998a; Urwyler & Bohrmann, 1998).

In another pair-feeding experiment, female Wistar rats were given 20 g/day of feed enriched with lycopene beadlets (Lycopene 10% CWS; batch no. 511821, containing 11% lycopene of a 72% all-*trans* and 22% 5-*cis* configuration) at target concentrations of 50 or 100 mg lycopene/kg diet for 4 weeks (equivalent to approximately 5 or 10 mg/kg bw per day), followed by a depletion period of up to 21 weeks. Accumulation and depletion of lycopene were monitored in liver, spleen and plasma of six rats of each group per time point after 2 and 4 weeks of supplementation and after 1, 2, 3, 5, 7, 10, 13, 17 and 21 weeks of depletion. Control animals received control diet and were examined after 4, 6, 11, 17 and 25 weeks (three rats per time point). The liver was also examined histopathologically. After 4 weeks of treatment, rats in both treated groups had accumulated lycopene in liver (72 µg/g, 61% all-*trans*-lycopene, at 5 mg/kg bw per day; and 105 µg/g, 53% all-*trans*-lycopene, at 10 mg/kg bw per day) and spleen (38 µg/g, 72%

all-*trans*-lycopene, at 5 mg/kg bw per day; and 35 µg/g, 64% all-*trans*-lycopene, at 10 mg/kg bw per day). Plasma concentrations were approximately 30 ng/ml at both doses, with approximately 50% and 41% all-*trans*-lycopene at 5 and 10 mg/kg bw per day, respectively. After 1 week of depletion, lycopene was no longer detectable in the plasma. During the depletion period, a steady decrease of total lycopene in liver and spleen was observed, while the proportion of the all-*trans* isomer steadily increased in both tissues. Histological examination showed intracellular brown-orange gritty pigments in livers of treated animals (average grading 1), up to 7 weeks (5 mg/kg bw per day) or 10 weeks (10 mg/kg bw per day) of depletion. Thereafter, deposits were no longer observed. In polarized light, the pigments occurred as birefringent deposits. There was no indication of treatment-related morphological changes in the liver (Glatzle et al., 1998b).

The occurrence of liver deposits after administration of tomato concentrate or beadlets was investigated in a pair-feeding experiment in which female Wistar rats were fed lycopene at doses of approximately 20 mg/kg bw per day as tomato concentrate mixed with feed for 4, 8 or 13 weeks, followed by a depletion period of 4, 8 or 12 weeks for those rats that were treated for 13 weeks. Other rats were fed lycopene at a dose of approximately 4 mg/kg bw per day (intended dose 5 mg/kg bw per day) as beadlets (Lycopene 10% CWS; batch no. 511821, containing lycopene of a 72% all-*trans* and 22% 5-*cis* configuration at 113 mg/g) mixed with feed for 4 or 8 weeks. Accumulation and depletion of lycopene were monitored in rat liver (six rats per time point), and liver was examined histopathologically. Liver concentrations of lycopene were comparable in the groups fed tomato concentrate (39, 45 and 43 µg/g after 4, 8 and 13 weeks) and in the groups fed beadlets (42 and 49 µg/g after 4 and 8 weeks), just like the presence of birefringent deposits (in all but one animal of the groups receiving tomato concentrate and in all animals of the groups receiving beadlets, with average gradings of 1.3 and 1.6, respectively). However, the proportion of all-*trans*-lycopene was higher in the groups receiving tomato concentrate (68–72%, with 25% 5-*cis*-lycopene) than in the groups receiving beadlets (55–59%, with 38% 5-*cis*-lycopene). During the depletion period for the group receiving tomato concentrate, the concentration of lycopene in the liver steadily decreased, with an increase in the proportion of all-*trans*-lycopene (up to 82%). The birefringent deposits, however, did not decrease appreciably (Glatzle et al., 1998c).

Ten female rats from the 64-week experiment by Glatzle et al. (1998a) were transferred into a new study protocol to investigate the long-term effects of continuous feeding with lycopene at 10 mg/kg bw per day over a total of 2 years (Urwyler & Riss, 1999; see also section 2.2.3). Compared with the liver lycopene content of 248 µg/g (55% all-*trans*-lycopene) observed after 64 weeks, a decrease in liver lycopene content was observed from week 64 to week 104 of treatment (78–215 µg/g), whereas no change in the proportion of all-*trans*-lycopene was noted (50–57%). The authors attributed this decrease to the relationship between age, general health status and feed consumption. Comparing this decrease with the observations on liver lycopene content in the long-term studies of toxicity by Smith et al. (2005) and Edwards et al. (2006) (see section 2.2.3), a similar reduction between 1 year and 2 years of treatment was found in female rats at a dose of 50 mg/kg bw per day (from 709 µg/g after 1 year to 442 µg/g after 2 years), but not

at 10 mg/kg bw per day (from 172 to 154 µg/g). In male rats, no reduction was observed at either 10 mg/kg bw per day (from 47 to 80 µg/g) or 50 mg/kg bw per day (from 176 to 193 µg/g). Pigment deposition was observed in the hepatocytes of all rats, with an average grading of 5 in five prematurely sacrificed rats and 3 in the remaining five rats. Pigments appeared as brown-orange gritty deposits in the cytoplasm with normal light and as birefringent crystals with polarized light. Despite the high pigment load and the long duration of exposure, there was no indication of associated microscopic changes in the liver.

2.2 Toxicological studies

2.2.1 Acute toxicity

One study of acute toxicity has been performed with synthetic lycopene. In this study, three male and three female Wistar rats received Lycopene 10 CWD (batch no. OD-12-04 6.Spr.A; 9.81% synthetic lycopene) as a single oral dose at 5000 mg/kg bw by gavage. This study followed Organisation for Economic Co-operation and Development (OECD) Test Guideline 423 (Acute Oral Toxicity – Acute Toxic Class Method; 1996) and was certified for compliance with GLP and QA. No animals died, and the median lethal dose (LD_{50}) was >5000 mg/kg bw (BASF, 2001a). Studies of acute toxicity have also been provided for lycopene extracted from tomatoes, administered via the oral, subcutaneous or intraperitoneal route to mice (Milani et al., 1970). These studies have been summarized in Table 2 (Annex 1, reference *186*). Lycopene extracted from tomatoes was not acutely toxic (oral LD_{50} >3000 mg/kg bw).

Table 2. Studies of acute toxicity with lycopene

Lycopene preparation	Species	Sex	Route	LD_{50} (mg/kg bw per day)	Reference
Lyc-O-Mato 6%[a] (natural tomato extract)	Rat	M, F	Oral	>5000	Matulka et al. (2004)
Lyc-O-Mato 6% (natural tomato extract)	Rat	M, F	Dermal	>2000	Matulka et al. (2004)
Lycopene from tomatoes	Mouse	M	Oral	>3000	Milani et al. (1970)
Lycopene from tomatoes	Mouse	M	Subcutaneous	>3000	Milani et al. (1970)
Lycopene from tomatoes	Mouse	M	Intraperitoneal	>3000	Milani et al. (1970)

F, female; M, male.

[a] A 6% lycopene solution in tomato extract, derived from food-processing tomatoes.

2.2.2 Short-term studies of toxicity

(a) Rats

In a new study submitted to the Committee at the current meeting, crystallized lycopene (90% purity; formulated as a 10% powder with 45–55% gum arabic, 25–45% lactose, 2–3% D,L-α-tocopherol and 1–2.5% sodium ascorbate) from *Escherichia coli* was suspended in water and administered daily to groups of Sprague-Dawley (Crl:CD (SD) IGS BR strain) rats (10 of each sex) by gavage at a concentration of 0, 200, 500 or 2000 mg/kg bw per day for 28 days. These administered doses correspond to approximate doses of 0, 20, 50 and 200 mg lycopene/kg bw per day. The study was reported to comply with OECD Test Guideline 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents; 1995). The rats were observed twice per day for mortality and clinical signs. Body weight measurements and food consumption were performed weekly. All rats were given an ophthalmoscopic examination at the beginning and end of the study. Haematology and clinical chemistry evaluations were performed on blood collected at the conclusion of the study. Water consumption was measured 16 h before sacrifice. Urine was collected over the same time period and subjected to a urinalysis. Animals found dead or killed at study termination were examined for gross pathological effects; selected organs were weighed, and histopathology was performed on selected tissues. No deaths, clinical signs or ophthalmoscopic abnormalities were observed. Although food consumption was reduced in females at 200 mg lycopene/kg bw per day during week 4, there was no overall reduction in food consumption or body weight among all groups at the conclusion of the study. Similarly, there were no consistent changes in measured haematological or clinical chemistry parameters that were attributable to treatment. No gross lesions or statistically significant changes in relative or absolute organ weights were observed at necropsy. Microscopic examination of tissues revealed only slight tubular mineralization and basophilia in the kidneys of controls and high-dose rats. Based on the absence of any observed adverse effects, the no-observed-adverse-effect level (NOAEL) was the highest tested dose of 200 mg lycopene/kg bw per day (Jian et al., 2008).

The possible toxicity of lycopene biomass (biomass of *B. trispora*; batch no. LC-411; 4.4% lycopene), extracted from the fermentation manufacturing process of lycopene, was examined in a short-term study in male and female Wistar rats. Based on the results of a 21-day range-finding study, groups of 20 rats of each sex were given diets containing the dried lycopene biomass at 0%, 0.1%, 0.3% or 1.0% by weight for 28 days, corresponding to mean daily intakes of 0, 90, 272 and 906 mg/kg bw in males and 0, 87, 260 and 868 mg/kg bw in females. The study followed OECD Test Guideline 407 (1995) and was certified for compliance with GLP and QA. Observations included clinical signs, neurobehavioural observations (including motor activity assessment), growth, food consumption, food conversion efficiency, haematology, clinical chemistry, organ weights, gross necropsy and histopathology. Haematological measurements revealed statistically significant decreases in mean corpuscular volume and prothrombin time in male rats of the group at 1.0%. The decreases were, however, very small (3% and 6%, respectively),

and other erythrocyte or coagulation variables were not significantly affected. Therefore, these findings were considered to be of no toxicological relevance. The only finding upon histopathology was a statistically significantly decreased incidence of "increased hyaline droplet nephropathy" in males of the group at 1.0% (0/20 versus 5/20 in the control group), which is of no toxicological relevance. All other observations did not reveal treatment-related changes (Jonker, 2000).

F344 rats received diets containing lycopene at a concentration of 0%, 0.005%, 0.012%, 0.024%, 0.05% or 0.124% (equivalent to 0, 2.5, 6, 12, 25 or 60 mg/kg bw per day, respectively) for 10 weeks in a study investigating the uptake and tissue disposition of lycopene. There were 10 and 20 animals of each sex in the treated and control groups, respectively, and lycopene was administered as a tomato extract (Betatene) containing 5.7% carotenoids (3.7% lycopene and 2% others) in medium-chain triglyceride. No adverse effects of lycopene treatment on weight gain, behaviour or coat appearance were noted, with the exception of a brownish discoloration of the tail in a few experimental animals (Zhao et al., 1998).

Groups of 20 male and 20 female Wistar rats were given diets containing lycopene, as a suspension in sunflower seed oil (20% by weight), at a concentration of 0%, 0.25%, 0.50% or 1.0% for 90 days. The lycopene examined in this study was derived from *B. trispora*. The sunflower seed oil was used to balance the level of added fat in the experimental diets (i.e. about 4–5%). The corresponding mean intake of lycopene was calculated to be 0, 145, 291 and 586 mg/kg bw per day for males and 0, 156, 312 and 616 mg/kg bw per day for females. The study followed OECD Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents; 1998) and was stated to be certified for compliance with GLP. Plasma concentrations of lycopene were not determined in this study. Clinical signs related to treatment with lycopene were limited to pink discoloration of the fur of all animals at the highest dose and many at the intermediate dose, resulting from direct contact with the red-staining lycopene present in the dietary admixture. Neurobehavioural testing, including functional observational battery and motor activity assessment, and ophthalmological examinations revealed no treatment-related effects. There were no statistically significant or consistent differences in body weights, food or water consumption, organ weights or parameters of haematology, clinical chemistry or urine analysis between the treated and control groups. The only treatment-related finding upon gross necropsy was a reddish discoloration of the contents of the gastrointestinal tract of all treated rats. This discoloration did not extend to any tissue. Histopathological examination did not reveal lycopene-related lesions. In the absence of toxicologically relevant findings, the NOAEL was a dietary concentration of 1.0% lycopene, the highest dose tested, equal to 586 and 616 mg/kg bw per day for males and females, respectively (Jonker et al., 2003).

The short-term toxicity of a natural lycopene extract derived from processing tomatoes was investigated in a 13-week study of toxicity in CD rats. Groups of 20 male and 20 female rats were given the natural tomato extract (Lyc-O-Mato 6%, containing 6% lycopene) at a dose of 0, 45, 450 or 4500 mg/kg bw per day diluted in peroxide-free corn oil and administered by gavage. These doses correspond to intakes of lycopene of 0, 2.7, 27 and 270 mg/kg bw per day, respectively. Body weight, food consumption and clinical signs were observed throughout the study.

Analyses of haematology, clinical chemistry, ophthalmoscopy and urine analysis were conducted at various time points throughout the study. At the end of the study, plasma concentration determination, organ weight measurements, and macroscopic and microscopic evaluation of a number of tissues were conducted. The study was stated to be certified for compliance with GLP. Absorption was demonstrated by the presence of lycopene in plasma, with similar levels for the groups receiving the intermediate or highest dose and a lower level for the group at the lowest dose. Treatment-related signs were restricted to staining of the faeces (orange-coloured at the lowest dose [males only] and intermediate dose, red-coloured at the highest dose). Body weight gain, food intake and food conversion efficiency of treated animals were similar to those of the controls. There were neither ocular lesions nor significant treatment-related differences between groups in urine analysis parameters, organ weights, or macroscopic and microscopic findings. Plasma alkaline phosphatase activities of treated animals were lower (not statistically significantly) than those of the controls, which was thought to have been the consequence of the smaller volume of corn oil used in the treatment groups. Slightly higher blood urea concentration and leukocyte count and lower bilirubin concentration in females at the highest dose and slightly increased platelet values in all treated females (no dose–response relationship) were not considered to be toxicologically relevant. The highest dose tested, 4500 mg/kg bw per day, equivalent to 270 mg lycopene/kg bw per day, was reported to be the NOAEL for natural tomato extract (Matulka et al., 2004).

Groups of six male and six female Wistar rats were given diets mixed with Lycopene 10% CWS (batch no. 20/93-2; 9.9% synthetic lycopene, <0.001% apo-12′-lycopenal) at concentrations intended to provide lycopene at 1000 mg/kg bw per day for 4 weeks. A control group of six males and six females received a diet mixed with the beadlet formulation without lycopene (batch no. 19/93-3; placebo group), and another control group of six males and six females received the pure diet for rodents (standard control group). To compare the toxicity of this Lycopene 10% CWS formulation containing <0.001% of the impurity apo-12′-lycopenal (i.e. <0.01% relative to lycopene) with that of a Lycopene 10% CWS beadlet formulation containing 0.2% of this impurity (i.e. 2% relative to lycopene), an additional group of six males and six females received a diet providing an intended dose of 1000 mg lycopene + 20 mg apo-12′-lycopenal/kg bw per day for 4 weeks. The latter formulation was obtained by mixing a Lycopene 10% CWS formulation (batch no. 7/95-1; 11.5% synthetic lycopene, 0.025% apo-12′-lycopenal) with a lycopene-C25-aldehyde 2% CWS formulation (batch no. RS2/95-69; 0.305% synthetic lycopene, 2.16% apo-12′-lycopenal). Owing to a mistake in feed preparation, this group received only 0.3% instead of the intended 2% apo-12′-lycopenal, so two additional groups of six males and six females were introduced, receiving either a diet providing 1000 mg lycopene + 20 mg apo-12′-lycopenal/kg bw per day or a placebo diet. Observations included clinical signs, body weight, food consumption, ophthalmoscopy, haematology, clinical chemistry, urine analysis, organ weights, macroscopy and microscopy, and plasma concentrations of lycopene, apo-12′-lycopenal, vitamin A and vitamin E. The study was certified to comply with GLP and QA. The only treatment-related changes observed were a red discoloration of the faeces and a brown-orange discoloration of the liver in most

animals receiving either lycopene only or lycopene supplemented with apo-12′-lycopenal. Histologically, the discoloration of the liver correlated with deposits of brown-yellow fine granulated pigment in hepatocytes, with no other associated histopathological alterations. The quantity of pigment was significantly higher in females than in males, the average grading being moderate in females and slight in males. No consistent and toxicologically relevant changes were observed in haematology, clinical chemistry or urine analysis parameters. Except for statistically significantly increased relative liver weight (15%) and relative kidney weight (12%) in males of the group receiving 1000 mg lycopene + 20 mg apo-12′-lycopenal/kg bw, organ weights were not affected. In the absence of any histopathological changes, these weight changes were not considered to be toxicologically relevant. The analysis of plasma samples collected on the day of necropsy from non-fasted rats showed group mean concentrations of lycopene of 120–250 µg/l in males and 140–190 µg/l in females in the groups treated with lycopene, with concentrations being significantly higher with increasing supplementation with apo-12′-lycopenal. This effect was more pronounced in males than in females. Apo-12′-lycopenal was detectable only in plasma of rats receiving 1000 mg lycopene + 20 mg apo-12′-lycopenal/kg bw, with group mean concentrations of 10 and 8 µg/l in males and females, respectively (or approximately 4% of the lycopene content). Vitamin A concentrations in plasma were not consistently affected, whereas vitamin E concentrations were consistently higher in all groups receiving feed together with formulation (approximately 2.3-fold in males and 3-fold in females). This effect was most probably due to the high concentration of vitamin E in the basic formulation. It was concluded that the lycopene beadlet formulation was well tolerated and that in the absence of histopathological liver damage, the treatment-related pigment deposition was not considered to be toxicologically relevant. The NOAEL was 1000 mg lycopene/kg bw per day, the only dose tested. The presence of apo-12′-lycopenal impurity at a concentration of 2% in the formulation (relative to lycopene) did not result in additional findings (Niederhauser et al., 1996; McClain & Bausch, 2003).

In a 14-week study of toxicity, Wistar rats were treated with Lycopene 10% CWS (batch no. 20/93-2; 9.9% synthetic lycopene) as a dietary admixture at concentrations intended to provide lycopene at 50, 150 or 500 mg/kg bw per day. A control group received a diet mixed with the beadlet formulation without lycopene (batch no. 19/93-3; placebo group), and another control group received the pure diet for rodents (standard control group). There were 26 males and 26 females per group, with 6 males and 6 females assigned to recovery groups for a 5-week treatment-free period. The study was certified for compliance with GLP and QA. Observations included clinical symptoms and mortality (daily), body weight development and food consumption (weekly), ophthalmoscopy (before dosing on all animals and in week 9 on animals in the control groups and the group receiving the highest dose), haematology and clinical chemistry (in blood taken from 10 animals per group before dosing and in weeks 7 and 14) and urine analysis (in urine sampled from 10 animals per group in weeks 6 and 13). In addition, blood was taken from all animals in the recovery groups just before the start and at the end of the recovery period for clinical chemistry determinations and from six males and six females of each group in weeks 2, 8, 12 and 17 for plasma concentration

determinations. At necropsy, a macroscopic examination was performed on all animals, and absolute and relative weights of adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and thyroid were determined. Microscopy was carried out on a number of organs and tissues from 20 males and 20 females in the control groups and in the group receiving the highest dose, on the liver from 20 males and 20 females in the groups receiving the lowest and intermediate doses, and on all gross lesions. There were no treatment-related deaths or adverse effects on general health and behaviour, eyes or urine analysis parameters. The only sign associated with the intake of lycopene was a reddish discoloration of the faeces of all animals from the group receiving the highest dose and to a lesser extent from the groups at the intermediate and lowest doses. Food consumption was comparable in all groups. Body weight gain was slightly increased (7–10%) in females treated with lycopene when compared with standard controls, but not when compared with females treated with placebo. In the recovery period, females at the intermediate and highest doses also had an increased body weight gain, compared with the standard controls (6–18% difference) and the females treated with placebo (12–25% difference). Statistically significant increases in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were observed in male, but not female, rats at all doses after 93 days of treatment (AST, 2-fold at the lowest dose and 3-fold at the intermediate and highest doses; ALT, no change at the lowest dose and 2-fold at the intermediate and highest doses). A trend for elevated AST and ALT activities was still seen in male rats after 5 weeks of recovery, although this was not statistically significant. Also, statistically significant changes were observed in some other biochemical parameters as well as in some haematological parameters after 93 days of treatment. However, as these changes were small, not dose related or found in one sex only, they were considered to be not related to treatment. In all groups, the plasma concentration of lycopene was continuously high, with higher mean concentrations in females (133–344 µg/l) than in males (102–197 µg/l). In male rats, plasma concentrations were comparable between the three dose groups, whereas in female rats, plasma concentrations increased non-proportionally with dose. After 2 weeks of recovery, complete elimination of lycopene in plasma was shown in all animals. Statistically significant changes in organ weights were observed only in animals receiving the highest dose, where females showed a decrease of 15% in relative thyroid weight and males a 2% increase in relative brain weight. These changes were not associated with morphological changes and did not persist beyond the recovery period. Necropsy revealed an orange-red discoloration of the liver (in males at 500 mg/kg bw per day and in females at 50, 150 and 500 mg/kg bw per day) and the adipose tissue (in males and females at 150 and 500 mg/kg bw per day). There were no microscopic findings that were considered to be related to treatment, except for the deposition of orange-brown, birefringent pigment in the hepatocytes of animals of all lycopene-treated groups, with females more severely affected than males (average grading, severe in females and slight to moderate in males). There was, however, no histopathological evidence of any hepatocellular damage. The pigment deposits were still present after 5 weeks of recovery. In the absence of histopathological liver damage, the relatively small increase in AST and ALT activities and the pigment deposition were not considered to be toxicologically relevant. The NOAEL was

500 mg lycopene/kg bw per day, the highest dose tested (Buser & Urwyler, 1996; McClain & Bausch, 2003).

In a 3-month study of toxicity, groups of 10 male and 10 female Wistar rats were dosed daily by gavage with one of the following: water, Lycopene 10 CWD (500, 1500 or 3000 mg/kg bw per day), Lycopene 10 CWD formulation matrix (3000 mg/kg bw per day), LycoVit 10% (3000 mg/kg bw per day) or LycoVit 10% formulation matrix (3000 mg/kg bw per day). These doses correspond to approximate doses of lycopene of 50, 150 and 300 mg/kg bw per day from Lycopene 10 CWD (batch no. OD-12-04 6.Spr.A, containing 9.81% synthetic lycopene) and 300 mg/kg bw per day from LycoVit 10% (batch no. 20014372, containing 10.8% synthetic lycopene). Additional satellite groups of five males and five females received water or 3000 mg/kg bw per day of either Lycopene 10 CWD or LycoVit 10% for an interim evaluation at 4 weeks. The study followed OECD Test Guideline 408 (1998) and was certified to comply with GLP and QA. Plasma concentrations of lycopene were not determined in this study. There were no significant differences in body weight, food consumption, organ weights or haematological evaluations between treatment groups, and there were no treatment-related deaths or effects seen in ophthalmoscopic evaluations. Similarly, a battery of behavioural and reflex tests, motor activity assessments, clinical chemistry evaluations and urine analysis did not reveal any biologically relevant findings. Treatment-related clinical findings were limited to a red discoloration of the faeces of animals in all treatment groups. Consistent with this finding was a red discoloration in the gastrointestinal tract (jejunum, caecum and, at the highest dose, stomach) of these animals, which was not associated with changes in gross pathology or histopathology. No other remarkable or substance-related abnormalities were observed in any of the other tissues examined, nor were there any significant histopathological findings. Given the absence of relevant toxicological findings with both lycopene formulations, the NOAEL was approximately 300 mg lycopene/kg bw per day, the highest dose tested (BASF, 2001b; Mellert et al., 2002).

A 100-day feeding study was conducted in which 10 male and 10 female Wistar rats were given synthetic lycopene (purity, 97–98.5%) at a dose of 1000 mg/kg bw per day, mixed with a small amount of food. A control group of 20 animals was also included. No clinical signs were observed, and treatment did not affect weight gain or haematological parameters (haemoglobin, erythrocyte and leukocyte counts, and differential blood counts). Liver, kidneys, adrenals, spleen, pancreas, testes, ovaries, skin, bone and bone marrow were histologically normal. Signs of tissue storage of lycopene were not detected by microscopic examination (Zbinden & Studer, 1958).

(b) Dogs

In a study evaluating the pharmacokinetics and tissue distribution of lycopene, six male Beagle dogs were given a 5% lycopene-containing granular reddish powder in gelatine capsules providing lycopene at a dose of 30 mg/kg bw per day for 28 days. Food consumption and body weight were not affected by treatment, and, other than red material in faeces, no clinical signs were observed (Korytko et al., 2003).

One female mongrel dog was treated orally with gelatine capsules containing synthetic lycopene (purity, 97–98.5%) at a daily dose of 100 mg/kg bw for 192 days. In total, the animal received 272 g of lycopene without displaying signs of intolerance. Body weight increased from 13 to 15 kg, and haematology, clinical chemistry and urine analysis parameters investigated were normal. Macroscopy did not reveal treatment-related changes. Chemical analysis showed the presence of lycopene in the liver and, to a much lesser extent, kidneys. The pigment was also detectable histologically in liver cells. Otherwise, the liver was histologically normal, as were other tissues and organs (Zbinden & Studer, 1958).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rats

Zbinden & Studer (1958) investigated the toxicity of lycopene as part of a two-generation study of reproductive toxicity. Groups of 15 male and 15 female Wistar rats were found to develop normally when given a diet containing 0% or 0.1% synthetic lycopene (purity, 97–98.5%) for 200 days. There were no mortalities or great differences in food consumption and body weight gain, and there were no remarkable haematological findings. Histopathological examinations in tissues and organs of two rats of each sex per group did not reveal abnormalities, with the exception of pigment deposition (marked in females and slight in males) in the liver of animals treated with lycopene. Continuation of treatment of the remaining 13 rats of each sex per group for a further 200 days was reported not to result in any sign of toxicity or spontaneous tumours (Zbinden & Studer, 1958).

In a 52-week study of toxicity, groups of 20 male and 20 female Wistar rats were treated with Lycopene 10% WS beadlets (batch no. UT02070001; 13.2% synthetic lycopene) mixed in the diet at a target dose of 10, 50 or 250 mg lycopene/kg bw per day. A control group received a diet mixed with placebo beadlets (batch no. UT02111005; placebo group), and another control group received the pure diet for rodents (standard control group). Five rats of each sex in the standard control group and the group at the highest dose were assigned to recovery groups for a 13-week treatment-free period. The study followed OECD Test Guideline 452 (Chronic Toxicity Studies; 1981) and was certified to comply with GLP and QA. In addition to the standard observations, a functional observational battery and motor activity assessment were included, plasma and liver samples were analysed for total lycopene content (i.e. sum of all determined isomers) and livers from all animals in the study were examined microscopically. Analysis of plasma and liver samples demonstrated systemic absorption of lycopene in all groups receiving Lycopene 10% WS beadlets (see section 2.1.3). Discoloured faeces or red staining was noted in both sexes in all groups given lycopene. There were no treatment-related deaths, ophthalmic changes or neurological changes. Body weight gain, food consumption and food conversion efficiency were unaffected by treatment after 52 weeks. Haematological parameters were not consistently affected by treatment, nor were clinical chemistry parameters other than AST and ALT activities. At week 13, slight increases in the group mean activities of AST and ALT were recorded at the highest dose only. At weeks 26 and 52, group mean activities of these enzymes were

increased in a dose-dependent manner in males (up to 1.7-fold and 2.6-fold, respectively) and females (up to 1.4-fold and 2-fold, respectively) at the lowest, intermediate and highest doses, although not always statistically significantly and generally without real progression between week 26 and week 52. At the end of the 3-month treatment-free period, AST and ALT activities had declined, but still tended to be higher in the group at the highest dose than in the standard control group. In males, the increased ALT levels were still between 1.5-fold and 1.9-fold higher than for controls throughout the treatment-free period. At week 51, there was an increase in urinary volume and decrease in urinary specific gravity in both sexes at 250 mg/kg bw per day. These findings were not accompanied by related changes in the kidney or increased water consumption. The weights of the adrenals, kidneys, liver, brain, testes and epididymides, and ovaries were unaffected by treatment. Macroscopy revealed abnormal contents (often a thick, red substance) in the stomach and caecum and yellow connective tissue in the abdominal cavity of animals receiving the intermediate and highest doses at terminal kill, but not at the end of the treatment-free period. These findings did not correlate with any microscopic observation and were probably associated with the colour of the test article. The macroscopic finding of mottled liver in both control and treated animals, especially males, correlated with agonal congestion/haemorrhage seen microscopically. Treatment-related findings upon microscopy were confined to the liver, particularly of females. In females at terminal kill, there were increases in the incidences and severity of hepatocyte pigment (in treated and placebo-fed groups, without an obvious dose–response relationship) and pigmented histiocytes (in a dose-related manner in all groups treated with lycopene) compared with those of standard controls. In females at the intermediate and highest doses, there were also a greater incidence and a greater severity of basophilic foci. After the treatment-free period, these findings were still observed, although the incidences of hepatocyte pigment and basophilic foci were slightly lower and similar, respectively, in females at the highest dose compared with the standard controls. In males, hepatocyte pigment was rarely seen, and the incidences of pigmented histiocytes and basophilic foci were only marginally increased in groups treated with lycopene. The pigment in hepatocytes and histiocytes was seen as fine golden-brown granular deposits, which stained variably positive for both haemosiderin and lipofuscins. It was concluded that treatment with lycopene for 52 weeks resulted in liver pigmentation with associated histopathological alterations of basophilic foci (in females at the intermediate and highest doses), whereas the latter were not observed in the short-term studies of toxicity. The significance of these treatment-related alterations for humans is unclear, given that there was no apparent sign of liver dysfunction and that, in contrast to humans, basophilic foci are commonly found at high incidences in the ageing rat. The latter is, for example, reported for F344 rats by Eustis et al. (1990) and was also observed in the present study in female control animals (standard and placebo). Basophilic foci were also observed in control animals (especially females) of other (26-week and 104-week) studies by the same laboratory using the same strain of rats (see also the 104-week study below). However, the Committee at its sixty-seventh meeting also noted that the alterations were observed in a single, non-standardized liver section investigated,

which would not necessarily be representative of other sections of the liver (Smith et al., 2005).

In a study of carcinogenicity, groups of 50 male and 50 female Wistar rats were treated with Lycopene 10% WS beadlets (batch no. UT02070001; 13.2% synthetic lycopene) mixed in the diet at target doses of 2, 10 or 50 mg lycopene/kg bw per day for 104 weeks. Two control groups of 50 males and 50 females received either a diet mixed with placebo beadlets (batch no. UT02111005; placebo group) or the pure diet (standard control group). The study followed OECD Test Guideline 451 (Carcinogenicity Studies; 1981) and was certified for compliance with GLP and QA. In addition to the standard observations, plasma and liver samples were analysed for total lycopene content (i.e. sum of all determined isomers), and liver, mesenteric and mandibular lymph nodes from all animals in the study, as well as kidney from all female rats, were examined microscopically.

Analysis of plasma and liver samples demonstrated systemic absorption of lycopene in all groups receiving Lycopene 10% WS beadlets (see section 2.1.3). Survival was acceptable, being 86%, 80%, 74%, 66% and 82% for males and 78%, 80%, 78%, 84% and 74% for females from the standard control group to the group at the highest dose, respectively. There was no influence of treatment on incidences and causes of morbidity and mortality or on incidences of clinical signs commonly seen in laboratory rats. Red discoloured faeces were noted in both sexes at 10 and 50 mg/kg bw per day, from week 67 and from the onset of treatment, respectively. Food consumption, overall body weight gain and food conversion efficiency of males and females given lycopene in the diet were similar to those of the standard control and placebo groups. Erythrocyte and leukocyte counts were not consistently affected by treatment.

Macroscopically, several findings in the gastrointestinal tract (e.g. abnormal or dark contents in the stomach and caecum, red discoloration of jejunum) and yellow connective tissue, especially in males and females at the highest dose, were probably associated with the colour of the test article. Likewise, treatment with lycopene resulted in dark red discoloration in the kidney (especially in females at the highest dose) and lymph nodes and variable discolorations in the liver, corresponding with non-neoplastic findings observed microscopically. In the kidney of females at the highest dose, the incidence and severity of tubular pigment were increased compared with values for controls and other treatment groups. In males, renal tubular pigmentation was significantly less than in females, and there was no difference between control and treated groups. In the mesenteric lymph node, all animals, including controls, displayed pigment or pigmented histiocytes, but the severity of pigmentation was increased in the placebo group (females only) and in lycopene-treated animals. In the mandibular lymph node, the incidence of pigment or pigmented histiocytes was increased in the placebo and treated groups, with the severity also being increased in females. The increases in lymph node pigmentation were not consistently dose dependent. Pigmentation, seen as fine granular or globular golden-brown deposits, was also observed in the liver, especially in females. In females treated with placebo or lycopene, there were increases in the incidences and severity of hepatocyte pigment (without an obvious dose–response relationship) and pigmented histiocytes (severity increased only in the group at the

highest dose) above that in standard controls. In males, hepatocyte pigment was rarely seen, but the incidence of pigmented histiocytes was also increased in the groups receiving placebo or lycopene above that in standard controls. Other non-neoplastic findings in the liver included greater incidence and severity of multinucleate cells (dose related, in all treated males, albeit marginally at the lowest dose) and of histologically altered cell foci. Altered cell foci of the eosinophilic type were of greater severity, but their incidence was without an obvious dose–response relationship in groups of males given placebo or lycopene. The incidence and severity of basophilic, normochromic and clear cell foci were greater in females at the intermediate dose and/or highest dose (although for basophilic foci, the incidences were only slightly above the high background incidence). In placebo controls and in treated animals at all doses, the incidence of vacuolated foci was greater than that in controls receiving pure diet only, but was without an obvious dose–response relationship. For eosinophilic and basophilic foci, it was stated by the authors that there was no evidence of an increase in cellular pleomorphism, distorted architecture or increased mitotic activity, but no data were provided to support this statement.

No liver carcinomas were observed in this study, and there was no treatment-related increase in liver adenomas. In fact, the types and incidences of tumours observed were generally similar in control and treated groups. The highest incidences were found for pituitary adenomas, mesenteric lymph node haemangiomas, uterus adenocarcinomas and stromal polyps, and mammary gland fibroadenomas (the incidence of which seemed to be slightly increased in females treated with lycopene). It was concluded that there were no indications to suggest an association between carcinogenicity and administration of lycopene.

As to the non-neoplastic findings, it was concluded that treatment with lycopene for 104 weeks, as with treatment for 52 weeks, resulted in liver pigmentation with associated histopathological alterations of hepatocellular foci, in particular eosinophilic, normochromic and basophilic foci, mainly at the intermediate and highest doses. Again, the significance of these treatment-related alterations for humans is unclear, given that there was no apparent sign of liver dysfunction, they were without a consistent dose–response relationship and placebo controls were in some cases also affected. Moreover, in contrast to humans, hepatocellular foci are commonly found in the ageing rat at high incidences; although experimental models suggest that some foci may be precursors of hepatocellular neoplasia, it is also known that only a very small proportion of foci progress to neoplasia even after continued administration. Indeed, treatment with lycopene did not result in an increase in liver tumours. However, the Committee at its sixty-seventh meeting also noted that the alterations were observed in a single, non-standardized liver section investigated, which would not necessarily be representative of other sections of the liver (Edwards et al., 2006).

Ten female Wistar rats from a 64-week exploratory study investigating the time course of lycopene concentrations in liver, spleen and plasma under steady-state conditions (see study by Glatzle et al., 1998a in section 2.1.6) were transferred to a study investigating the long-term effects of continuous feeding with lycopene over a total of 2 years. The rats were pair-fed a diet enriched with lycopene

(Lycopene 10% CWS beadlets; batch no. 511821; 113 mg synthetic lycopene/g) at a concentration of 113 mg/kg. The amount of feed served was adapted weekly to the body weights measured so as to provide lycopene at a dose of approximately 10 mg/kg bw per day throughout the study. Animals were observed daily for clinical signs and weekly for body weight development. At 18 and 21 months, blood was collected from non-fasted rats and analysed for AST, ALT, alkaline phosphatase, triglycerides, phospholipids and total cholesterol. At necropsy, the liver and all tissues with macroscopic changes were removed and examined microscopically. Except for the pathological data, the results for the observations performed were described with little detail or not at all, as was the case for body weight development. It was stated that no clinical signs were recorded throughout the study and that five rats were sacrificed prematurely owing to deterioration of their general condition. In the absence of a concurrent control group, clinical chemistry values for the non-fasted rats were compared with historical control values for fasted female Wistar rats. Clinical chemistry parameters were elevated, which was most likely caused by the non-fasting state of the animals. Upon macroscopy, staining of the mammary gland and abdominal fat tissue (yellow) and liver (orange) was noted in some animals, as well as enlarged pituitary glands (in 4/5 prematurely sacrificed rats and in 2/5 rats surviving to the end). Histopathology showed brown-orange gritty deposits in hepatocytes of all rats, with the amount of pigment deposited higher in prematurely sacrificed animals than in animals at terminal kill. No microscopic changes associated with the liver pigmentation were observed, nor was there discoloration of other tissues. Pituitary adenomas were found in all animals with enlarged pituitary glands and were the cause of the moribund condition of prematurely sacrificed animals (Urwyler & Riss, 1999).

2.2.4 Genotoxicity

The results of genotoxicity studies with lycopene are summarized in Table 3. The studies followed OECD Test Guidelines 471 (Bacterial Reverse Mutation Test; 1983 + 1997 update), 473 (In Vitro Mammalian Chromosome Aberration Test; 1983 + 1997 update), 474 (Mammalian Erythrocyte Micronucleus Test; 1987 + 1997 update), 476 (In Vitro Mammalian Cell Gene Mutation Test; 1984 + 1997 update) or 486 (Unscheduled DNA Synthesis Test with Mammalian Liver Cells In Vivo; draft 1997) and were certified to comply with GLP and QA. Lycopene from all sources gave negative results in all studies. Unformulated synthetic lycopene is sensitive to air and light, thereby forming mutagenic degradation products. When formulated and, as such, protected against oxidative processes, synthetic lycopene gave predominantly negative results in the genotoxicity studies.

Table 3. Studies of genotoxicity with lycopene from all sources

End-point	Test system	Test substance	Concentration	Result	References
Synthetic lycopene					
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, *Escherichia coli* WP2 uvrA	Lycopene 10 CWD[a] (in water)	20–5000 pg/plate, ±S9	Negative[b]	BASF (2000)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA1535, TA1537, *E. coli* WP2 uvrA	Lycopene[c] (in DMSO)	1.6–1000 pg/plate, ±S9	Negative[d]	Gocke (1996); McClain & Bausch (2003)
Reverse mutation	*S. typhimurium* TA97, TA100	Lycopene, degraded[e] (in DMSO)	250–1000 pg/plate, ±S9	Positive[f]	Gocke (1996); McClain & Bausch (2003)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA1535, TA1537, *E. coli* WP2 uvrA	Lycopene 10% CWS[g] (in water)	100–10 000 pg/plate, ±S9	Negative[h]	Gocke (1996); McClain & Bausch (2003)
Reverse mutation	*S. typhimurium* TA97, TA100	Lycopene 10% CWS, aged[i] (in water)	100–10 000 pg/plate, ±S9	Negative[h]	Gocke (1996); McClain & Bausch (2003)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA1535	Lycopene 10% WS[j] (in water)	100–10 000 pg/plate, ±S9	Negative[k]	Gocke (1999); McClain & Bausch (2003)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA1535	Lycopene 10% FS[l] (in acetone)	100–10 000 pg/plate, ±S9	Negative[m]	Gocke (2000); McClain & Bausch (2003)
Gene mutation	Mouse lymphoma L1578Y $Tk^{+/-}$ cells	Lycopene 10% CWS (in treatment medium)	6.25–400 pg/ml, −S9; 12.5–800 pg/ml, +S9	Negative[n]	Muster (1996a); McClain & Bausch (2003)

Table 3 (contd)

End-point	Test system	Test substance	Concentration	Result	References
Chromosomal aberration	Chinese hamster V79 cells	Lycopene 10 CWD (in water)	First experiment: 56.25, 312.5 or 625 pg/ml, −S9; 625, 1250 or 2500 pg/ml, +S9	Negative[o]	BASF (2001c)
			Second experiment: 300, 600 or 1200 pg/ml, −S9[p]; 1200 pg/ml, −S9[q]; 600, 1200 or 2400 pg/ml, +S9[o]	Weak positive[r]	
			Third experiment: 1000, 1200 or 1400 pg/ml, −S9	Positive[s]	
Chromosomal aberration	Human lymphocytes	Lycopene 10% CWS (in culture medium)	250–1000 pg/ml, −S9; 250–2500 pg/ml, +S9	Negative[t]	Miller (1996a); McClain & Bausch (2003)
In vivo					
Micronucleus formation	Mouse bone marrow	Lycopene 10 CWD (in water)	500, 1000 or 2000 mg/kg bw, 2× intraperitoneal dose with an interval of 24 h	Negative[u]	BASF (2001d)
Micronucleus formation	Mouse bone marrow	Lycopene 10% CWS (in water)	1070, 2140 or 4280 mg/kg bw, 2× oral dose with an interval of 24 h	Negative[v]	Miller (1996b); McClain & Bausch (2003)
Micronucleus formation	Mouse bone marrow	Lycopene 10% CWS, aged[w] (in water)	2140 or 4280 mg/kg bw, 2× oral dose with an interval of 24 h	Negative[v]	Miller (1996b); McClain & Bausch (2003)

Table 3 (contd)

End-point	Test system	Test substance	Concentration	Result	References
Micronucleus formation	Mouse peripheral blood	Lycopene 10% CWS in soft drink	50 mg lycopene/l, ad libitum for 14 days[x]	Negative	Muster (1999); McClain & Bausch (2003)
Micronucleus formation	Mouse peripheral blood	Lycopene 10% CWS in soft drink, aged[y]	25 or 50 mg lycopene/l, ad libitum for 14 days[z]	Negative	Muster (1999); McClain & Bausch (2003)
Unscheduled DNA synthesis	Rat hepatocytes	Lycopene 10 CWD (in water)	1000 or 2000 mg/kg bw, single oral dose	Negative[aa]	BASF (2001e)
Unscheduled DNA synthesis	Rat hepatocytes	Lycopene 10% CWS (in water)	6000 mg/kg bw, single oral dose	Negative[ab]	Muster (1996b); McClain & Bausch (2003)
Unscheduled DNA synthesis	Rat hepatocytes	Lycopene 10% CWS, aged[w] (in water)	6000 mg/kg bw, single oral dose	Negative[ab]	Muster (1996b); McClain & Bausch (2003)
Lycopene from *Blakeslea trispora* and from other natural sources					
In vitro					
Reverse mutation	*S. typhimurium* TA98, TA100	Lycopene from tomato paste (in DMSO)	100 pg/plate, ±S9	Negative[ac]	He & Campbell (1990)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA	Lycopene from tomato paste (in DMSO)	0.05–5000 pg/plate, ±S9	Negative[ac]	Aizawa et al. (2000)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538, *E. coli* WP2 uvrA	Lyc-O-Mato 6%[ad] (natural tomato extract; in water)	312.5–5000 pg/plate, ±S9	Negative[ae]	Matulka et al. (2004)

Table 3 (contd)

End-point	Test system	Test substance	Concentration	Result	References
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA	Lycopene 20% CWD[af] (in water)	Up to 5000 pg/plate, ±S9	Negative[ag]	CTBR Bio-Research Inc. (2003a)
Chromosomal aberration	Human lymphocytes	Lycopene 20% CWD (in water)	1280, 2560 or 5000 pg/ml, ±S9	Negative[ah]	CTBR Bio-Research Inc. (2003b)
In vivo					
Micronucleus formation	Mouse peripheral blood	Lycopene in tomato juice (1:1 diluted with water)	54 mg lycopene/l, ad libitum for 14 days[ai]	Negative	McClain & Bausch (2003)
DNA damage (comet assay)	Human lymphocytes	Lycopene in tomato juice	40 mg lycopene/day, for 2 weeks	Negative[aj]	Pool-Zobel et al. (1997)
DNA damage (comet assay)	Human lymphocytes	Lycopene in tomato puree	16.5 mg lycopene/day, for 21 days	Negative[ak]	Riso et al. (1999)
Spontaneous mutation	LacZ mouse DNA from lung, colon and prostate	Lycopene-rich tomato extract[al]	7 or 14 g/kg diet, for 9 months	Negative	Guttenplan et al. (2001)

DMSO, dimethyl sulfoxide; S9, 9000 × *g* supernatant from rat liver.

[a] Batch no. OD-12–04 6.Spr.A, containing 9.81% lycopene, except for the assay for unscheduled DNA synthesis in vivo, where the same batch was reported to contain 9.3% lycopene.

[b] With and without metabolic activation from S9, using both the plate incorporation method and the preincubation method. No cytotoxicity or precipitation was observed.

[c] Batch no. DV-111255; purity, 99.74%.

Table 3 (contd)

[d] With and without metabolic activation from S9, using both the plate incorporation method and the preincubation method. No cytotoxicity was observed. Precipitation was observed at 200 and 1000 pg/plate. Previous experiments with a less pure batch of crystalline lycopene (purity, 96%) showed positive effects, especially with strains TA100 and TA97. This was due to a mutagenic impurity, as demonstrated by the negative results for pure crystalline lycopene.

[e] Batch no. DV-111255; purity, 99.74%, degraded by exposure to air or a combination of air, light and room temperature.

[f] Only without metabolic activation from S9, and more pronounced in TA100 than in TA97. P reincubation method was used, with precipitation observed at all concentrations, but no cytotoxicity. Additional experiments showed that the formation of mutagenic degradation products by oxidative processes can be reduced by adding antioxidants such as DL-α-tocopherol.

[g] Batch no. 7/95-1, containing 11.5%/10.9% lycopene (by ultraviolet [UV]/HPLC, respectively).

[h] With and without metabolic activation from S9, using the preincubation method. No cytotoxicity was observed. Red particles observed at 10 000 pg/plate did not allow evaluation of background growth.

[i] Batch no. 7/95-1, containing 11.5%/10.9% lycopene (by UV/HPLC, respectively), after stress storage (under air in dark bottle at 45 °C for 1, 2, 4 or 8 weeks).

[j] Batch no. RS2/99-105, containing 10.4% lycopene and 0.19% lycopyl C25-aldehyde (by HPLC).

[k] With and without metabolic activation from S9, using the preincubation method. Reddish precipitation was observed at 10 000 pg/plate, not allowing evaluation of background growth and necessitating manual counting. Cytotoxicity was observed only at 3160 pg/plate for TA102 without S9.

[l] Batch no. UE00002004, containing 10.5% lycopene (by UV).

[m] With and without metabolic activation from S9, using both the plate incorporation method and the preincubation method. Reddish precipitation was observed at 316 pg/plate and greater. Cytotoxicity was not observed.

[n] With and without metabolic activation from S9. Cytotoxicity was observed at 400 pg/ml (without S9) and 800 pg/ml (with S9).

[o] With and without metabolic activation from S9. The cell cultures were treated for 4 h without and with S9 and were harvested 14 h later. Effects on mitotic index were observed only without S9, at 625 pg/ml.

[p] The cells were exposed continuously for 18 h and were then harvested. Effects on mitotic index were observed at 600 and 1200 pg/ml.

[q] The cells were exposed continuously for 18 h (without S9) or 4 h (with S9) and were harvested at 28 h. No effects on mitotic index were observed.

[r] Only without metabolic activation from S9, at a harvest time of 28 h. A slight, not statistically significant increase in structural aberrations was observed.

[s] The cell cultures were treated for 18 h and were harvested 10 h later. A dose-dependent, statistically significant increase in structural (but not numerical) aberrations was observed when precipitation of the test substance occurred. Effects on mitotic index were observed at 1400 pg/ml.

Table 3 (contd)

[t] With and without metabolic activation from S9. In the first experiment, cells were treated continuously for 22 h without S9 and then harvested or were treated for 3 h with S9 and harvested 19 h later. In the second experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 19 h later. Cytotoxicity was obvious at the highest concentrations tested, as indicated by a reduction of the mitotic index and the number of cells that could be evaluated.

[u] No toxic signs or symptoms were observed, and there were no effects on the ratio of polychromatic to normochromatic erythrocytes.

[v] No toxic signs or symptoms were observed, but the ratio of polychromatic to normochromatic erythrocytes was slightly reduced.

[w] Batch no. 7/95-1, containing 11.5%/10.9% lycopene (by UV/HPLC, respectively), after stress storage (under air in dark bottle at 45 °C for 8 weeks).

[x] Equivalent to a dose of 3.5–11.5 mg lycopene/kg bw per day, estimated based on an average water intake of 3–10 ml/day for growing mice and an average body weight of 43.5 g. No toxic effects were observed, and the ratio of polychromatic to normochromatic erythrocytes was not affected.

[y] After storage at 45 °C for 4 weeks in the dark.

[z] Equivalent to a dose of 1.7–5.8 or 3.5–11.5 mg lycopene/kg bw per day, estimated based on an average water intake of 3–10 ml/day for growing mice and an average body weight of 43.5 g. No toxic effects were observed, and the ratio of polychromatic to normochromatic erythrocytes was not affected.

[aa] No toxic signs or symptoms were observed

[ab] No signs of toxicity or cytotoxicity were observed.

[ac] With and without metabolic activation from S9. No further details are available.

[ad] A 6% lycopene solution in tomato extracts, derived from food-processing tomatoes.

[ae] With and without metabolic activation from S9, using the plate incorporation method. No cytotoxicity was observed. Precipitation was seen at 5000 pg/plate.

[af] Batch no. 154C1, containing 21.2% lycopene in an octenyl succinic anhydride starch matrix with α-tocopherol.

[ag] With and without metabolic activation from S9, using both the plate incorporation method and the preincubation method. No cytotoxicity was observed. Red precipitate was present at all doses but did not interfere with scoring.

[ah] With and without metabolic activation from S9. The cells were exposed for 4 h without and with S9 and were harvested 17 h later; or for 21 h without S9 and harvested immediately thereafter. No effects on relative mitotic index were observed.

[ai] Equivalent to 3.7–12.4 mg lycopene/kg bw per day, estimated based on an average water intake of 3–10 ml/day for growing mice and an average body weight of 43.5 g. No toxic effects were observed.

[aj] Lycopene suppressed the level of DNA strand breaks, but not the level of oxidized pyrimidine bases.

[ak] Lycopene reduced lymphocyte DNA damage as induced by hydrogen peroxide.

[al] Betatene, a 5.7% carotenoid suspension (3.7% lycopene and 3% others) in medium-chain triglycerides.

2.2.5 Reproductive toxicity

(a) Multigeneration study

(i) Rats

In a two-generation study of reproductive toxicity, groups of 15 male and 15 female Wistar rats received a diet containing 0% or 0.1% synthetic lycopene (purity, 97–98.5%) for 14–16 weeks before mating and subsequently throughout pregnancy and beyond, for a total of 238 treatment days. After weaning, pups of the first generation were fed either the same diet containing lycopene (13 male and 18 female rats) or the control diet (38 animals) for 50–80 days. One of the dams treated with lycopene aborted; the number of offspring was slightly, but not significantly, lower than in the control group, in which two animals aborted. The duration of gestation was normal, and no malformations were seen. Pup weight at birth was slightly, but not significantly, higher in the treated group than in the control group. Continuation of treatment of the first generation for a further 200 days, while producing a second generation, was reported to have resulted in normal reproduction (Zbinden & Studer, 1958).

Sprague-Dawley rats were treated with diets mixed with Lycopene 10% WS beadlets (batch no. UT02070001; 13.2% synthetic lycopene) to provide lycopene at a target dose of 50, 150 or 500 mg/kg bw per day for two consecutive generations (F_0 and F_1). F_0 animals (30 of each sex per dose) received the test diet 10 weeks before pairing, throughout mating and gestation, and through to necropsy after weaning. F_1 animals (25 of each sex per dose) were mated after 10 weeks of exposure, and females were allowed to rear their young to weaning. Two control groups were included: one (standard) control group received the basic powdered diet, and the other (placebo) control group received a diet mixed with placebo beadlets (batch no. UT02111005). The study followed OECD Test Guideline 416 (Two-Generation Reproduction Toxicity Study; 2001). Additionally, blood and liver samples from selected F_0 and F_1 adults (five of each sex per group) and their weanlings (two of each sex per litter) were taken shortly before sacrifice for determination of lycopene content. The study was certified to comply with GLP and QA.

The achieved intakes during premating, gestation and lactation were consistent with the target doses. Data on exposure showed detectable concentrations of lycopene in the plasma of rats in all groups treated with Lycopene 10% WS beadlets, with higher values in adult females than in adult males in each generation. There was no dose dependency for adult males in either generation; for adult females, concentrations increased with dose, but proportionally to a lesser degree than the dose. Plasma concentrations in the weaned pups were greater than those in the adults for both generations, were consistent between the sexes and increased with dose, but proportionally to a lesser degree than the dose. All treated groups of adult males and females from each generation had detectable concentrations of lycopene in the liver. As noted for plasma, levels in females were much higher than in males, with no dose dependency for males and an increase with dose for females, but proportionally to a lesser degree than the dose. Liver

concentrations for the weaned pups were again consistent between the sexes for both generations. Values for the group at the lowest dose were comparable with those noted for the adult males and then increased slightly with dose, but proportionally to a lesser degree.

Observations in adults not related to reproduction included red-coloured faeces in all groups receiving lycopene throughout the study and orange-stained fur or skin for F_0 and F_1 animals at 500 mg/kg bw per day and for F_0 males at 150 mg/kg bw per day. In the F_0 generation, treatment with lycopene generally had no effect on mean body weight change or food consumption. In the F_1 generation, males and females at 500 mg/kg bw per day occasionally had marginally lower food consumption during premating, resulting in a marginally lower body weight during the first 7 or 2 weeks, respectively, but overall there was little or no effect on body weight or body weight gain. F_1 females at 500 and 150 mg/kg bw per day also had slightly reduced food consumption during days 7–20 of gestation and days 1–7 of lactation. Compared with standard controls, mean body weight gain was reduced in all groups given beadlets, including placebo, during the last week of gestation and the first week of lactation, but it recovered thereafter, resulting in only slightly lower body weights (<5%) at day 21 of lactation. In the groups receiving lycopene, macroscopic examination showed dark adipose tissue in some animals of the F_0 generation and yellow discoloration of fat and organs of the abdominal cavity and liver streaks in some animals of the F_1 generation. Treatment-related histological changes were, however, not observed.

Some occasional small changes (generally less than 10% change) in absolute and/or relative organ weights were observed (e.g. for coagulating glands/seminal vesicles and uterus), without histopathological correlates. With respect to reproductive findings, no treatment-related effects on mating performance or fertility were observed. Gonadal function, as assessed by determination of estrus cycle, detailed sperm analysis (motility, counts, morphology) and microscopic examination of ovaries, testes and epididymides, was also unaffected. The survival and growth of F_0 offspring were not affected by treatment, whereas the number of F_1 offspring dying during days 1–4 increased (13, 21 and 14 in groups treated with lycopene, respectively, versus 5 in control groups). Live litter sizes, however, were unaffected and comparable with those for controls throughout lactation. F_2 pup body weights were not affected by treatment, except for a slightly decreased weight for female pups at the highest dose at day 1. Although some changes in physical development of F_1 and F_2 pups (measured as mean day of attainment of pinna unfolding, incisor eruption and eye opening) reached statistical significance, they were of a minor nature only. During lactation, an increased number of F_2 pups at the highest dose showed clinical signs (thin bodies, lacrimation, hair loss, pale skin), but only one or two litters were affected. Aside from red-coloured faeces and fur, skin, fat or abdominal organs stained yellow-orange owing to the colour of lycopene, it can be concluded that in the parental generation, treatment with lycopene was associated only with marginal effects on body weight and food consumption (F_1 only), with no influence on mating performance or fertility. Hence, the NOAEL for parental and reproductive toxicity was 500 mg lycopene/kg bw per day, the highest dose tested. The NOAEL for effects on growth and development of the offspring was also

500 mg lycopene/kg bw per day, as the clinical findings in F_2 pups at the highest dose can be considered to be incidental (Edwards et al., 2005).

(b) Developmental toxicity

(i) Rats

In order to establish the influence of the impurity apo-12′-lycopenal on the potential developmental toxicity of lycopene, groups of up to 14 pregnant female Wistar rats were treated with Lycopene 10% CWS containing a low concentration of apo-12′-lycopenal (batch no. 20/93-2) or Lycopene 10% CWS with an approximate 10-fold excess of apo-12′-lycopenal (mixture of batch nos 7/95-1 and RS2/95-70). The formulations were given as a dietary admixture at concentrations calculated to provide lycopene at a dose of 1000 mg/kg bw per day or 1000 mg lycopene + 20 mg apo-12′-lycopenal/kg bw per day, respectively, from day 6 to day 18 of gestation, followed by caesarean section on day 21 of gestation. A control group receiving a placebo beadlet formulation (batch no. 19/93-3) was also included, following the same protocol. Observations included clinical signs and measurement of body weight and food consumption. At necropsy, uterus and ovaries were examined for reproductive parameters, and internal organs were examined macroscopically. Fetuses were examined for external, visceral and skeletal deviations. The study followed OECD Test Guideline 414 (Prenatal Developmental Toxicity Study; 1981), but with less than the recommended number of pregnant animals, and was certified to comply with GLP and QA.

No effects on body weight or food consumption were observed. In 13 of 14 females of the group receiving lycopene + apo-12′-lycopenal, yellowish fat in the abdominal cavity was noted. Reproductive parameters evaluated (numbers of corpora lutea, implantations, resorptions and live/dead fetuses, fetal body weight and sex ratio) were not affected. Fetal examination showed an increased incidence of extrathoracic (14th) ribs in the lycopene group compared with the group receiving lycopene + apo-12′-lycopenal and the control group (26.1%, 16.8% and 14.7%, respectively). Co-administration of apo-12′-lycopenal at a concentration of 2% in the formulation (relative to lycopene) thus did not result in substance-related effects (Eckhardt, 1996; McClain & Bausch, 2003).

In a study to examine the effects of lycopene on embryonic and fetal development, groups of mated female Wistar rats were given diets admixed with Lycopene 10% WS beadlets (batch no. UT02070001; 13.2% synthetic lycopene) from day 5 (implantation) through to day 21 postcoitum (the day of scheduled caesarean section), at a target dose of 50, 150 or 500 mg lycopene/kg bw per day. There were two control groups, one receiving standard diet and the other receiving placebo control beadlets (batch no. UT02111005). Each group consisted of 22 mated female rats, with an additional 3 mated female rats per group in the three groups receiving lycopene for exposure monitoring. From the latter animals, blood was taken on days 6 and 21 postcoitum. The study followed OECD Test Guideline 414 (2001) and was certified to comply with GLP and QA.

Analysis of plasma samples showed systemic absorption of Lycopene 10% WS beadlets in all three treated groups, without clear dose dependency. For all groups, mean values in the plasma were higher on day 20 than on day 6. No mortalities occurred, and no clinical signs were observed, except for discoloured faeces in all animals treated with lycopene. No treatment-related effects on body weight or food consumption were observed. In beadlet controls and females at the highest dose, postimplantation loss (which was caused by embryonic rather than fetal resorptions) was slightly increased above control and historical control values. Although examination of fetuses showed some differences in individual visceral and skeletal findings between treated groups and (historical) controls (among which were left-sided umbilical artery, abnormally shaped sternebrae and vertebral bodies, and non-ossified cervical vertebral bodies), treatment with lycopene did not increase the overall number of external, visceral and skeletal abnormalities and variations. Most findings were common variations or abnormalities for the rat strain used. The NOAEL for maternal and developmental toxicity was 500 mg/kg bw per day, the highest dose tested (Edwards et al., 2004b).

In a study of developmental toxicity, groups of 25 pregnant Sprague-Dawley rats were given water, Lycopene 10 CWD (500, 1500 or 3000 mg/kg bw per day), Lycopene 10 CWD formulation matrix (3000 mg/kg bw per day), LycoVit 10% (3000 mg/kg bw per day) or LycoVit 10% formulation matrix (3000 mg/kg bw per day) by gavage on day 6 to day 19 postcoitum. These doses correspond to approximate lycopene dosages of 50, 150 and 300 mg/kg bw per day for Lycopene 10 CWD (batch no. OD-12-04 6.Spr.A, containing 9.81% synthetic lycopene) and 300 mg/kg bw per day for LycoVit 10% (batch no. 20014372, containing 10.8% synthetic lycopene). The animals were sacrificed on day 20 postcoitum. The study followed OECD Test Guideline 414 (draft 1999) and was certified for compliance with GLP and QA.

Red-coloured faeces were observed from day 7 or 8 postcoitum for all females receiving Lycopene 10 CWD or LycoVit 10%. Some other clinical observations (e.g. regurgitation) reflected systemic responses to the high viscosity and large volumes of the test and control substances administered. At necropsy, the animals treated with lycopene showed thick reddish contents in the intestines (with a dose-dependent increase) or stomach. Maternal food consumption, body weight gain and gravid uterus weight were not affected by treatment. At caesarean section, some small, not statistically significant changes were observed in the numbers of implantation sites and fetuses per dam (slight decrease) and in early resorptions and postimplantation loss (slight increase) in females receiving Lycopene 10 CWD (no dose–response relationship) and LycoVit 10% when compared with placebo and vehicle controls. Fetal body weights were not affected by treatment. No treatment-related effects on external and soft tissue malformations and variations were observed. Treatment with lycopene also did not affect skeletal malformations. There were a large number of skeletal variations in fetuses from all groups, mostly related to incomplete or absent ossification. Generally, fetal and litter incidences were similar in treated and control groups and were consistent with historical control data. Despite some differences in individual findings (including incomplete ossification and absence of cartilage of the supraoccipital, parietal and

hyoid bones), the overall incidence of skeletal variations did not differ between groups. Given the absence of relevant toxicological findings with both lycopene formulations, the NOAEL for both maternal and developmental toxicity was approximately 300 mg lycopene/kg bw per day, the highest dose tested (BASF, 2001f; Christian et al., 2003).

(ii) Rabbits

In order to find appropriate doses for the main study, a preliminary study of prenatal developmental toxicity was conducted in groups of five mated Himalayan rabbits receiving either placebo beadlets (batch no. UT02111005) or Lycopene 10% WS beadlets (batch no. UT02070002; 13.2% synthetic lycopene) at a daily dose of 0 or 400 mg lycopene/kg bw (divided between two doses), respectively, by gavage from day 6 until day 27 of gestation. As analysis of plasma samples and liver samples at termination demonstrated the presence of systemic absorption, it was concluded that the maximum practical dose caused no apparent maternal or embryo-fetal toxicity and was an appropriate highest dose for the main study (Edwards et al., 2004c).

In the main study, groups of 20 mated female Himalayan rabbits were given Lycopene 10% WS beadlets (batch no. UT02070001; 13.2% synthetic lycopene) at a dose of 0 (beadlet control), 50, 150 or 400 mg lycopene/kg bw per day by gavage (twice daily) from day 6 until day 27 postcoitum, with caesarean section carried out on day 28 postcoitum. Exposure was monitored by blood sampling from four animals per group on days 6 and 27 postcoitum. The study followed OECD Test Guideline 414 (2001) and was certified to comply with GLP and QA.

Systemic absorption of lycopene was demonstrated in all groups receiving Lycopene 10% WS beadlets, on both day 6 and day 27 postcoitum, without clear dose dependency. For all groups, the mean plasma concentrations of lycopene were slightly higher on day 6 than on day 27 postcoitum. At 50, 150 and 400 mg/kg bw per day, red faeces were noted from day 6 postcoitum until termination. During necropsy, reddish stomach content was noted for one female at 50 mg/kg bw per day and for most females at 150 and 400 mg/kg bw per day. At 50 and 400 mg/kg bw per day, a single female in each group was found dead; these deaths were assumed to be caused by oesophageal reflux and aspiration of the very viscous suspension of the test item. Treatment with lycopene had no apparent effect on food consumption or body weight gain. One female at the highest dose delivered preterm on day 28 postcoitum. One female in the beadlet control group and one female at the highest dose had total postimplantation loss. Excluding these two females, postimplantation loss (which concerned embryonic rather than fetal resorptions) was slightly increased in females at the lowest and intermediate doses when compared with the control group and with historical controls, but not in females at the highest dose. The number of fetuses per dam and fetal weights were not affected by treatment. Identical to the preliminary study, examination of the fetuses did not reveal any differences in total numbers of litters and fetuses with external and fresh visceral findings, although some individual findings differed between groups (including sutural bone between frontals, absence of accessory lung lobe and additional ossification of forelimb and hindlimb). Given the absence of relevant toxicological findings, the NOAEL for maternal and developmental toxicity was 400 mg lycopene/kg bw per day, the highest dose tested (Edwards et al., 2004d).

In a study of the potential developmental toxicity of two synthetic lycopene formulations (Lycopene 10 CWD and LycoVit 10%), groups of 25–34 pregnant New Zealand White rabbits were given water, Lycopene 10 CWD (500, 1500 or 2000 mg/kg bw per day), Lycopene 10 CWD formulation matrix (2000 mg/kg bw per day), LycoVit 10% (2000 mg/kg bw per day) or LycoVit 10% formulation matrix (2000 mg/kg bw per day) by gavage from day 6 until day 28 postcoitum. These correspond to approximate lycopene dosages of 50, 150 and 200 mg/kg bw per day from Lycopene 10 CWD (batch no. OD-13-10 Fass 1, containing 10% synthetic lycopene) and 200 mg/kg bw per day from LycoVit 10% (batch no. 80000007, containing 10% synthetic lycopene). The animals were sacrificed on day 29 postcoitum. The study followed OECD Test Guideline 414 (draft 2000) and was certified to comply with GLP and QA.

Red-coloured faeces were observed for all females receiving Lycopene 10 CWD or LycoVit 10%. There was a high rate of mortality in the groups given Lycopene 10 CWD, which was, in part, related to regurgitation after gavage and accidental aspiration of stomach contents into the lungs, owing to the high viscosity, large volume and possibly bad taste or odour of the test substance. Other observations in the animals treated with lycopene included breathing problems and altered gastointestinal motility and, at necropsy, red material in stomach, intestines and, in some decedent animals, lungs. Compared with vehicle controls, food consumption was reduced in the group receiving Lycopene 10 CWD at the highest dose during days 6–25 of gestation, accompanied by a slightly reduced body weight gain (9%) over the whole treatment period in this group. The reduced food consumption was most probably associated with the high viscosity of the preparation at the highest dose. Food consumption was also slightly reduced in both matrix control groups during days 6–29, resulting in a slight decrease in body weight gain (10%) only for the LycoVit 10% matrix group. No treatment-related effects on external and soft tissue malformations and variations were observed. There were a large number of skeletal variations in fetuses from all groups, including controls, mostly related to incomplete or absent ossification. For most findings, there was no relationship with lycopene treatment. Although the overall incidences of skeletal variations among groups were similar, some individual findings (e.g. unossified or incomplete ossification of the fifth and sixth sternebrae) had a slightly increased incidence in the group treated with LycoVit 10%. Given the inherent variability in ossification in term fetuses, the skeletal findings are not considered to be related to treatment. It can be concluded that in rabbits, more than in rats, gavage administration of the viscous, gelatinous suspensions of Lycopene 10 CWD or LycoVit 10% at maximum dosage volumes affected the animals' ability to retain the intubated test material and altered the animals' gastrointestinal motility, contributing to maternal stress and, in some cases, maternal deaths. Despite these difficulties, no direct toxic effect of lycopene on maternal animals or fetuses was observed. For both formulations, the NOAEL for both maternal and developmental toxicity was approximately 200 mg lycopene/kg bw per day, the highest dose tested (BASF, 2001g; Christian et al., 2003). It was noted that the amount and mode of administration are not representative for human exposure to lycopene from food or dietary supplements.

2.2.6 Additional toxicological data on impurities/reaction by-products

As most of the available toxicological studies were performed with formulations of synthetic lycopene complying with the specifications, the safety of any impurities or reaction by-products present (if any) has been implicitly tested at their maximum permissible levels. Additional data were, however, available for apo-12′-lycopenal and TPPO. Three studies were available that were conducted with 10% synthetic lycopene formulations containing an enhanced content of apo-12′-lycopenal (up to 2% relative to lycopene). These studies—i.e. a 4-week study of toxicity in rats (Niederhauser et al., 1996), a test for gene mutations in vitro in *Salmonella typhimurium* (Gocke, 1999) and a study of developmental toxicity in rats (Eckhardt, 1996)—have been summarized in sections 2.2.2, 2.2.4 and 2.2.5(b), respectively.

2.2.7 Levels relevant for risk assessment

NOAELs from the preceding toxicological studies that are relevant for risk assessment are summarized in Table 4.

Table 4. Levels relevant for risk assessment

Species	Study	Effect	NOAEL	LOAEL
Rat	Three-month studies of toxicity[a,b,c]	Toxicity	1%, equal to 586 mg/kg bw per day[d]	–
	One-year study of toxicity[a]	Toxicity	1.3%, equal to 250 mg/kg bw per day	–
	Two-year studies of toxicity and carcinogenicity[a]	Toxicity	50 mg/kg bw per day	–
		Carcinogenicity	50 mg/kg bw per day	–
	Multigeneration reproductive toxicity study[a,b]	Parental toxicity	500 mg/kg bw per day[d]	–
		Offspring toxicity	500 mg/kg bw per day[d]	–
	Developmental toxicity study[a,b,c]	Maternal toxicity	1000 mg/kg bw per day[d]	–
		Embryo/fetotoxicity	1000 mg/kg bw per day[d]	–
Rabbit	Developmental toxicity study[b,c]	Maternal toxicity	400 mg/kg bw per day[d]	–
		Embryo/fetotoxicity	400 mg/kg bw per day[d]	–

[a] Dietary administration.
[b] Two or more studies combined.
[c] Gavage administration.
[d] Highest dose tested.

2.3 Observations in humans

2.3.1 Case-studies

Reich et al. (1960) reported a case of lycopenodermia occurring in a female aged 61 years who consumed 2 litres of tomato juice per day for several years (exact duration not specified). This is equivalent to a daily lycopene dose of up to 160 mg, or 2.3 mg/kg bw per day. The subject suffered from recurrent bouts of abdominal pain associated with nausea, vomiting and diarrhoea and presented with orange-yellow discoloration of the skin on the hands, forearms, face and soles of the feet. She had a previous history of the same condition. Clinical and chemical investigation revealed unusually high serum lycopene concentrations and hepatic storage of lycopene pigments, as evidenced by large, round vacuolated parenchymal cells and the presence of fatty cysts and fine yellow masses. The authors concluded that the subject suffered from lycopenodermia caused by high intakes of lycopene from tomato juice (modified from original data in Annex 1, reference *186*; Reich et al., 1960).

Similar symptoms were reported for a female aged 19 years who consumed four or five large tomatoes plus pasta with tomato sauce daily for 3 years. A yellow-orange pigmentation was observed on the forehead, nasolabial folds, palms of the hands and soles of the feet, and recurrent abdominal pain was reported. Hepatic echographia revealed liver alterations caused by deposits of lycopene; when dietary intake of tomatoes was restricted, there was a complete regression of pigmentation, and the abdominal pain disappeared. Based on clinical features and dietary history, the authors diagnosed the subject with lycopenodermia (La Placa et al., 2000).

Additional case-studies documenting incidences of orange-yellow skin discoloration (e.g. carotenodermia) in individuals consuming diets rich in tomatoes or tomato products (e.g. tomato soup) have been reported (Bonnetblanc et al., 1987; Gandhi et al., 1988), and both have identified the lycopene content of the tomatoes or tomato products as the probable cause of the discoloration.

2.3.2 Clinical trials

A number of clinical trials were provided, mostly with a focus on investigating the antioxidant properties of lycopene supplementation. Safety-related end-points were addressed in only a few of these studies (see short summaries below), but were limited to reporting of adverse symptoms and/or measurement of some haematology and clinical chemistry parameters.

In a dietary intervention study in 23 healthy male volunteers, administration of 40 mg of lycopene (as tomato juice) for 2 weeks was tolerated well and did not significantly affect blood haemoglobin, leukocyte count, serum electrolytes or serum cholesterol (Müller et al., 1999).

In a crossover dietary intervention study, 19 healthy subjects (10 males and 9 females) received daily 39.2 mg of lycopene from spaghetti sauce, 50.4 mg of lycopene from tomato juice, 75 mg of lycopene from tomato extract capsules and a placebo. All four treatments lasted 1 week, followed by a 1-week washout phase.

Treatment with lycopene had no effect on plasma lipid (total cholesterol, high-density lipoprotein [HDL] cholesterol, LDL cholesterol and triglyceride) concentrations, and no adverse symptoms were reported throughout the duration of the study (Agarwal & Rao, 1998). No adverse effects were reported in groups of 12 men and 12 women consuming daily 15 mg of encapsulated synthetic (LycoVit 10%) or tomato-derived lycopene (Lyc-O-Mato Beads 5%) in a 28-day study (Hoppe et al., 2003).

After 34 healthy female volunteers were placed on a lycopene-rich diet for 1 week (providing approximately 40 mg lycopene/day), no changes were observed in leukocyte count, lipid profile, liver function tests or serum creatine kinase activity (Chopra et al., 2000).

No effects on concentrations of total cholesterol, HDL cholesterol or triglycerides were found in groups of female volunteers (6–8 per group) treated for 6 weeks with a daily dose of 5 mg of lycopene obtained from three different sources (i.e. tomato extract soft gel capsules, tomato juice or raw tomatoes) (Böhm & Bitsch, 1999).

In a multicohort study in healthy French (38 males and 37 females), Irish (72 males and 66 females), Dutch (33 males and 39 females) and Spanish volunteers (32 males and 32 females), serum responses to carotenoids and tocopherols were measured upon supplementation with capsules containing 15 mg of carotenoids (carotene-rich palm oil, lutein or lycopene) in corn oil or corn oil only (placebo), with or without 100 mg of α-tocopherol. The volunteers, divided into four groups of each sex per cohort, received supplements over 20 weeks. In the first 4 weeks, they received α-tocopherol only; in the next 12 weeks, carotene-rich palm oil, lutein, lycopene or placebo; and in the final 4 weeks, the carotenoids/placebo with α-tocopherol. Only in the Spanish cohort was monitoring carried out for biochemical and haematological indices, plasma total cholesterol, HDL cholesterol and LDL cholesterol concentrations, and any incidences of adverse events. Results of haematological analysis and clinical chemistry in this cohort were unremarkable, and no significant changes in plasma lipid concentrations were observed. Carotenodermia was reported in 25% of the subjects in the Spanish cohort supplemented with lycopene, compared with 95% of those supplemented with carotene and 40% of those supplemented with lutein (Olmedilla et al., 2002).

In a double-blind study conducted by Postaire et al. (1997), healthy subjects (10 per group) received either 26 mg β-carotene + 4 mg lycopene/day or 6 mg β-carotene + 6 mg lycopene/day in capsules for 8 weeks. A slight change in the yellow, but not red, pigmentation of the skin (i.e. carotenodermia) was observed in the group receiving β-carotene at the high dose. A significant increase in skin melanin concentration was found in both treatment groups. Prostate cancer patients ($n = 32$) received approximately 30 mg lycopene/day from tomato sauce–based pasta dishes during a 3-week intervention segment of a clinical trial. Patients were asked to record any adverse gastrointestinal effects (i.e. constipation, burping, gas and/or flatulence, nausea, bloating, diarrhoea, cramping and heartburn). With the exception of three patients who reported minor gastrointestinal disturbances, which resolved within a few days, the dietary intervention was well accepted (Chen et al., 2001).

In a randomized clinical trial in 15 patients with prostate cancer, subjects were treated with 15 mg of lycopene (as Lyc-O-Mato capsules) twice per day for 3 weeks, during which time subjects were asked to report any adverse events and were subjected to full physical examinations and a complete blood count and chemistry profile assessment. Another group of 11 patients that did not receive lycopene was also monitored. None of the subjects reported any adverse events, and no abnormalities in blood counts or clinical chemistry were observed (Kucuk et al., 2001).

Taken together, these studies show that administration of dietary lycopene is generally well tolerated, with adverse effects limited to minor gastrointestinal disturbances in prostate cancer patients and dermal discoloration in healthy people treated with lycopene for several weeks.

3. DIETARY EXPOSURE

At the sixty-seventh meeting in 2006, the Committee was advised that the background dietary exposure to lycopene from tomatoes and tomato products was up to 10 mg/day, based on published estimates from eight countries (Canada, Finland, France, Germany, Hungary, the Netherlands, United Kingdom, USA). The Committee at the current meeting considered this background exposure estimate to still be valid.

Lycopene used as a food colour can be derived from a number of different sources, such as synthetic lycopene, lycopene derived from *Blakeslea trispora* and lycopene extract from tomato. However, it is likely that lycopene from these sources will be substituted for one another as food colours when used in accordance with Good Manufacturing Practice. Therefore, the dietary exposure to lycopene from all these sources will be similar.

The dietary exposure to lycopene used as a food colour can be estimated based on the proposed maximum use levels of additive proposed for lycopene extract from tomato in various food categories—namely, cereal-based mixed dishes, 50 mg/kg; fruit and vegetable juices, 25 mg/kg; soft drinks, 25 mg/kg; dairy-based products, 25 mg/kg; sugar and sugar products, including chocolate, 25 mg/kg; and vegetable and animal fats, 20 mg/kg.

These maximum use levels were combined with food consumption data for the relevant food categories from Australia (1995 Australian National Nutrition Survey; Food Standards Australia New Zealand, 2009), Europe (European Food Safety Authority's Concise European Food Consumption Database; European Food Safety Authority, 2009) and the USA (2003–2004 National Health and Nutrition Examination Survey; Foods Analysis and Residue Evaluation Program, 2009). Table 4 shows the contribution to dietary exposure to lycopene for each food category, based on consumers only. A conservative estimate of the mean dietary exposure was performed by summing these individual contributions. It results in a total dietary exposure between 10 and 50 mg/day, with a median value of 21.5 mg/day. Results of this estimation are comparable with those obtained at the sixty-seventh meeting of the Committee.

Table 4. Contribution of relevant food categories to the dietary exposure to lycopene based on maximum concentrations and consumption for consumers only

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
USA	24-h recall	1	Cereal-based mixed dishes	37	28	70[a]	–	50.0	1.85
			Fruit and vegetable juices	240	180	500[a]	–	25.0	6.00
			Soft drinks	510	370	1060[a]	–	25.0	12.75
			Dairy-based products	300	230	660[a]	–	25.0	7.50
			Sugar & sugar products, including chocolate	58	–	102[a]	–	25.0	1.45
			Fats (vegetable and animal)	8	5	16[a]	–	20.0	0.16
Total									**29.71**
Australia	24-h recall	2	Cereal-based products and dishes	158	98	489	64	50.0	7.90
			Fruit and vegetable juices and drinks	342	263	999	36	25.0	8.55

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Soft drinks, flavoured mineral waters and electrolyte drinks	569	391	1500	29	25.0	14.23
			Milk products and dishes	306	241	832	94	25.0	7.65
			Confectionery and health bars	43	30	125	21	25.0	1.08
			Fats and oils	16	12	42	76	20.0	0.32
Total									**39.72**
Austria	24-h recall	1	Cereal-based mixed dishes	256.6	210.0	570.0	44.0	50.0	12.83
			Fruit and vegetable juices	408.4	250.0	1000.0	35.3	25.0	10.21
			Soft drinks with percentage of fruits lower than *n*	323.2	200.0	1000.0	27.3	25.0	8.08
			Dairy-based products	211.3	200.0	500.0	38.9	25.0	5.28

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Sugar & sugar products, including chocolate	38.7	30.0	108.0	59.7	25.0	0.97
			Fats (vegetable and animal)	20.2	14.2	50.0	61.2	20.0	0.40
Total									**37.77**
Belgium	24-h recall	2	Cereal-based mixed dishes	53.1	42.5	112.5	10.7	50.0	2.66
			Fruit and vegetable juices	180.8	125.0	500.0	41.1	25.0	4.52
			Soft drinks with percentage of fruits lower than *n*	476.0	330.0	1340.0	62.9	25.0	11.90
			Dairy-based products	111.7	80.0	253.8	54.8	25.0	2.79
			Sugar & sugar products, including chocolate	37.6	29.0	98.4	80.3	25.0	0.94

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Fats (vegetable and animal)	47.9	33.6	140.4	94.8	20.0	0.96
Total									**23.77**
Bulgaria	24-h recall	1	Cereal-based mixed dishes	–	–	–	–	50.0	0.00
			Fruit and vegetable juices	264.7	200.0	700.0	15.7	25.0	6.62
			Soft drinks with percentage of fruits lower than *n*	346.2	250.0	800.0	32.9	25.0	8.66
			Dairy-based products	69.3	50.0	200.0	8.6	25.0	1.73
			Sugar & sugar products, including chocolate	59.4	40.0	180.0	66.8	25.0	1.49
			Fats (vegetable and animal)	42.0	37.5	89.3	93.4	20.0	0.84
Total									**19.33**

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
Czech Republic	24-h recall	2	Cereal-based mixed dishes	121.0	110.0	275.0	6.5	50.0	6.05
			Fruit and vegetable juices	215.9	150.0	550.0	15.9	25.0	5.40
			Soft drinks with percentage of fruits lower than *n*	282.3	173.3	950.0	43.4	25.0	7.06
			Dairy-based products	91.8	75.0	250.0	63.0	25.0	2.30
			Sugar & sugar products, including chocolate	41.3	30.9	113.8	94.5	25.0	1.03
			Fats (vegetable and animal)	48.3	41.6	109.0	99.1	20.0	0.97
Total									**22.80**
Denmark	Food diary	7	Cereal-based mixed dishes	–	–	–	–	50.0	0.00
			Fruit and vegetable juices	140.2	102.9	360.0	51.7	25.0	3.50

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Soft drinks with percentage of fruits lower than *n*	259.3	151.4	850.0	78.1	25.0	6.48
			Dairy-based products	46.8	21.6	178.1	93.8	25.0	1.17
			Sugar & sugar products, including chocolate	44.0	37.8	100.7	98.9	25.0	1.10
			Fats (vegetable and animal)	35.5	31.4	75.0	100.0	20.0	0.71
Total									**12.97**
France	Dietary record	7	Cereal-based mixed dishes	73.7	58.6	192.9	84.1	50.0	3.69
			Fruit and vegetable juices	114.8	85.7	300.0	49.6	25.0	2.87
			Soft drinks with percentage of fruits lower than *n*	127.9	71.4	426.3	38.3	25.0	3.20

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Dairy-based products	114.7	103.6	260.7	92.2	25.0	2.87
			Sugar & sugar products, including chocolate	33.4	26.8	87.2	93.6	25.0	0.84
			Fats (vegetable and animal)	27.8	25.1	55.1	100.0	20.0	0.56
Total									**14.01**
Germany	Dietary history	28	Cereal-based mixed dishes	69.7	56.9	171.1	97.0	50.0	3.48
			Fruit and vegetable juices	157.7	72.6	601.9	89.6	25.0	3.94
			Soft drinks with percentage of fruits lower than *n*	369.5	171.4	1442.9	41.5	25.0	9.24
			Dairy-based products	94.7	70.3	268.5	98.8	25.0	2.37
			Sugar & sugar products, including chocolate	45.1	33.6	126.9	99.6	25.0	1.13

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Fats (vegetable and animal)	29.1	27.1	53.6	100.0	20.0	0.58
Total									**20.74**
Hungary	Dietary record	3	Cereal-based mixed dishes	–	–	–	–	50.0	0.00
			Fruit and vegetable juices	80.7	16.7	335.3	58.8	25.0	2.02
			Soft drinks with percentage of fruits lower than *n*	223.2	166.7	600.0	42.7	25.0	5.58
			Dairy-based products	71.8	50.5	209.5	89.1	25.0	1.80
			Sugar & sugar products, including chocolate	39.9	35.0	93.4	97.3	25.0	1.00
			Fats (vegetable and animal)	53.5	48.0	102.2	100.0	20.0	1.07
Total									**11.46**

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
Iceland	24-h recall	1	Cereal-based mixed dishes	158.2	115.0	430.0	25.6	50.0	7.91
			Fruit and vegetable juices	295.6	250.0	900.0	29.4	25.0	7.39
			Soft drinks with percentage of fruits lower than *n*	674.0	500.0	1700.0	50.3	25.0	16.85
			Dairy-based products	204.9	183.0	500.0	53.3	25.0	5.12
			Sugar & sugar products, including chocolate	45.3	22.5	165.0	68.1	25.0	1.13
			Fats (vegetable and animal)	35.6	26.0	103.0	91.9	20.0	0.71
Total									**39.12**
Ireland	Dietary record	7	Cereal-based mixed dishes	41.3	31.2	111.1	43.6	50.0	2.07
			Fruit and vegetable juices	59.1	35.7	187.0	57.0	25.0	1.48

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Soft drinks with percentage of fruits lower than *n*	203.6	136.8	635.9	71.4	25.0	5.09
			Dairy-based products	39.3	27.0	113.1	74.4	25.0	0.98
			Sugar & sugar products, including chocolate	42.4	34.6	109.3	96.6	25.0	1.06
			Fats (vegetable and animal)	36.4	32.6	74.8	99.7	20.0	0.73
Total									**11.41**
Italy	Dietary record	7	Cereal-based mixed dishes	38.2	28.6	92.9	9.3	50.0	1.91
			Fruit and vegetable juices	47.0	18.7	172.1	37.2	25.0	1.18
			Soft drinks with percentage of fruits lower than *n*	114.9	75.0	347.1	31.4	25.0	2.87

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Dairy-based products	50.8	35.7	150.0	64.3	25.0	1.27
			Sugar & sugar products, including chocolate	21.0	16.0	55.0	92.9	25.0	0.52
			Fats (vegetable and animal)	35.7	33.1	64.1	99.9	20.0	0.71
Total									**8.47**
The Netherlands	Dietary record	2	Cereal-based mixed dishes	48.3	37.5	112.5	3.6	50.0	2.41
			Fruit and vegetable juices	194.8	140.0	550.0	35.7	25.0	4.87
			Soft drinks with percentage of fruits lower than *n*	338.8	232.5	990.0	54.5	25.0	8.47
			Dairy-based products	143.6	125.0	325.0	72.4	25.0	3.59
			Sugar & sugar products, including chocolate	48.2	39.2	120.0	89.6	25.0	1.20

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Fats (vegetable and animal)	48.5	41.0	112.5	98.4	20.0	0.97
Total									**21.52**
Norway	Quantitative food frequency questionnaire	0	Cereal-based mixed dishes	189.6	174.9	332.8	100.0	50.0	9.48
			Fruit and vegetable juices	100.9	54.0	313.1	85.3	25.0	2.52
			Soft drinks with percentage of fruits lower than *n*	375.6	203.0	1300.0	87.9	25.0	9.39
			Dairy-based products	49.1	27.2	167.2	96.8	25.0	1.23
			Sugar & sugar products, including chocolate	47.2	37.6	121.0	98.9	25.0	1.18
			Fats (vegetable and animal)	40.9	32.4	98.7	100.0	20.0	0.82
Total									**24.62**

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
Poland	24-h recall	1	Cereal-based mixed dishes	156.7	115.0	400.0	18.1	50.0	7.83
			Fruit and vegetable juices	288.5	210.0	750.0	12.1	25.0	7.21
			Soft drinks with percentage of fruits lower than *n*	370.1	250.0	1000.0	11.5	25.0	9.25
			Dairy-based products	46.7	25.0	200.0	47.9	25.0	1.17
			Sugar & sugar products, including chocolate	59.9	50.0	145.1	95.2	25.0	1.50
			Fats (vegetable and animal)	61.1	51.1	143.5	98.1	20.0	1.22
Total									**28.18**
Slovakia	24-h recall	1	Cereal-based mixed dishes	321.6	210.0	700.0	38.3	50.0	16.08
			Fruit and vegetable juices	455.8	300.0	1200.0	7.3	25.0	11.40

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Soft drinks with percentage of fruits lower than *n*	613.6	400.0	2000.0	20.2	25.0	15.34
			Dairy-based products	176.8	150.0	400.0	26.9	25.0	4.42
			Sugar & sugar products, including chocolate	143.1	42.5	240.0	48.3	25.0	3.58
			Fats (vegetable and animal)	58.9	20.0	150.0	49.3	20.0	1.18
Total									**51.99**
Sweden	Dietary record	7	Cereal-based mixed dishes	55.3	40.0	126.7	69.1	50.0	2.77
			Fruit and vegetable juices	148.3	114.3	428.6	58.4	25.0	3.71
			Soft drinks with percentage of fruits lower than *n*	243.4	142.9	685.7	73.8	25.0	6.08

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Dairy-based products	–	–	–	–	25.0	0.00
			Sugar & sugar products, including chocolate	30.8	24.4	80.7	92.1	25.0	0.77
			Fats (vegetable and animal)	24.7	19.7	66.4	96.6	20.0	0.49
Total									**13.82**
United Kingdom	Dietary record	7	Cereal-based mixed dishes	180.7	165.9	342.7	99.5	50.0	9.04
			Fruit and vegetable juices	106.0	78.0	297.9	44.8	25.0	2.65
			Soft drinks with percentage of fruits lower than *n*	292.5	192.3	891.6	72.9	25.0	7.31
			Dairy-based products	50.1	35.7	137.6	61.7	25.0	1.25
			Sugar & sugar products, including chocolate	30.8	21.6	88.0	86.4	25.0	0.77

Table 4 (contd)

Country	Method	No. of days	Subcategory	Mean (g/day)	Median (g/day)	95th percentile (g/day)	% consumers	Maximum concentration (mg/kg)	Mean exposure (mg/day)
			Fats (vegetable and animal)	20.5	16.4	50.7	96.1	20.0	0.41
Total									**21.43**
Median dietary exposure, all countries									**21.5**

[a] 90th percentile.

4. COMMENTS

In addition to a 28-day toxicity study that had not been previously assessed, the Committee reconsidered all the toxicity studies that had been available at the sixty-seventh meeting. Most of the studies had been performed with formulations of lycopene that complied with the specifications as established at the sixty-seventh and current meetings and met appropriate standards for study protocol and conduct.

4.1 Toxicological data

When lycopene was administered orally to rats as a formulation containing 10% synthetic lycopene, its LD_{50} was more than 5000 mg/kg bw.

The toxicity of synthetic lycopene, lycopene extract from tomato and lycopene derived from *Blakeslea trispora* was evaluated in short-term toxicity studies in rats and dogs and long-term studies in rats. In most studies of toxicity, there were no statistically significant or consistent differences in body weights, food or water consumption, or organ weights or in parameters of haematology, clinical chemistry or urine analysis between the treated and control groups. In the absence of any toxicologically relevant effects, the NOAELs were always established at the highest dose tested (up to 586 mg/kg bw per day in a 90-day rat study). A 100-day toxicity study in the rat at a lycopene dose of 1000 mg/kg bw per day (only one dose level tested) that was completed in 1958 and a more recent (1996) limited 1-month toxicity study in the rat at the same dose provided supporting evidence for the absence of any adverse effects at high doses.

In a 52-week study of toxicity in rats, slight increases in the group mean activities of AST and ALT were recorded at week 13 at the highest dose only (250 mg/kg bw per day). At weeks 26 and 52, group mean activities of these enzymes were increased in a dose-dependent manner in males (up to 1.7-fold and 2.6-fold, respectively) and females (up to 1.4-fold and 2-fold, respectively) at the lowest, intermediate and highest doses (10, 50 and 250 mg/kg bw per day, respectively), although increases were not always statistically significant and were generally without progression between week 26 and week 52. At the end of a 13-week treatment-free period, AST and ALT activities had declined, but they still tended to be higher in the highest dose group than in the control group.

Slightly elevated levels of AST and ALT, which achieved statistical significance among male rats after 3 or 12 months of exposure to the highest tested dose (500 and 250 mg/kg bw per day, respectively), were considered not to be adverse because of the lack of concordance with other important measures usually associated with liver damage, such as increased organ weight and histopathological lesions.

As the doses of lycopene were increased in short-term studies in rats, a discoloration of the faeces owing to excretion of the test substance was observed. Macroscopically, all treated animals in the feeding studies showed a discoloration of the liver and adipose tissue. The observed discoloration in the liver was associated with pigment deposits in the hepatocytes; however, there was no histopathological evidence of liver damage. Observations made in short-term

studies of toxicity in dogs were consistent with the findings in rats. The Committee considered that the changes observed in the repeated-dose studies of toxicity did not represent adverse effects.

In a study of carcinogenicity in which rat diets were mixed with synthetic lycopene at doses up to 50 mg/kg bw per day, the treatment resulted in a discoloration of the faeces, gastrointestinal tract and connective tissue at the intermediate and/or highest dose (10 and 50 mg/kg bw per day, respectively). Pigment deposits were observed in the liver, kidneys (especially in females at the highest dose) and mesenteric and mandibular lymph nodes (at all doses). The liver pigmentation was observed mainly in hepatocytes and histiocytes in females and, to a lesser degree, in histiocytes in males. Histopathologically, the liver pigmentation was associated with a greater incidence and severity of eosinophilic foci in males and of normochromic and basophilic foci in females, especially at the intermediate and highest doses, albeit without a consistent dose–response relationship. There was no apparent evidence of hepatotoxicity. Also, no increase in the incidence of liver tumours was observed, nor was treatment with lycopene associated with an increase in the incidence of tumours in any other tissue or organ. The histopathological alterations of liver foci, observed mainly at the intermediate and highest doses, were considered to be treatment related but of no toxicological consequence because they did not progress to tumours.

On the basis of the results of the carcinogenicity study in rats and an adequate range of genotoxicity tests, the Committee concluded that lycopene has no genotoxic or carcinogenic potential when protected against oxidative processes.

In a two-generation study of reproductive toxicity in rats, adults receiving lycopene at a dietary dose of up to 500 mg/kg bw per day showed no evidence of toxicity. The discoloured faeces and staining of fur/skin/fat/abdominal organs observed were attributed to the presence of lycopene in the diet. Mating performance, fertility, and survival and growth of the pups were not affected by treatment with lycopene. The NOAELs for parental, reproductive and offspring toxicity were all 500 mg/kg bw per day, the highest dose tested.

In studies of developmental toxicity in rats and rabbits, no teratogenicity was observed. Similarly, there were no treatment-related increases in the overall number of external, visceral or skeletal abnormalities and variations. In all developmental studies, the dams showed discoloured faeces, and the contents of the gastrointestinal tract appeared discoloured in the gavage studies. Given the absence of any adverse toxicological findings, the NOAELs for maternal and developmental toxicity were at the highest tested doses in both rats and rabbits, 1000 and 400 mg/kg bw per day, respectively.

The toxicity of lycopene extract from tomato was assessed in 10-week and 13-week studies in rats and a 6-week study in human volunteers. As the major non-lycopene constituents present in the extract from tomato were naturally occurring fatty acids (72%), it was anticipated that the toxicity profile of this lycopene extract would be similar to those of synthetic lycopene and lycopene derived from *Blakeslea trispora*. Consistent with the results of all toxicity studies on synthetic lycopene and lycopene derived from *B. trispora*, no toxicologically relevant effects were observed in the studies using lycopene extract from tomato.

Other studies in humans, although not specifically designed to assess the safety of lycopene, revealed no adverse effects after administration of dietary lycopene. There are, however, case reports of skin discoloration (lycopenodermia) and/or gastrointestinal discomfort after prolonged high intakes of lycopene-rich food and/or supplements, those effects being reversible upon cessation of lycopene ingestion.

4.2 Assessment of dietary exposure

Dietary exposure to lycopene naturally present in food is likely to be up to 10 mg/day in adults. Lycopene used as a food colour can be derived from a number of different sources, such as synthetic lycopene, lycopene derived from *Blakeslea trispora* and lycopene extract from tomato. However, it is likely that lycopene from these sources will be substituted for one another as food colours when used in accordance with Good Manufacturing Practice. Therefore, the dietary exposure to lycopene from all these sources will be similar. Based on the proposed use levels, dietary exposure to lycopene added as a food colour is estimated to be up to 5 times higher than the upper end of the range of the background exposure, 10 mg/day.

5. EVALUATION

Lycopene is a normal constituent of the human diet, and the background dietary exposure to lycopene from vegetables and fruits is up to 10 mg/person per day. The available data indicate that dietary lycopene is generally well tolerated in humans. After prolonged high intake of lycopene-rich food and/or supplements, the effects were limited to skin discoloration and/or gastrointestinal discomfort. The Committee reconsidered the available toxicological data, including a new 28-day study, together with the dietary exposure to lycopene that occurs naturally in food and lycopene used as a food colour from all sources. The Committee concluded that, based on lycopene's very low toxicity, there was no need to establish a numerical ADI. The Committee decided to revise the group ADI established at the sixty-seventh meeting and replace it with a group ADI "not specified" for lycopene from all sources. Hence, the previous group ADI of 0–0.5 mg/kg bw for lycopene has been withdrawn.

The group ADI "not specified" applies to synthetic lycopene, lycopene derived from the fungus *Blakeslea trispora* and lycopene extract from tomato that comply with the specifications established at the sixty-seventh and the current meetings, when used in accordance with Good Manufacturing Practice.

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OCTENYL SUCCINIC ACID MODIFIED GUM ARABIC

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1. EXPLANATION

At the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008), the Committee evaluated octenyl succinic acid (OSA) modified gum arabic, which it had not evaluated previously. OSA modified gum arabic (gum arabic hydrogen octenylbutanedioate, Chemical Abstracts Service No. 455885-22-0) is produced by controlled esterification of the polysaccharide in gum arabic with octenyl succinic acid anhydride, analogous to the production of starch sodium octenyl succinate (OSA modified food starch).

The Committee considered safety data on OSA modified gum arabic together with safety data on the related food additives gum arabic and starch sodium octenyl succinate. Gum arabic was evaluated by the Committee at its thirteenth, seventeenth, twenty-sixth and thirty-fifth meetings (Annex 1, references *19*, *32*,

59 and *88*). At the thirteenth and seventeenth meetings, the Committee did not impose a limit on acceptable daily intake (ADI) except for Good Manufacturing Practice. An ADI "not specified" was allocated at the twenty-sixth meeting (replacing the previous terminology "not limited"), and this was confirmed at the thirty-fifth meeting. Starch sodium octenyl succinate was evaluated by the Committee at its twenty-sixth meeting (Annex 1, reference *59*), at which it was allocated an ADI "not specified".

1.1 Chemical and technical considerations

OSA modified gum arabic is produced by esterifying gum arabic *Acacia seyal* or gum arabic *Acacia senegal* in aqueous solution with not more than 3% of octenyl succinic acid anhydride. It is subsequently spray dried. The degree of esterification of OSA modified gum arabic is not more than 0.6%, and residual octenyl succinic acid is not more than 0.3%.

OSA modified gum arabic is a cold water–soluble hydrocolloid used as an emulsifier in a wide range of food products. The introduction of lipophilic groups to the polysaccharide in gum arabic results in enhanced emulsifying properties for OSA modified gum arabic relative to the parent compound.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

No absorption, distribution, metabolism or excretion data are available for OSA modified gum arabic. Gum arabic consists mainly of high molecular weight polysaccharides and their calcium, magnesium and potassium salts. If any hydrolysis of gum arabic occurs, it will yield arabinose, galactose, rhamnose and glucuronic acid, which will be metabolized in normal biochemical pathways. Gum arabic is not significantly digested by laboratory animals or humans, but experiments with rats and humans show that it can be fermented by bacteria in the caecum/colon (Ross et al., 1983, 1984; Phillips, 1998). According to the sponsor, OSA modified gum arabic is expected to be de-esterified in the stomach to gum arabic and to be fermented in the colon as well; however, there are no experimental data available on the de-esterification of OSA modified gum arabic, such as in vitro data on hydrolysis under simulated gastric conditions.

2.2 Toxicological studies

2.2.1 Acute toxicity

Two studies of acute oral toxicity with OSA modified gum arabic were available, both certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). In a limit test, five male and five female Sprague-Dawley albino rats were administered OSA modified gum arabic (100% pure, specific gravity 1.080 g/ml, degree of esterification and residual OSA not specified) as a 30% by weight suspension in distilled water at a dose of 2000 mg/kg body weight (bw) via gavage. The oral median lethal dose (LD_{50}) in this test was >2000 mg/kg bw (Moore,

2002). In a test that followed the up-and-down procedure, OSA modified gum arabic (specific gravity 0.893 g/ml, degree of esterification and residual OSA not specified) was administered as a 30% by weight suspension in distilled water to five female Sprague-Dawley albino rats at a dose of 2000 mg/kg bw via gavage. The oral LD_{50} for this OSA modified gum arabic was also >2000 mg/kg bw (Moore, 2003).

These findings of low acute oral toxicity for OSA modified gum arabic support the findings of low acute toxicity for traditional gum arabic (Annex 1, reference *60*).

2.2.2 Short-term studies of toxicity

In a 14-day dietary palatability/range-finding study, groups of five male and five female Hsd:SD rats were administered OSA modified gum arabic (degree of esterification 0.18%, residual OSA 0.11%) at a dietary level of 0, 15 000, 30 000 or 50 000 mg/kg feed (equal to mean daily intakes of 0, 1240, 2530 and 4200 mg/kg bw for males and 0, 1330, 2670 and 4480 mg/kg bw for females, respectively). Animals were observed twice daily for mortality and daily for signs of gross toxicity and behavioural changes. Detailed clinical observations were recorded weekly, and body weights and food consumption were recorded on days 0, 4, 7, 11 and 14. Gross necropsies were performed on all animals at termination, including examination of the external surface of the body, all orifices and the thoracic and abdominal cavities and their contents. No treatment-related effects were observed, with the possible exception of a slight dark discoloration noted in the kidney and adrenal tissue in females of the 30 000 mg/kg feed group and in males and females of the 50 000 mg/kg feed group (Merkel, 2006). However, as this discoloration was not observed in the subsequent 90-day study (see below), it was considered to be of no toxicological relevance.

In the subsequent 90-day study of toxicity, groups of 10 male and 10 female Hsd:SD rats were given OSA modified gum arabic at a dietary level of 0, 10 000, 25 000 or 50 000 mg/kg feed, equal to mean daily intakes of 0, 680, 1720 and 3410 mg/kg bw for males and 0, 800, 2030 and 4050 mg/kg bw for females, respectively. The highest dose was set based on the results of the 14-day range-finding study. The study was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents; 1998) and was certified for compliance with GLP (except for the serology analysis) and QA. Observations included mortality, clinical signs, behaviour, body weight, food intake, water consumption, a functional observational battery, motor activity, ophthalmoscopy, haematology, clinical chemistry, urinalysis, organ weights, and macroscopic and microscopic pathology.

One high-dose male and one control female died during the study, but these deaths were unrelated to treatment. There were no treatment-related clinical signs. Ophthalmoscopic examinations and functional observational and motor activity tests did not reveal treatment-related effects. Mean body weight gain was statistically significantly increased in males of the 50 000 mg/kg feed group in week 6 of the study only. Statistically significant increases in mean daily food consumption were observed in weeks 2 and 8 (males) and weeks 3 and 12 (females) of the 25 000 mg/kg feed group. A statistically significant decrease in food conversion

efficiency was observed in week 1 in females of the 50 000 mg/kg feed group. These deviations in body weight, food consumption and food conversion efficiency were incidental and unrelated and therefore were not considered to be toxicologically relevant. Haematology, clinical chemistry and urinalysis revealed a few incidental findings, including changes in neutrophil counts, activated partial thromboplastin time, activities of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, serum concentration of potassium and bilirubin, and urinary pH. However, as each change was observed only in one sex at one dose level, they were not considered to be of toxicological relevance. No differences in organ weights and no treatment-related macroscopic or microscopic effects were observed upon necropsy. The dark discoloration in the kidney and adrenal tissue noted in the range-finding study was not observed in the 90-day study. Overall, it can be concluded that the no-observed-effect level (NOEL) is 50 000 mg/kg feed (equal to 3410 mg/kg bw per day), the highest dose tested in this study (Merkel, 2007).

The finding of low subchronic toxicity for OSA modified gum arabic was also observed for gum arabic and starch sodium octenyl succinate. In studies with gum arabic reviewed by the Committee at its twenty-sixth meeting (Annex 1, reference *60*) and an additional 13-week study of toxicity with gum arabic in rats by Anderson et al. (1982), no untoward effects were observed at dietary concentrations below that which caused nutritional imbalance (8%, approximately 5 g/kg bw per day). At higher dietary concentrations (20%, approximately 14 g/kg bw per day), gum arabic caused caecal enlargement and a reduction in feed consumption and weight gain.

In short-term feeding studies with starch sodium octenyl succinate reviewed by the Committee at its twenty-sixth meeting (Annex 1, reference *60*), the only significant lesion observed in rats exposed to dietary concentrations up to 30% (equivalent to 15 000 mg/kg) was corticomedullary mineralization of the kidney. This effect occurred in animals fed either modified or unmodified starch and was concluded to be possibly related to a marginal magnesium deficiency when carbohydrate comprises a major proportion of the diet.

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available on OSA modified gum arabic.

In carcinogenicity studies with gum arabic reviewed by the Committee at its twenty-sixth meeting (Annex 1, reference *60*), no significant adverse effects were observed in rats or mice administered gum arabic at dietary concentrations up to 5% (equivalent to 2500 mg/kg bw in rats and 7500 mg/kg bw in mice) for 103 weeks.

2.2.4 Genotoxicity

The results of one bacterial reverse mutation assay (Ames test) with OSA modified gum arabic (degree of esterification 0.14%, residual OSA 0.57%) are summarized in Table 1. The study followed OECD Test Guideline 471 (Bacterial Reverse Mutation Test; 1997) and was certified for compliance with GLP and QA (although the statements were not signed).

Table 1. Results of a study of genotoxicity in vitro with OSA modified gum arabic

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA97a, TA98, TA100 and TA1535; *Escherichia coli* WP2 uvrA	1st experiment: 5–5000 µg/plate, ±S9 2nd experiment: 100–5000 µg/plate, ±S9	Negative[a]	Totaro (2006)

S9, × 9000 *g* supernatant from rat liver.

[a] With and without metabolic activation (S9), by the direct plate incorporation method (first experiment) and the preincubation method (second experiment).

From studies of genotoxicity in vitro and in vivo with gum arabic, as reviewed by the Committee at its twenty-sixth meeting (Annex 1, reference *60*), it was concluded that mutagenicity studies in a number of test systems, including host-mediated assay, Ames test, *Saccharomyces cerevisiae*, dominant lethal test and *Drosophila*, were negative. The results from another series of genotoxicity assays (Sheu et al., 1986) that were not considered at the previous meeting are summarized in Table 2. Gum arabic induced dominant lethal effects in male rats (although no clear dose–response was observed) but not in male mice at considerably higher doses. In addition, gum arabic did not cause heritable chromosomal effects in male mice. Overall, the Committee concluded that gum arabic is not genotoxic, but it did not extend this conclusion to OSA modified gum arabic.

2.2.5 Reproductive toxicity

No information was available on OSA modified gum arabic.

In oral developmental toxicity studies with gum arabic reviewed by the Committee at its twenty-sixth and thirty-fifth meetings (Annex 1, references *60* and *89*), no teratogenic or other adverse effects on the offspring were observed in rats, mice, hamsters or rabbits, although maternal toxicity was observed at high dose levels in the rabbit.

2.3 Observations in humans

No information was available on OSA modified gum arabic.

According to the evaluation of gum arabic by the Committee at its twenty-sixth meeting (Annex 1, reference *60*), sensitivity reactions to gum arabic have been reported to occur in some cases, whereas repeated intravenous administration of acacia to patients with nephrotic oedema did not result in liver enlargement or other complications. In an additional study with five healthy male volunteers reported by Ross et al. (1983), daily oral administration of 25 g of gum arabic for 3 weeks was well tolerated; it had no effect on glucose tolerance, stool weight, faecal fat, bile acids, neutral sterols or volatile fatty acids, but it decreased the serum cholesterol.

Table 2. Genotoxicity of gum arabic in vivo

End-point	Test system	Concentration	Result	Reference
Dominant lethal test	Sprague-Dawley rats	0%, 0.4%, 1.3% and 4.0% of the diet (reported to be equal to 0, 500, 1500 and 5000 mg/kg bw per day)	Positive[a]	Sheu et al. (1986)
Dominant lethal test	1st experiment: Female (SEC × C57BL)F1 mice and male (101 × C3H)F1 mice	0%, 3%, 5%, 10% and 20% of the diet (equivalent to 0, 4500, 7500, 15 000 and 30 000 mg/kg bw per day)	Negative[b]	Sheu et al. (1986)
	2nd experiment: Female (SEC × C57BL)F1 and (CH3 × C57BL)F1 mice and male (101 × C3H)F1 mice	0% and 15% of the diet (equivalent to 0 and 22 500 mg/kg bw per day)	Negative[c]	
Heritable translocation test	Female (SEC × C57BL)F1 and (CH3 × C57BL)F1 mice and male (101 × C3H)F1 mice	0% and 15% of the diet (equivalent to 0 and 22 500 mg/kg bw per day)	Negative[d]	Sheu et al. (1986)

[a] Males were exposed to gum arabic in the diet for 10 weeks. Twenty males from each group were selected, and each male was caged with two virgin females per week for 2 successive weeks. At 14 days after the midweek of mating, pregnant females were sacrificed, and the numbers of live and dead implants were counted. The data were analysed considering the females mated in the first week and the females mated in the second week as different groups. The number of dead implants per pregnant female was significantly increased in the mid-dose group in the first-week females (9% versus 4%) and in the high-dose group in both the first-week females (8% versus 4%) and the second-week females (9% versus 4%). Although not significant, a higher incidence of dead implants was observed in females in the second week of mating in the low-dose group (10% versus 4%).

[b] Males (12 per group) were exposed to gum arabic in the diet for 8 weeks. Males were caged with untreated (SEC × C57BL)F1 females for 1 week. Mated females were sacrificed for uterine analysis 12–15 days after observation of vaginal plugs.

[c] Males (36 per group) were exposed to gum arabic in the diet for 8 weeks. Males were caged with one (SEC × C57BL)F1 female and one (CH3 × C57BL)F1 female for 1 week. Females with vaginal plugs were replaced with new ones each morning. Mated females were sacrificed for uterine analysis 12–15 days after observation of vaginal plugs.

[d] Untreated males (50–75) were each caged with three (SEC × C57BL)F1 females for 1 week. The females were allowed to produce progeny. Thereafter, males (50 per group) were fed test diets for 8 weeks and remated with the same females. The fertility of the male progeny obtained from the mating before and after the treatment was determined. All progeny classified as having reduced fertility were subjected to cytological analysis for the confirmation of translocation heterozygosity. The male progeny obtained before treatment served as the negative controls as well as for determining the possible existence of translocations in the parental mice. No difference in newly arisen translocations was observed between control and treated animals (0/433 versus 1/450).

3. DIETARY EXPOSURE

OSA modified gum arabic is intended to replace gum arabic in a number of food applications. It can be used at lower concentrations than gum arabic (approximately half) in the encapsulation of flavouring substances used in foods, so the overall dietary exposure to gum arabic is lowered by the use of OSA modified gum arabic in the food flavourings industry.

The Committee received one dietary exposure analysis for OSA modified gum arabic from a sponsor (TIC Gums, 2008). Additionally, the Committee evaluated information submitted to the United States Food and Drug Administration (USFDA) as part of a Generally Recognized as Safe (GRAS) Notice for the use of OSA modified gum arabic (in the USFDA letter to the notifier, the name of the ingredient was "modified gum acacia").

3.1 Use in food

OSA modified gum arabic is proposed for use as an emulsifier for flavouring agents in baked goods, beverages (non-alcoholic and alcoholic), breakfast cereals, processed cheese, chewing gum, flour confectionery and icings, egg products, fish products, frozen dairy, fruit ices, gelatines and puddings, gravies, imitation dairy products, instant coffee and tea, jams and jellies, meat products, milk products, other grains, processed poultry, processed fruit juices, processed vegetable juices, snack foods, soft candy, soups and sweet sauces at levels up to 500 mg/kg of the food. OSA modified gum arabic is also proposed for use as an emulsifier in some fruit-flavoured drinks, some beverages (carbonated juice and energy drinks), fruit juices, salad dressing, sauces, icing, some breads (whole-grain and high-fibre breads) and some cereals (high-fibre, low-sugar and low-fat adult cereals) at levels up to 10 000 mg/kg of the food. See Table 3 for specific proposed food uses and levels.

Table 3. OSA modified gum arabic use levels and applicable foods

Food category	Levels as a flavouring emulsion (mg/kg)	Levels for other emulsifier uses (mg/kg)	Cumulative levels[a] (mg/kg)
Baked goods	500	–	500
Beverages type I, non-alcoholic (other emulsifier uses only in carbonated juice and energy drinks)	220	780	Up to 1 000
Beverages type II, alcoholic	220	–	220
Breakfast cereals	300	–	300
Cheese (processed)	120	–	120

Table 3 (contd)

Food category	Levels as a flavouring emulsion (mg/kg)	Levels for other emulsifier uses (mg/kg)	Cumulative levels[a] (mg/kg)
Chewing gum	60	–	60
Confectionery & icings (includes icing portion of iced baked goods, peanut butter at 240 mg/kg for flavouring use only)	300	10 000	10 300
Egg products	140	–	140
Fish products	500	–	500
Frozen dairy	500	–	500
Fruit ices	500	–	500
Gelatines & puddings	500	–	500
Gravies	400	–	400
Imitation dairy products	240	–	240
Instant coffee & tea	240	–	240
Jams & jellies	240	–	240
Meat products	240	–	240
Milk products	220	–	220
Other grains	240	–	240
Processed poultry	500	–	500
Processed fruits (juices) (other emulsifier uses in vitamin- or mineral-fortified juice–containing drinks only)	400	600	Up to 1 000
Beverages containing fruit juice	–	1 000	1 000
Processed vegetable juice	400	–	–
Salad dressing	–	10 000	10 000
Snack foods	440	–	440
Soft candy	300	–	300
Soups	240	–	240
Sauces	–	10 000	10 000

Table 3 (contd)

Food category	Levels as a flavouring emulsion (mg/kg)	Levels for other emulsifier uses (mg/kg)	Cumulative levels[a] (mg/kg)
Sweet sauce	400	–	400
Whole-grain and high-fibre breads (>5 g per serving)	–	400	400
Some cereals (low-fat, low-sugar and high-fibre health foods, meeting three conditions: high in fibre (≥5 g per serving), low in fat (≤3 g per serving) and low in sugar (≤20 g per serving); use level of 350 mg/kg for foods with large serving size (55 g/serving) and 650 mg/kg for foods with smaller serving size (30 g/serving)	–	350 or 650	Up to 650

[a] If used as both general and flavouring emulsifier in the same foodstuffs.

3.2 Dietary exposure estimates

The dietary exposure analysis received by the Committee used individual dietary records from both the United Kingdom and the USA. The food consumption data from the United Kingdom were taken from the 2000–2001 National Diet and Nutrition Survey, which examines a nationally representative sample drawn from adults aged 19–64 years and provides 7-day weighted food records. Use levels from Table 3 were combined with the appropriate food consumption data and summed for each individual. The results on both a milligram per day and a milligram per kilogram body weight per day basis are shown in Tables 4 and 5, respectively.

A parallel analysis was completed using USA food consumption data from the 2003–2004 National Health and Nutrition Examination Survey, which collects 2 days of individual dietary records for infants, children and adults. As before, food consumption data were combined with the use levels shown in Table 3. Using these data, the mean dietary exposure to OSA modified gum arabic from general and flavouring emulsification uses obtained for the USA population aged 2+ years was 524 mg/person per day (9 mg/kg bw per day), and the 90th-percentile exposure was 964 mg/person per day (17 mg/kg bw per day).

The USFDA evaluated a GRAS Notice for OSA modified gum arabic, which contained a dietary exposure assessment for its use as a flavouring substance

Table 4. Average daily dietary exposure of United Kingdom adults (19–64 years) to OSA modified gum arabic

	Per capita consumption (mg/day)				All users consumption (mg/day)			
		Percentile				Percentile		
Uses	Mean	90	95	97.5	Mean	90	95	97.5
Flavouring emulsions	189	350	459	534	189	350	459	534
Other emulsifier uses	137	324	433	519	163	346	453	543
All uses: cumulative	326	608	729	859	326	609	729	859

Table 5. Average daily dietary exposure of United Kingdom adults (19–64 years) to OSA modified gum arabic on a body weight basis

	Per capita consumption (mg/kg bw per day)				All users consumption (mg/kg bw per day)			
		Percentile				Percentile		
Uses	Mean	90	95	97.5	Mean	90	95	97.5
Flavouring emulsions	2.5	4.5	5.7	6.8	2.5	4.5	5.7	6.8
Other emulsifier uses	1.9	4.3	5.7	7.3	2.2	4.6	6.1	7.8
All uses: cumulative	4.3	7.9	10	12	4.3	7.9	10	12

emulsifier. The published letter from the USFDA lists the mean and 90th-percentile exposures for all users for this subset of uses as 270 and 524 mg/day, consistent with the above analyses (USFDA, 2008).

The Committee concluded that the estimated dietary exposure to OSA modified gum arabic from the proposed uses would be less than 20 mg/kg bw per day.

4. COMMENTS

4.1 Toxicological data

No absorption, distribution, metabolism or excretion data are available for OSA modified gum arabic. Gum arabic is not significantly digested by laboratory animals or humans, but experiments with rats and humans show that gum arabic can be fermented by bacteria in the caecum/colon. According to the sponsor, OSA modified gum arabic is expected to be de-esterified in the stomach to gum arabic

and to be fermented in the colon as well. However, there are no experimental data available on the de-esterification of OSA modified gum arabic, such as in vitro data on hydrolysis under simulated gastric conditions.

Toxicological studies have been performed with different batches of OSA modified gum arabic, which can be considered to be representative of the OSA modified gum arabic under evaluation. OSA modified gum arabic is of low acute oral toxicity in rats. In a 90-day study of toxicity in rats, no significant treatment-related effects were seen when OSA modified gum arabic was administered at dietary concentrations up to 50 000 mg/kg feed. Therefore, 50 000 mg/kg feed (equal to 3410 mg/kg bw per day), the highest dose tested, was taken to be the NOEL.

OSA modified gum arabic was not mutagenic in an assay for mutagenicity in bacteria in vitro. It was not tested in any assay of genotoxicity with mammalian cells. From studies of genotoxicity in vitro and in vivo with gum arabic, reviewed by the Committee at its twenty-sixth meeting (Annex 1, reference *60*), it was concluded that mutagenicity studies in a number of test systems, including host-mediated assay, Ames test, *Saccharomyces cerevisiae,* dominant lethal test and *Drosophila*, were negative. In another series of in vivo genotoxicity assays that were not considered in the previous evaluation, gum arabic was reported to induce dominant lethal effects in male rats (although no clear dose–response was observed), but not in male mice at considerably higher doses. In addition, gum arabic did not cause heritable chromosomal effects in male mice. Overall, the Committee concluded that gum arabic is not genotoxic, but it did not extend this conclusion to OSA modified gum arabic.

4.2 Assessment of dietary exposure

The Committee received one dietary exposure analysis for OSA modified gum arabic. Additionally, the Committee evaluated information submitted to the USFDA as part of a GRAS Notice for the use of OSA modified gum arabic.

OSA modified gum arabic is intended to replace gum arabic in a number of food applications. It is proposed for use as an emulsifier for flavouring agents in baked goods, beverages (non-alcoholic and alcoholic), breakfast cereals, processed cheese, chewing gum, flour confectionery and icings, egg products, fish products, frozen dairy, fruit ices, gelatines and puddings, gravies, imitation dairy products, instant coffee and tea, jams and jellies, meat products, milk products, other grains, processed poultry, processed fruit juices, processed vegetable juices, snack foods, soft candy, soups and sweet sauces at levels up to 500 mg/kg of the food. OSA modified gum arabic is also proposed for use as an emulsifier in some fruit-flavoured drinks, fruit juices and some other beverages (carbonated juice and energy drinks), salad dressing, sauces, icing, some breads (whole-grain and high-fibre breads) and some cereals (high-fibre, low-sugar and low-fat adult cereals) at levels up to 10 000 mg/kg of the food.

The dietary exposure analysis received by the Committee used individual dietary records from both the United Kingdom and the USA. The food consumption data from the United Kingdom were taken from the 2000–2001 National Diet and Nutrition Survey. The food consumption data from the USA were taken from the

2003–2004 National Health and Nutrition Examination Survey. The mean dietary exposure estimated using the data from the United Kingdom was 326 mg/person per day (4.3 mg/kg bw per day), with an estimated 97.5th-percentile dietary exposure of 859 mg/person per day (12 mg/kg bw per day). In the parallel analysis using the food consumption data from the USA, the mean dietary exposure was 524 mg/person per day (9 mg/kg bw per day), and the 90th-percentile exposure was 964 mg/person per day (17 mg/kg bw per day).

The Committee concluded that the estimated dietary exposure to OSA modified gum arabic from the proposed uses would be less than 20 mg/kg bw per day.

5. EVALUATION

Only limited data were available for OSA modified gum arabic. The Committee concluded that the available data on OSA modified gum arabic indicate a very low toxicity, comparable with the toxicities of traditional gum arabic and starch sodium octenyl succinate (OSA modified food starch), both of which were previously reviewed by the Committee and allocated ADIs "not specified".

Comparing the exposure estimate of 20 mg/kg bw per day with the NOEL from the 90-day study of oral toxicity in rats (3410 mg/kg bw per day, the highest dose tested), the margin of exposure is at least 170. The Committee decided to allocate a temporary ADI "not specified" to OSA modified gum arabic, used in the applications specified and in accordance with Good Manufacturing Practice. The Committee decided to make the ADI temporary pending submission of data by the end of 2011 showing hydrolysis of OSA modified gum arabic to confirm the validity of using gum arabic data in the evaluation of OSA modified gum arabic.

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SODIUM HYDROGEN SULFATE

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1. EXPLANATION

At the present meeting, the Committee evaluated sodium hydrogen sulfate for use as an acidifier, at the request of the Codex Committee on Food Additives at its fortieth session (FAO/WHO, 2008). The Committee was asked for a safety assessment and revision of specifications. At its sixty-eighth meeting, the Committee considered sodium hydrogen sulfate for use in the preparation of acidified sodium chlorite, an antimicrobial washing solution, and established specifications, but did not evaluate it for safety (Annex 1, reference *187*). At its ninth and twenty-third meetings, the Committee evaluated a large number of food acids and salts and was of the opinion that acceptable daily intakes (ADIs) for ionizable salts should be based on previously accepted recommendations for the constituent cations and anions (Annex 1, references *11* and *50*).

The sulfate ion was evaluated at the twenty-ninth meeting of the Committee (Annex 1, reference *70*), when an ADI "not specified" was established, as sulfate is a natural constituent of food and is a product of sulfur metabolism in animals. Sodium sulfate was evaluated at the fifty-third, fifty-fifth and fifty-seventh meetings

(Annex 1, references *144*, *149* and *154*), when an ADI "not specified" was established.

The Committee decided to assess sodium hydrogen sulfate in terms of the sulfate component because of its dissociation to the constituent ions and given that sodium and hydrogen ions are ubiquitous and natural constituents of foods.

1.1 Chemical and technical considerations

Sodium hydrogen sulfate is manufactured by mixing sodium chloride with sulfuric acid at elevated temperatures to form molten sodium hydrogen sulfate. The molten sodium hydrogen sulfate is sprayed and cooled to form a solid product with uniform particle size.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

Renal clearance data for the sulfate anion were included in the Committee's evaluation of sodium sulfate at its fifty-third meeting (Annex 1, reference *144*). No additional information was located.

2.2 Toxicological studies

2.2.1 Studies on sodium hydrogen sulfate

Groups of male and female Sprague-Dawley rats were gavaged with sodium hydrogen sulfate at a single oral dose of 1750, 2000, 2250, 2500, 3000 or 3500 mg/kg body weight (bw) to determine its acute oral toxicity. Fifty-five animals were treated in total. Control rats were similarly dosed with deionized water. Surviving animals were killed after 14 days. The oral median lethal dose (LD_{50}) was determined to be 2800 mg/kg bw in males and >2500 mg/kg bw in females. Fewer females than males died. As the test progressed, it was decided to stop dosing the females, as it was clear that the LD_{50} was above 2500 mg/kg bw. Effects observed during the study included weight loss, dehydration, scruffy coats, lethargy and death. Gross abnormalities observed in the animals that died during the study included mottled red lungs, pale mottled livers and stomach lesions or ruptures (Northview Pacific Laboratories, 1990).

2.2.2 Studies on sulfate

Artificially reared neonatal piglets were used as a model to evaluate the effect of inorganic sulfate on bowel function in human infants. Two experiments were conducted. The first evaluated the effect of high levels of sulfate on growth, feed intake and consistency of faeces, and the second determined the dose at which at least 50% of the pigs developed non-pathogenic diarrhoea. Following a 5-day acclimatization period, 40 piglets were distributed into four groups for each experiment. Piglets were fed liquid diets only via an Autosow and did not have access to drinking-water. Inorganic sulfate was added to the diets as anhydrous

sodium sulfate at levels of 0, 1200, 1600 and 2000 mg/l for experiment 1 (18-day study) and 0, 1800, 2000 and 2200 mg/l for experiment 2 (16-day study). Piglets were individually caged and weighed daily, and the volume of diet for each piglet was adjusted according to its body weight. Feed intake and consistency of faeces were recorded 3 times daily. Rectal swabs were taken from those piglets with soft or liquid stools and analysed for haemolytic *Escherichia coli* and rotavirus. At the end of each experiment, piglets were sedated and killed. Urine samples were taken, and the kidneys were removed. The levels of added sulfate did not affect the growth of the piglets or their feed intake. Levels of 2000 and 2200 mg sulfate/l resulted in practically all (90–100%) piglets having diarrhoea, beginning 2 days after the start of the trial and persisting throughout the experimental period. Rectal swabs were negative, from which the authors concluded that the piglets had non-pathogenic diarrhoea. Kidney weight was not affected by added sulfate. Sulfate concentrations in the urine reached a maximum in the piglets fed diets with 1600 and 1800 mg sulfate/l in experiments 1 and 2, respectively ($P < 0.05$), but declined at higher levels. Based on the results, the authors concluded that the concentration of added sulfate at which 50% of piglets develop non-pathogenic diarrhoea is between 1600 and 1800 mg/l (Gomez et al., 1995).

2.3 Observations in humans

In 1999, the United States Environmental Protection Agency and Centers for Disease Control and Prevention conducted a study on the health effects from exposure to high levels of sulfate in the drinking-water in two sensitive populations (infants and transient adults). For the infant study, the authors intended to conduct a prospective cohort study of newborn infants whose mothers planned to feed their infants formula mixed with tap water. However, a pilot study involving a self-administered questionnaire to all women attending 32 clinics to determine how many women planned to use tap water to mix infant formula for their babies revealed that very few infants were exposed to tap water containing high levels of sulfate.

One hundred and five adult volunteers were randomly assigned to one of five sulfate groups: 0 mg/l ($n = 24$), 250 mg/l ($n = 10$), 500 mg/l ($n = 10$), 800 mg/l ($n = 33$) or 1200 mg/l ($n = 28$). Bottled water was provided for the volunteers for 6 days. The bottled water for days 1, 2 and 6 was unsupplemented, whereas the bottles for days 3–5 contained water with added sulfate. Bottles were returned to estimate how much water was consumed each day. Volunteers recorded the number of bowel movements each day. There were no statistically significant differences in the bowel movements among the groups on days 3–6, nor were there any statistically significant differences in the bowel movements when comparing days 1 and 2 with days 3–5, within each dose group. The authors concluded that there was no statistically significant increase in reports of diarrhoea with increasing dose of sulfate in the drinking-water (United States Environmental Protection Agency, 1999).

3. DIETARY EXPOSURE

Sodium hydrogen sulfate is an acid and can be added to foods to lower pH, to improve shelf life and/or improve flavour. Typically, sodium hydrogen sulfate may be added to beverages, confectionery, fillings, syrups, salad dressings and sauces. It is stronger than organic acids such as citric acid, so lower amounts are required to reach the same pH. Because it does not impart a sour or citric taste, as do other acidifiers, it can be used in products where these are not desirable—for example, in non-citrus-flavoured soft drinks, tea, chocolate-flavoured drinks and coffee-flavoured drinks (personal communication from C. Kneuven, Jones-Hamilton Co., to WHO, 2008).

Typical use levels for a variety of food categories and poundage data were given by the food industry (personal communication from C. Kneuven, Jones-Hamilton Co., to WHO, 2008). Although most uses were at 2000 mg/kg or less, the highest use level reported was 4000 mg/kg for processed cheeses, soup and soup mixes (Table 1).

Table 1. Typical use levels for sodium hydrogen sulfate

	Typical use level	
Food category	mg/kg	%
Beverages	600	0.06
Confectionery, fillings and syrups	1000	0.1
Processed cheeses	4000	0.4
Dressings and sauces	2000	0.2
Jams and jellies	800	0.08
Processed vegetables and vegetable juices	3000	0.3
Soups and soup mixes	4000	0.4
Salsa	500	0.05

3.1 Screening by the budget method

As no ADI has been allocated to sodium hydrogen sulfate and as sodium sulfate has an ADI “not specified”, it was not possible to undertake a budget method calculation.

3.2 Poundage data

The annual poundage of sodium hydrogen sulfate sold into the North American and European markets was reported in the food industry submission to be approximately 2000 tonnes, with 1900 tonnes being used in North America and 100 tonnes in Europe (personal communication from C. Kneuven, Jones-Hamilton Co., to WHO, 2008). It was noted that production volumes could potentially increase to a total of 5000 tonnes in the future.

Per capita dietary exposures can be calculated by applying a correction factor of 0.8 for under-reporting of the amount of additive produced. In this case, it was assumed that all consumers may be exposed to the additive, as non-alcoholic beverages, confectionery, sauces, soups and cheese products are widely consumed. Per capita dietary exposure to sodium hydrogen sulfate for the USA was estimated to be between 22 and 54 mg/day and for Europe between 0.4 and 1 mg/day (USA population 30×10^7 in 2006, European population 80×10^7 in 2008), for current and projected production volumes, respectively, assuming the same proportion of use between the USA and Europe.

3.3 Dietary exposure based on national nutrition surveys

Potential dietary exposures to sodium hydrogen sulfate were estimated for several European countries using information on diets from the European Food Safety Authority's Concise European Food Consumption Database (European Food Safety Authority, 2009), derived from national nutrition surveys. Potential mean dietary exposures and high-consumer exposures to sodium hydrogen sulfate were calculated for the whole adult population aged 16–64 years for the 19 countries in the database, assuming that sodium hydrogen sulfate was used at typical use levels in broad food categories where use is proposed. High-consumer dietary exposures were estimated by taking consumption for two food categories with the highest dietary exposure at the 95th percentile plus mean exposures for the whole population for all other food categories (European Food Safety Authority, 2008). Potential mean dietary exposures for the whole adult population for each country ranged from 400 to 1160 mg/day; for high consumers of sodium hydrogen sulfate, potential dietary exposures ranged from 1090 to 6340 mg/day (Table 2). Major contributors to total dietary exposure were fruit and vegetable juices, vegetable soups, non-alcoholic beverages with a low percentage of fruit, tea and coffee, and cheese.

Table 2. Potential dietary exposure to sodium hydrogen sulfate for adults in 19 European countries (based on food consumption data sourced from the Concise European Food Consumption Database)[a]

Country	Survey	Model	Dietary exposure (mg/day)
Austria	2005–2006 Austrian Study on Nutritional Status 19–64 years; 24-h recall (2123 respondents)	Mean all	1010
		High consumer	5530
Belgium	2004 Belgian Food Consumption Survey 16–64 years, 24-h recall over 2 days (1723 respondents)	Mean all	1130
		High consumer	4190

Table 2. (contd)

Country	Survey	Model	Dietary exposure (mg/day)
Bulgaria	2004 National Survey of Food Intake and Nutritional Status 16–64 years, 24-h recall (853 respondents)	Mean all	460
		High consumer	2910
Czech Republic	2003–2004 Individual Food Consumption Study 16–64 years, 24-h recall (1751 respondents)	Mean all	690
		High consumer	2600
Denmark	200–2002 Danish National Dietary Survey (DK2002) 4–65 years, diary over 7 days (4439 respondents)	Mean all	1000
		High consumer	2550
Estonia	1997 Estonian Adult Nutrition Survey 16–64 years, 24-h recall (2018 respondents)	Mean all	420
		High consumer	1090
Finland	2002 National Findiet Study 25–64 years, 24-h recall over 2 days (2007 respondents)	Mean all	520
		High consumer	1280
France	1999 Enquête Individuelle et Nationale sur les Consommations Alimentaires (INCA) 15+ years over 7 days (1474 respondents)	Mean all	820
		High consumer	2660
Germany	1998 German Nutrition Survey 18+ years, diet history over 28 days (4030 respondents)	Mean all	1160
		High consumer	3140
Hungary	2003–2004 Hungarian National Dietary Survey 18+ years, dietary record over 3 days (1179 respondents)	Mean all	440
		High consumer	1600
Iceland	2002 The Diet of Icelanders 15–80 years, 24-h recall (1075 respondents)	Mean all	990
		High consumer	4990

Table 2. (contd)

Country	Survey	Model	Dietary exposure (mg/day)
Ireland	1997–1998 North/South Ireland Food Consumption Survey (NSIFCS) 15–80 years, dietary record over 7 days (1369 respondents)	Mean all	760
		High consumer	1630
Italy	1994–1996 Nationwide Nutritional Survey of Food Behaviour (INN-CA) 16–64 years, dietary record over 7 days (1544 respondents)	Mean all	400
		High consumer	1090
The Netherlands	1997–1998 Dutch National Food Consumption Survey All ages, dietary record over 2 days (6250 respondents)	Mean all	1080
		High consumer	3040
Norway	1993–1997 Norwegian National Dietary Survey 16+ years, food frequency survey (2352 respondents)	Mean all	1030
		High consumer	2240
Poland	2000 Household Food Consumption and Anthropometric Survey (HFCAAS) 1–96 years, 24-h recall (4134 respondents)	Mean all	770
		High consumer	3560
Slovakia	2006 Monitoring of Nutritional Status of Adult Population 19–54 years, 24-h recall (2208 respondents)	Mean all	1160
		High consumer	6340
Sweden	1997–1998 Dietary Habits and Nutrient Intake in Sweden 17+ years, dietary record over 7 days (1210 respondents)	Mean all	860
		High consumer	2240

Table 2. (contd)

Country	Survey	Model	Dietary exposure (mg/day)
United Kingdom	2000–2001 National Diet and Nutrition Survey (NDNS) 19–64 years over 7 days (1724 respondents)	Mean all	930
		High consumer	2220

[a] Assumptions for all countries:

1. Summary statistics for 16- to 64-year age group only, where data available.
2. Typical use levels for food categories applied to broad food group: "Sugar & sugar products including chocolate" at 1000 mg/kg, "Vegetable soups" at 4000 mg/kg, "Fruit & vegetable juice" at 3000 mg/kg, "Soft drinks" at 600 mg/kg, "Coffee, tea, cocoa" at 600 mg/kg, "Cheese" at 4000 mg/kg, "Miscellaneous foods including foods for special dietary uses" at 2000 mg/kg.
3. High-consumer estimate derived from consumption for two food groups with highest dietary exposure at the 95th percentile plus mean for population for all other food groups.

It should be noted that basing potential dietary exposures on the amounts of food consumed for 15 broad food categories given in the European diets will overestimate the dietary exposure to sodium hydrogen sulfate. The actual use of sodium hydrogen sulfate would be restricted to subgroup categories within the broader food group and to foods within these subgroups where a low pH is required and an acidic or citric taste is undesirable. For example, in these estimates, the typical use level for processed cheese was assigned to all cheeses, thus overestimating the potential contribution from cheese; the typical use level for beverages was assigned to all tea and coffee, thus overestimating the potential contribution from these beverages, as in reality the food additive would be used only in some flavoured teas and coffees. In addition, for the European Food Safety Authority food group 2, "sugar and sugar-containing products", the higher of two concentrations given for products within this category was used (2000 mg/kg for confectionery, fillings and syrups), which would overestimate the contribution from the "jams and jellies" subcategory, where typical use was reported at 800 mg/kg.

A more accurate dietary exposure estimate for the Australian population was also determined, based on individual dietary records and typical levels of use for specific food groups within broader food categories, as notified in the food industry submission (Table 3). For example, the typical use level for beverages was assigned to flavoured teas, soft drinks, fruit and vegetable juices, fruit drinks and dry beverage preparations, rather than to all non-alcoholic beverages. Potential mean dietary exposures for the whole population were 700 mg/day. For consumers of foods containing sodium hydrogen sulfate only, potential mean dietary exposures of 740 mg/day were similar to those for the whole population, as the additive can be used in a wide range of foods; dietary exposures for high consumers at the 90th percentile were 1210 mg/day. The major contributors to total potential dietary exposure were soups and soup mixes (45%), water-based flavoured drinks (22%) and fruit and vegetable preparations (15%).

Table 3. Potential dietary exposure to sodium hydrogen sulfate for the Australian population (based on individual dietary records)

Survey	Assumptions	Model	Dietary exposure	
			mg/day	mg/kg bw per day[a]
1995 National Nutrition Survey aged 2+ years 24-h recall (13 858 respondents, of whom 94% were consumers)	Typical levels of use for all listed food categories (Table 1)	Mean all	700	11.8
		Mean consumers	740	12.6
		90th-percentile consumers	1210	21.9

[a] Individual body weights were used in the calculations (mean body weight for the Australian population was 67 kg).

4. COMMENTS

4.1 Toxicological data

When sodium hydrogen sulfate is added to food products containing water or after ingestion of sodium hydrogen sulfate, it ionizes to sodium ions, hydrogen ions and sulfate ions. The Committee received a submission containing unpublished studies on sodium hydrogen sulfate, including a study on its acute toxicity and studies on inhalation toxicity, skin irritation and corrosivity, and freshwater ecotoxicity. A literature search identified no published studies of the toxicity of sodium hydrogen sulfate. Additional information identified by a literature search related to sulfate, as the Committee decided to assess sodium hydrogen sulfate in terms of the sulfate component because of its dissociation to the constituent ions and given that sodium and hydrogen ions are ubiquitous and natural constituents of foods.

In an acute toxicity study, the oral LD_{50} of sodium hydrogen sulfate in rats was determined to be 2800 mg/kg bw in males and >2500 mg/kg bw in females. The additional studies received as part of the submission were not considered relevant to the evaluation of the oral toxicity of sodium hydrogen sulfate.

In studies evaluating the effect of inorganic sulfate on bowel function, the body weight and kidney weight of neonatal pigs administered up to 2000 mg/l in a liquid diet for 18 days were unaffected. In a 16-day study, the concentration of added sulfate in the diet at which 50% of the piglets developed non-pathogenic diarrhoea was estimated to be between 1600 and 1800 mg/l. No differences in bowel movements were noted in adult volunteers receiving sulfate in the drinking-water at concentrations up to 1200 mg/l for 3 consecutive days.

The additional studies identified on sulfate did not raise concern about its toxicity.

4.2 Assessment of dietary exposure

Sodium hydrogen sulfate is typically added to beverages, confectionery, fillings, syrups, processed cheeses, salad dressings, sauces, jams and jellies, and processed vegetable products at levels ranging from 500 to 4000 mg/kg. For beverages, sodium hydrogen sulfate is generally used in non-citrus-flavoured soft drinks, tea, and chocolate-flavoured and coffee-flavoured drinks, as it does not impart a sour or citric taste, as do other acidifiers.

Based on poundage data for the USA, where the food additive has the highest reported production levels, mean per capita exposures for the population in the USA for current production volumes and for increased production volumes in the future, as predicted by the sponsor, were estimated to be 20 and 50 mg/day, respectively, assuming that all members of the population were consumers of products containing the additive.

From the limited data submitted by the sponsor on the proposed use of sodium hydrogen sulfate as a food acid, potential mean and high-consumer dietary exposures (derived from consumption for two food groups with highest dietary exposure at the 95th percentile plus mean for population for all other food groups) for 19 European populations (aged 16–64 years) were calculated based on typical use levels, assuming that the additive was used in all foods in each of the broad food categories identified above. Potential mean per capita dietary exposures for this "worst case" scenario ranged from 400 to 1160 mg/day for the whole population and from 1090 to 6340 mg/day for high consumers of foods containing sodium hydrogen sulfate. Potential dietary exposures based on individual dietary records and use of sodium hydrogen sulfate in food subcategories specified by the sponsor were submitted for the Australian population. Potential mean dietary exposures for Australians were lower than those for Europeans but of the same order of magnitude (mean per capita dietary exposure of 700 mg/day for the whole Australian population and 1210 mg/day for high consumers at the 90th percentile). The Committee considered that the predicted dietary exposures for the European and Australian populations were overestimates, a view supported by the much lower per capita estimates reported for the population in the USA. The actual use of sodium hydrogen sulfate would be restricted to subcategories within the broader food group and to foods within these subcategories where a low pH was required and/or for drinks where an acidic or citric taste was undesirable.

5. EVALUATION

Considering that the available evidence did not provide any indication of toxicity, the Committee allocated an ADI "not specified" for sodium hydrogen sulfate, in line with the principles established for ionizable salts at its twenty-ninth meeting, when used in the applications specified and in accordance with Good Manufacturing Practice.

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SUCROSE OLIGOESTERS TYPE I AND TYPE II

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1. EXPLANATION

At the request of the Codex Committee on Food Additives at its thirty-ninth session (FAO/WHO, 2007), the Committee evaluated sucrose oligoesters (SOE), which are separated into two types, SOE type I and type II. SOE type I and type II are produced by interesterification of sucrose with methyl esters of fatty acids derived from edible fats and oils, including hydrogenated fats and oils such as stearic acid and palmitic acid. A sucrose molecule has eight hydroxyl groups, and so it can produce mono- to octa-esters (Table 1). “Sucrose esters of fatty acids” consist mainly of sucrose mono- to tri-esters, whereas SOE type I consists mainly of sucrose tetra- to octa-esters and SOE type II consists of sucrose mono- to octa-esters. The lipophilic character of these constituents increases according to the

increasing degree of esterification and the increasing chain length of the fatty acids. Other physical and chemical properties of the products also vary depending on the degree of esterification and the chain length of the fatty acids (Mitsubishi Chemical Corporation & Dai-ichi Kogyo Seiyaku Co. Ltd, 2008).

Table 1. Classification of sucrose fatty acid esters

		Composition of esters (%)			
Property	Group	Mono–tri	Tetra–octa	Hepta+octa	Octa
Hydrophilic	Sucrose esters of fatty acids	80–100	0–20	–	–
↓	Sucrose oligoesters type II	20–80	20–80	0–20	0–10
↓	Sucrose oligoesters type I	0–20	80–100	0–50	0–20
Lipophilic	Olestra[a]	–	–	97–100	70–100

[a] The monograph on olestra in the sixth edition of the Food Chemicals Codex (United States Pharmacopeia, 2008) specifies the following distribution for the number of esters: octa-esters, not less than 70%; hexa-, hepta- and octa-esters, not less than 97%; hexa-esters, not more than 1%; and penta-esters, not more than 0.5%. Olestra is used as a replacement for fats in food.

SOE type I and type II are lipophilic emulsifiers as well as stabilizers and tableting aids for foods presented in tablet form. They are authorized for use in a number of countries, including Japan, the USA, China and the Republic of Korea.

The Committee had previously evaluated low-range sucrose esters of fatty acids, which principally include mono-, di- and tri-esters, at its thirteenth, seventeenth, twentieth, twenty-fourth, thirty-fifth, thirty-ninth, forty-fourth and forty-ninth meetings (Annex 1, references *19*, *32*, *41*, *53*, *88*, *101*, *116* and *131*). At the forty-ninth meeting, the Committee established a group acceptable daily intake (ADI) of 0–30 mg/kg body weight (bw) for sucrose esters of fatty acids and sucroglycerides on the basis of potential laxative effects in humans.

For the present evaluation of SOE type I and type II, the Committee considered the available data on the safety of SOE type I and type II in view of the Committee's previous evaluation of low-range sucrose esters of fatty acids.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Noker et al. (1997) studied the absorption, distribution and excretion of three sucrose esters of stearic acid. In this study, male Wistar rats that were acclimated to the gavage process were dosed by oral gavage with ^{14}C-labelled compounds at 250 mg/kg bw. The compounds, which were labelled in the stearate moiety or uniformly (U) in the sucrose moiety, were sucrose 1-[^{14}C]tetrastearate, sucrose 1-[^{14}C]hexastearate, sucrose 1-[^{14}C]octastearate, U-[^{14}C]sucrose tetrastearate, U-[^{14}C]sucrose hexastearate and U-[^{14}C]sucrose octastearate. The study was divided into three parts: 1) absorption and distribution experiments with blood and tissue collections; 2) an excretion experiment with collection of urine, faeces and carbon dioxide; and 3) a lymph collection experiment. Each experiment was performed with the stearate-labelled compounds, then repeated with the sucrose-labelled compounds.

In the absorption and distribution experiments, blood was collected from rats (n = 3) 4 and 120 h after dosing with labelled compounds, and the rats were sacrificed. At sacrifice, tissue samples were obtained from each rat. Blood was obtained from other groups of rats at selected times between 1 and 96 h, with a terminal sample taken at 120 h after dosing. The radioactivity in the blood was highest for rats dosed with sucrose-1-[^{14}C]tetrastearate; the amount detected was less than 10 µg equivalents/ml. The authors concluded that the tetra-ester was hydrolysed to absorbable radioactivity, presumably stearic acid, more readily than were the hexa- and octa-esters.

At 4 h after dosing with sucrose 1-[^{14}C]tetrastearate, most of the radioactivity was present in the stomach and upper segments of the small intestine. Outside the gastrointestinal tract, the highest level of radioactivity was in the liver (10 times higher than in the blood). Other tissues with relatively high levels of radioactivity were the heart, spleen, bone marrow and lymph nodes. At 120 h after dosing with the same compound, relatively little radioactivity remained in the gastrointestinal tract, and more radioactivity appeared in the tissues. After dosing with U-[^{14}C]-sucrose tetrastearate, lower levels of radioactivity were detected in the tissues. As with the stearate-labelled compound, the radioactivity was concentrated in the gastrointestinal tract at 4 h and increased in tissues outside the gastrointestinal tract at 120 h. Notably, however, the patterns of tissue distribution of radioactivity following administration of the sucrose-labelled compound were different from the patterns observed in rats dosed with the stearate-labelled compound. The authors concluded that the accumulation of radioactivity in the tissues over time may have been due to the incorporation of stearic acid, or its products, into cellular components.

In the excretion experiment, animals (n = 3 per compound) were fasted for 24 h, then dosed. They were maintained in metabolism cages for 120 h, during

which expired air, urine and faeces were collected. For each compound, the rats excreted between 96% and 115% of the dose in the faeces (excretion greater than 100% of the administered dose was attributed to evaporation of the solvent during homogenization of the faeces by the study authors). Analyses of the faeces of animals dosed with the tetrastearate showed that the excreted esters were 80% and 88% unchanged parent compound for the stearate-labelled and the sucrose-labelled compounds, respectively; most of the remaining radioactivity consisted of more polar products. Analyses of the faeces of animals dosed with hexastearate or octastearate showed that more than 95% and 98% of the extracted radioactivity was from the parent compounds for the stearate-labelled and sucrose-labelled compounds, respectively. For rats dosed with sucrose esters labelled in the fatty acid moiety, the percentages of the dose absorbed (calculated as the sum of radioactivity in expired carbon dioxide, urine and carcass) for the tetra-, hexa- and octa-esters were 5.9%, 2.5% and 2.4%, respectively. For rats dosed with sucrose esters labelled in the sucrose moiety, the percentages of the dose absorbed for the tetra-, hexa- and octa-esters were 3.0%, 0.8% and 0.8%, respectively. In each case, the amount of absorbed radioactivity was greatest for the tetra-ester. The authors concluded that only the tetra-ester was metabolized to any appreciable extent.

In the lymph collection experiment, rats with thoracic duct cannulae were dosed following a 24-h fast and placed in metabolism cages. Lymph was collected continuously for up to 120 h. No intact tetra-, hexa- or octa-esters were detected in lymph samples from rats given stearate-labelled compounds.

Olestra, which is a mixture of primarily hepta- and octa-esters, has few free hydroxyl groups on its sucrose moiety. It is essentially unabsorbed following oral administration (United States Food and Drug Administration, 1996).

2.1.2 Biotransformation

Noker et al. (1997) considered the metabolism of radiolabelled tetra-, hexa- and octa-esters of stearic acid in male Wistar rats. They attributed differences in the apparent degree of metabolism and absorption of the tetra-, hexa- and octa-esters to the rate at which these compounds are hydrolysed in the gastrointestinal tract. These data indicated that, relative to the hexa- and octa-esters, the tetra-ester is more readily hydrolysed. Jandacek & Holcombe (1991) also found that as the degree of esterification increases, sucrose esters of fatty acids are less easily hydrolysed, with essentially no hydrolysis occurring for the octa-ester. Other investigators have shown that sucrose esters are hydrolysed in the intestinal tract prior to absorption (Shigeoka et al., 1984).

The extent of absorption and subsequent disposition of radioactivity in various tissues, including lymph and liver, were detectably different when sucrose-labelled or stearate-labelled compounds were given to rats. This could occur only if the compounds were metabolized (hydrolysed) during or prior to absorption, yielding two different radioactive moieties to be absorbed (i.e. labelled sucrose and labelled stearate). The change in the pattern of distribution of radioactivity over time is consistent with a slow rate of metabolism (hydrolysis) (Noker et al., 1997).

Noker et al. (1997) noted that their data are consistent with other findings (Bloom et al., 1951) that water-insoluble fatty acids are preferentially transported to the lymph duct after absorption and subsequently distributed throughout the body. In contrast, sucrose is metabolized in the intestinal mucosa to glucose and fructose, which are transported by the portal vein to the liver, where they are rapidly metabolized.

Olestra is highly lipophilic and sequesters fat-soluble vitamins in the gastrointestinal tract. Comprehensive discussions concerning olestra can be found in a Federal Register document published in the USA that permits the use of olestra as a fat replacement in foods (United States Food and Drug Administration, 1996).

2.2 Toxicological studies

2.2.1 Acute toxicity

No data are available.

2.2.2 Short-term studies of toxicity

No toxicity studies are available for SOE type II emulsifiers.

A 28-day dietary range-finding study is available for SOE type I emulsifiers (Yoshida et al., 2004). In this study, groups of F344 rats (five animals of each sex per group) were fed diets containing Ryoto-Sugar Ester S-170, which is composed of approximately 75% tetra-, penta-, hexa- and hepta-esters, 14% mono-, di- and tri-esters and 11% octa-ester, at a dietary level of 0%, 1.25%, 2.5%, 5% or 10% (equivalent to 0, 625, 1250, 2500 and 5000 mg/kg bw per day). No toxicity was reported in this study. The no-observed-effect level (NOEL) was 5000 mg/kg bw per day, the highest dose tested (Yoshida et al., 2004; Mitsubishi Chemical Corporation & Dai-ichi Kogyo Seiyaku Co. Ltd, 2008).

In the subsequent 12-month study of toxicity, male and female F344 rats (10 animals of each sex per group) were fed diets containing Ryoto-Sugar Ester S-170 at a dietary level of 0%, 1.25%, 2.5% or 5.0% (calculated to provide doses of 0, 580, 1140 and 2370 mg/kg bw per day in males and 0, 630, 1330 and 2800 mg/kg bw per day in females). Food and water were available ad libitum. Body weight and food consumption were measured weekly up to 13 weeks and monthly thereafter. Clinical conditions (not defined) were checked daily throughout the study. After the 12-month treatment, urine and blood were collected from all of the surviving animals for urinalysis and haematological analysis. Necropsies were performed, and major organs and tissues were weighed and examined histopathologically. Body weight and food consumption were not affected by treatment. There were no differences in survival between the control and treated groups. Urinalysis and haematological analyses did not reveal any treatment-related effects. Elevations in alkaline phosphatase in females and platelet count in males (except for the low-dose group) were reported, but these results were not dose related. The organ weights of the rats did not exhibit any dose-related changes for either sex, with the exception of relative liver weights, which were marginally, but statistically significantly, decreased only in males in the high-dose group. There were no

macroscopic or microscopic correlates in the liver accompanying these findings. The apparent decrease in relative liver weight in the high-dose males was considered to be sporadic and unrelated to treatment. Macroscopic and microscopic examinations did not reveal any differences between control and treated animals. The NOEL was 2370 mg/kg bw per day, the highest dose tested (Yoshida et al., 2004; Mitsubishi Chemical Corporation & Dai-ichi Kogyo Seiyaku Co. Ltd, 2008).

2.2.3 Long-term studies of toxicity and carcinogenicity

In a 2-year carcinogenicity study, male and female F344 rats (50 animals of each sex per group) were fed diets containing Ryoto-Sugar Ester S-170 at a dietary level of 0%, 2.5% or 5.0% (calculated to provide doses of 0, 1030 and 2120 mg/kg bw per day in males and 0, 1160 and 2420 mg/kg bw per day in females) (Yoshida et al., 2004). After the 2-year treatment period, all surviving animals were euthanized, and all major organs and tissues were macroscopically and microscopically examined. Animals found moribund or dead were examined pathologically. Tumours were observed in all groups of animals, including the controls. In the male rats, testicular tumours were the most common tumours, followed by lesions of the thyroid, pituitary, haematopoietic organs, subcutaneous region and adrenals. In the female rats, the most common tumours were those of the pituitary, thyroid, uterus, haematopoietic organs and mammary glands. Histologically, all the tumours observed in the 2-year study are known to occur spontaneously in F344 rats (Haseman et al., 1998). There were no significant increases in tumour incidences, nor were there positive trends in the incidences of any specific tumours in treated rats compared with controls. The authors concluded that Ryoto-Sugar Ester S-170 produced no carcinogenic activity in F344 rats in this study (Yoshida et al., 2004). The NOEL from the 2-year carcinogenicity study was 2120 mg/kg bw per day, the highest dose tested.

2.2.4 Genotoxicity

A reverse mutation assay of Ryoto-Sugar Ester ER-290, which belongs to SOE type II, was conducted in compliance with United Kingdom Good Laboratory Practice standards. *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2uvrA$^-$ were treated with the test material using the Ames plate incorporation method at five dose levels in triplicate, both with and without the addition of a rat liver homogenate metabolizing system (10% liver S9 in standard co-factors). The dose ranged from 50 to 5000 µg/plate. The experiment was repeated on a separate day using fresh cultures of the bacterial strains and fresh test material formulations. The vehicle and positive control plates gave the expected results. The test material caused no visible reductions in the growth of the bacterial background lawns up to the maximum recommended dose level of 5000 µg/plate. No significant increases in the frequency of revertant colonies were recorded for any of the bacterial strains at any dose of the test material, either with or without metabolic activation. Ryoto-Sugar Ester ER-290 was considered to be non-mutagenic under the conditions of this test (Harlan Laboratories, 2009).

No genotoxicity data were available for SOE type I.

2.2.5 Reproductive toxicity

No studies of reproductive or developmental toxicity were available for SOE type I or type II.

In a two-generation reproductive and developmental toxicity study, sucrose polyester (a mixture of 8.2% hexa-, 33% hepta- and 58% octa-esters of edible-grade fatty acids with sucrose) was fed to Sprague-Dawley (Charles River) rats at up to 10% of their diet. The levels of vitamins A and E were controlled in this study, which had a reproductive and teratogenic phase in each generation. There were no significant effects on body weight or growth, although feed consumption increased in response to increasing levels of sucrose polyester (presumably causing caloric dilution of the diets). No deleterious effects were reported on mating, conception, embryonic development, fetal or postnatal viability, or postnatal growth. Histological studies of both generations revealed no treatment-related effect in any of the groups (data not presented). As no adverse effects on reproductive or developmental parameters were reported, the authors concluded that sucrose polyester would not present either a reproductive or a teratogenic hazard to humans (Nolen et al., 1987).

At its thirteenth meeting, the Committee reviewed the available data on reproductive toxicity, and no treatment-related effects were observed for low-range sucrose esters of fatty acids.

2.3 Observations in humans

No data are available for SOE type I or type II.

At its forty-ninth meeting, the Committee established a group ADI of 0–30 mg/kg bw for sucrose esters of fatty acids and sucroglycerides on the basis of potential to induce laxative effects at doses exceeding 30 mg/kg bw per day in humans.

The laxation and other gastrointestinal effects of olestra were thoroughly explored in children and adults prior to its clearance by the United States Food and Drug Administration. The NOEL for the stool-softening effect of olestra is close to 8 g/day (United States Food and Drug Administration, 1996).

3. DIETARY EXPOSURE

SOE type I are highly esterified and lipophilic, whereas SOE type II are less lipophilic and closer to the hydrophilic sucrose esters of fatty acids (see Table 1). However, the functional uses of SOE type I and type II are similar, and there is common use across some food categories, according to the food industry submission. Therefore, the dietary exposure was estimated for SOE type I and type II combined.

Typically, SOE type I and type II are used as emulsifiers in fats, oils and fat emulsions, chocolate, cream, seasonings, condiments and some tablet forms of hard sweets and dietary supplements. There are several other additives available

that perform the same function in foods, so SOE are expected to capture only a small proportion of the total market for emulsifiers (≤10%).

According to the food industry submission, poundage data and model diets are available for Japan and the USA.

Typical use levels for most food categories are 3000 mg/kg or less. The highest maximum use level reported was 20 000 mg/kg for dietary supplements and powdered seasonings (Table 2).

Table 2. Typical and maximum use levels for SOE

Food category		Codex numbering system	Typical use level		Maximum use level	
			mg/kg	%	mg/kg	%
Fats and oils, fat emulsions	Shortening	02.1.2	2 000	0.2	5 000	0.5
	Margarine, fat spread	02.2.1.2 (02.2.1.3)[a] 02.2.2	2 000	0.2	5 000	0.5
	Fats and oils used as raw materials in foods	02.1	3 000	0.3	5 000	0.5
Chocolate	Chocolate	05.1.4 05.1.5	3 000	0.3	5 000	0.5
Cream and similar	Creams for whipping, whipped cream	01.4.2 01.4.4 02.3	2 000	0.2	5 000	0.5
	Coffee whitener	01.3.2 02.3	2 000	0.2	5 000	0.5
	Ice cream	01.7	2 000	0.2	3 000	0.3
Tablet-form foods	Candy in pressed tablet form	05.2.1	10 000	1	20 000	2
	Dietary supplements in pressed tablet form	13.6	10 000	1	20 000	2
Seasonings and condiments	Solid-type sauce mixes	12.6.3	2 000	0.2	5 000	0.5
	Powdered seasonings	12.2.2	10 000	1	20 000	2

[a] As SOE would be used in margarine, 02.2.1.3 (Blends of margarine and butter) was included; however, it is noted that as this type of product does not exist in Japan, it is not included in their model diet.

3.1 Screening by the budget method

The budget method can be used to estimate the theoretical maximum level of SOE type I and type II in those foods and beverages that are likely to contain the food additive that would not result in the ADI being exceeded by the population (Hansen, 1979; FAO/WHO, 2001). Assuming an ADI of 0–30 mg/kg bw per day, that SOE are used in solid food only and that only 25% of the total amount of solid food in the food supply contains the food additive, the theoretical maximum level of SOE would be 4800 mg/kg food. As this theoretical maximum level for solid food was lower than the maximum reported use level of 20 000 mg/kg, data on dietary exposures were required, based on the typical and maximum use levels submitted by the industry.

3.2 Poundage data

Poundage data were submitted for Japan (4100 tonnes per year) and the USA (3.2 million tonnes per year). Estimates of per capita exposure ranged from 60 mg/day for the population in the USA (assuming that SOE captured 10% of the market for emulsifiers) to 110 mg/day for the Japanese population (includes SOE and sucrose esters of fatty acids). These data are summarized in Table 3.

Table 3. Per capita estimates of SOE type I and type II dietary exposure

			Estimated dietary exposure		
Country	Year	Assumptions	mg/day	mg/kg bw per day[a]	% of the ADI[b]
Japan	2006	4100 tonnes per year[c] 0.8 correction factor Population 12.8×10^7	110	1.8	6
USA	1987	3 195 000 tonnes per year[d] 0.6 correction factor Population 24.3×10^7 10% of all emulsifiers are SOE	60	1.0	3

[a] Assumes a 60-kg body weight for each population.
[b] ADI of 0–30 mg/kg bw.
[c] Includes SOE and sucrose esters of fatty acids.
[d] Reported as 71 million pounds per year for all emulsifiers (converted to tonnes assuming 0.45 kg per pound).

3.3 Dietary exposure based on national nutrition survey data

Dietary exposure estimates for SOE type I and type II were submitted by the industry for the populations of Japan and the USA based on typical and maximum use levels for SOE combined with mean food consumption amounts derived from

national nutrition surveys for the food categories listed in Table 2 (model diets). In some cases, where the amount of the food category containing SOE was not reported in the national survey, a reasonable mean food consumption amount was assumed, based on available literature. The data submitted are summarized in Table 4.

Table 4. Estimated dietary exposure to SOE

				Dietary exposure		
Country	Survey	Assumptions	Model	mg/day	mg/kg bw per day[a]	% of the ADI[b]
Japan	2005 survey on dietary exposure to food additives, Ministry of Health, Labor and Welfare	Use levels for all food categories listed in Table 2	Mean all, typical use level	115	1.9	6
		Per capita food consumption	Mean all, maximum use level	224	3.7	12
USA	1994–1996 Continuing Survey of Food Intakes by Individuals (CSFII)	Use levels for all food categories listed in Table 2	Mean all, typical use level	151	2.5	8
		Per capita food consumption	Mean all, maximum use level	274	4.6	15
USA	2005 United States Food and Drug Administration estimate, based on 1994–1996 CSFII	Maximum use level of 5000 mg/kg (0.5%) for all food categories permitted to add SOE in USA Individual dietary records	Mean all, maximum use level	45	0.8	3
			High consumer (90th percentile), maximum use level	98	1.6	5

[a] Assumes a 60-kg body weight for both populations.
[b] ADI of 0–30 mg/kg bw.

For Japan, the mean dietary exposure to SOE type I and type II for the whole population was estimated to be 115 and 224 mg/day (1.9 and 3.7 mg/kg bw per day) for typical and maximum use levels, respectively.

For the USA, the mean dietary exposure to SOE type I and type II for the whole population was estimated to be 151 and 274 mg/day (2.5 and 4.6 mg/kg bw per day) for typical and maximum use levels, respectively. An estimate of dietary exposure to SOE for the USA based on consumers only that was included in the industry submission is not presented here, as it was not considered valid to sum dietary exposure for consumers of each food category across the whole diet using

mean consumer food consumption amounts; "consumer only" estimates are best derived from analysis of data from individual dietary records.

Another estimation of dietary exposure to SOE type I and type II for the population and high consumers in the USA was evaluated, provided in a United States Food and Drug Administration (2005) ruling on SOE (see Table 4). The maximum use level was taken to be 5000 mg/kg across all food categories. As it was recognized that the additive SOE is a mixture, dietary exposure at the 90th percentile was also estimated for individual component esters of the mixture, using analysis of 50 different batches of the additive to obtain mean percentages of each component. These were reported as follows: 1.5 mg mono-ester/day, 4.2 mg di-ester/day, 10 mg tri-ester/day, 14 mg tetra-ester/day, 19 mg penta-ester/day, 23 mg hexa-ester/day, 20 mg hepta-ester/day and 9 mg octa-ester/day (United States Food and Drug Administration, 2005).

4. COMMENTS

4.1 Toxicological data

The Committee considered studies of the absorption, distribution, metabolism and elimination (ADME) of components of SOE type I—i.e. tetra-, hexa- and octa-esters of fatty acids, each separately radiolabelled in both the fatty acid and sucrose moieties. These studies indicated that the tetra-, hexa- and octa-esters of sucrose with stearic acid were very slowly hydrolysed prior to intestinal absorption of the hydrolysis products, but only the tetra-ester was hydrolysed and absorbed to any appreciable extent. These data were consistent with pharmacokinetic data on mono-, di- and tri-esters of sucrose with stearic and palmitic acids, which were reviewed at the forty-fourth meeting. Small amounts of mono-esters were absorbed intact, but the di- and tri-esters were not. Although specific ADME data for SOE type II were not available, their metabolic fate can be predicted by the demonstrated inverse relationship between hydrolysis and the degree of esterification.

Toxicological studies, including a 28-day range-finding study and a combined 12-month toxicity and 2-year carcinogenicity study, were available for SOE type I fed to rats at dietary concentrations of up to 50 000 mg/kg (equal to 2370 mg/kg bw per day in males and 2800 mg/kg bw per day in females in the toxicity study; equal to 2120 mg/kg bw per day in males and 2420 mg/kg bw per day in females in the carcinogenicity study). In these studies, no significant toxicological or tumorigenic effects were reported. The NOEL from the 12-month toxicity study was 2370 mg/kg bw per day, the highest dose tested. The NOEL from the 2-year carcinogenicity study was 2120 mg/kg bw per day, the highest dose tested.

No studies of reproductive or developmental toxicity were available for SOE type I or type II. However, in a two-generation reproductive and developmental toxicity study of sucrose polyester (a mixture of 8.2% hexa-, 33% hepta- and 58% octa-esters of edible-grade fatty acids with sucrose) fed to rats at up to 10% of their diet, no adverse effects on reproductive or developmental parameters were reported.

SOE type II tested negative in a reverse mutation assay in bacterial cells. No genotoxicity data were available for SOE type I.

4.2 Assessment of dietary exposure

SOE type I and type II are used as emulsifiers in fats and oils, chocolate, cream, seasonings and condiments, and some tablet forms of hard sweets and dietary supplements, with typical use levels ranging from 2000 to 10 000 mg/kg and a maximum use level of 20 000 mg/kg. There are several other additives available that perform the same function in foods, so SOE type I and type II are expected to capture only a small proportion of the total market for emulsifiers (≤10%). As the functional uses of SOE type I and type II are similar and there is common use across some food categories, the dietary exposure was estimated for SOE type I and type II combined.

Use of the budget method indicated that detailed dietary exposure estimates were required for SOE type I and type II, as the theoretical maximum permitted use level of 4800 mg/kg was less than that expected to be used in some food categories (maximum 20 000 mg/kg).

Per capita estimates of dietary exposure to SOE type I and type II from use as an emulsifier based on poundage data were 60 and 110 mg/day for the USA and Japan, respectively. The Committee noted that this was an overestimate for Japan owing to the inclusion of sucrose esters of fatty acids in the reported data. The estimate for the USA assumed 10% of all production of emulsifiers to be SOE type I and type II, which was supported by the industry submission that indicated that SOE type I and type II would capture no more than 10% of the emulsifier market in the USA.

From the limited data submitted by the sponsor on the dietary exposure to SOE type I and type II based on national nutrition survey food consumption data, estimated mean dietary exposures for two populations where a wide range of processed foods are available, Japan and the USA, ranged from 115 to 150 mg/day (1.9–2.5 mg/kg bw per day), assuming typical SOE use across different food categories. Estimated mean dietary exposures for Japan and the USA, assuming maximum SOE type I and type II use levels across different food categories, ranged from 220 to 270 mg/day (3.7–4.6 mg/kg bw per day). However, the Committee considered these dietary exposures predicted for Japan and the USA to be overestimates because of assumptions made in the calculations, as not all products in each category will contain SOE type I or type II, and consumers will not consistently select those foods containing SOE type I and type II over a lifetime.

Another estimation of dietary exposures based on individual dietary records for the population in the USA was evaluated, where mean dietary exposure to SOE type I and type II was 45 mg/day (0.8 mg/kg bw per day) and 90th-percentile exposure was 98 mg/day (1.6 mg/kg bw per day), assuming a maximum use level of 5000 mg/kg for all food categories included in the assessment. This mean dietary exposure estimate for the USA was of the same order of magnitude as the per capita estimates for the population in the USA based on poundage data.

5. EVALUATION

The available ADME data for the components of SOE type I and for low-range sucrose esters of fatty acids indicate that only the lower esters were hydrolysed to any appreciable extent. The Committee concluded that these data and the results of the newly available 12-month toxicity study and 2-year carcinogenicity study on SOE type I did not identify any effects of toxicological concern at the highest dose tested.

The Committee noted that some of the components of sucrose esters of fatty acids may be present in significant amounts in SOE type I and type II. The Committee also noted that the group ADI of 0–30 mg/kg bw allocated to sucrose esters of fatty acids and sucroglycerides was based on a potential laxative effect in humans. The Committee therefore considered that it was appropriate to include SOE type I and type II in a group ADI of 0–30 mg/kg bw for sucrose esters of fatty acids, sucroglycerides and SOE type I and type II. Estimated dietary exposures to SOE type I and type II combined for mean and high consumers, based on typical or maximum use levels, were well below the ADI of 0–30 mg/kg bw, with estimates ranging from 3% to 15% of the ADI.

The Committee emphasized that this evaluation is valid only for the material as specified.

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ANNEXES

ANNEX 1

REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

1. *General principles governing the use of food additives* (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. *Procedures for the testing of intentional food additives to establish their safety for use* (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. *Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)* (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. I. *Antimicrobial preservatives and antioxidants*, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. *Specifications for identity and purity of food additives (food colours)* (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. II. *Food colours*, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. *Evaluation of the carcinogenic hazards of food additives* (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. *Evaluation of the toxicity of a number of antimicrobials and antioxidants* (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. *Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents* (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. *Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants* (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).

10. *Specifications for identity and purity and toxicological evaluation of food colours*. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.
11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases* (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
12. *Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases.* FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.
13. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.
14. *Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non nutritive sweetening agents* (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.
15. *Toxicological evaluation of some flavouring substances and non nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.
16. *Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.
17. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics* (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.
18. *Specifications for the identity and purity of some antibiotics.* FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.
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20. *Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances.* FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.
21. *Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.
22. *Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and*

certain other substances; and a review of the technological efficacy of some antimicrobial agents (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. *Toxicological evaluation of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.
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25. *A review of the technological efficacy of some antimicrobial agents.* FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.
26. *Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants* (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.
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28. *Specifications for the identity and purity of some enzymes and certain other substances.* FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.
29. *A review of the technological efficacy of some antioxidants and synergists.* FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.
30. *Evaluation of certain food additives and the contaminants mercury, lead, and cadmium* (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.
31. *Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate.* FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.
32. *Toxicological evaluation of certain food additives with a review of general principles and of specifications* (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).
33. *Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents.* FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.
34. *Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers.* FAO Food and Nutrition Paper, No. 4, 1978.
35. *Evaluation of certain food additives* (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series,

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42. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 10, 1976.
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61. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 25, 1982.
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63. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 18, 1983.
64. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 28, 1983.
65. *Guide to specifications: General notices, general methods, identification tests, test solutions, and other reference materials.* FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.
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84. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 24. Cambridge University Press, 1989.
85. *Evaluation of certain veterinary drug residues in food* (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.
86. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 25, 1990.
87. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/2, 1990.
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104. *Evaluation of certain veterinary drug residues in food* (Fortieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 832, 1993.
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ANNEX 2

ABBREVIATIONS USED IN THE MONOGRAPHS

ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and elimination
ALT	alanine aminotransferase
AP	alkaline phosphatase
AST	aspartate aminotransferase
BEU	branching enzyme units
BROD	benzyloxyresorufin *O*-dealkylase
bw	body weight
CCFA	Codex Committee on Food Additives
CSFII	Continuing Survey of Food Intakes by Individuals (USA)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC_{50}	median effective concentration
EDTA	ethylenediaminetetraacetate
EFSA	European Food Safety Authority
EROD	ethoxyresorufin *O*-deethylase
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GEGR	glycerol ester of gum rosin
GEWR	glycerol ester of wood rosin
GLP	Good Laboratory Practice
GR	gum rosin
GRAS	Generally Recognized as Safe
GSFA	General Standard for Food Additives (Codex)
GST	glutathione transferase
HDL	high-density lipoprotein
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
HPLC	high-performance liquid chromatography
IQ	intelligence quotient
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	median lethal dose
LDL	low-density lipoprotein
LOAEL	lowest-observed-adverse-effect level
LSC	liquid scintillation counting
MROD	methoxyresorufin *O*-demethylase
NCE	normal chromatic erythrocyte
NDMAD	*N*-nitrosodimethylamine *N*-demethylase
NHANES	National Health and Nutrition Examination Survey (USA)
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
OECD	Organisation for Economic Co-operation and Development

OSA	octenyl succinic acid
PCE	polychromatic erythrocyte
PhIP	2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine
PROD	pentoxyresorufin *O*-dealkylase
QA	quality assurance
QR	quinone reductase
S9	9000 × *g* supernatant from rat liver
SCF	Scientific Committee on Food (European Commission)
SOE	sucrose oligoesters
TOR	tall oil rosin
TOS	total organic solids
TPPO	triphenyl phosphine oxide
U	uniformly
UDP	uridine 5′-diphosphate
UDPGT	uridine 5′-diphosphate glucuronosyltransferase
USA	United States of America
USFDA	United States Food and Drug Administration
UV	ultraviolet
WHO	World Health Organization
WR	wood rosin

ANNEX 3

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Geneva, 16–24 June 2009

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ANNEX 4

ACCEPTABLE DAILY INTAKES, OTHER TOXICOLOGICAL INFORMATION AND INFORMATION ON SPECIFICATIONS

1. FOOD ADDITIVES EVALUATED

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
Branching glycosyltransferase from *Rhodothermus obamensis* expressed in *Bacillus subtilis*	N	The Committee allocated an ADI "not specified" for branching glycosyltransferase from *Rhodothermus obamensis* expressed in *Bacillus subtilis* used in the specified applications and in accordance with Good Manufacturing Practice.
Cassia gum	N, T	The Committee allocated an ADI "not specified" for cassia gum that complies with the tentative specifications established at the current meeting, when used in the applications specified and in accordance with Good Manufacturing Practice. The Committee decided to make the specifications tentative pending submission of data on a suitable and validated method for determination of anthraquinones at a level of 0.5 mg/kg and below, by the end of 2010.
Cyclamic acid and its salts (dietary exposure assessment)		Of the four maximum use levels (250, 500, 750 and 1000 mg/kg) that the Committee considered at the request of the Codex Committee on Food Additives (CCFA) for cyclamates in beverages covered by General Standard for Food Additives (GSFA) Food Category 14.1.4, only the lowest level of 250 mg/kg was not likely to lead to dietary exposures exceeding the ADI for high consumers, including children. Moreover, it was noted that a maximum use level of 350 mg/kg also resulted in dietary exposures for high consumers, including children, that were less than the ADI.
Cyclotetraglucose and cyclotetraglucose syrup	R (cyclotetraglucose syrup)	The Committee removed the temporary designation and established an ADI "not specified" for cyclotetraglucose and cyclotetraglucose syrup. The specifications for cyclotetraglucose syrup were revised, and the tentative designation was removed.
Ferrous ammonium phosphate	N	The newly available information on the toxicity of iron did not indicate a need to revise the

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
		provisional maximum tolerable daily intake (PMTDI) of 0.8 mg/kg body weight (bw). Consideration of the toxicity of ammonium and phosphate did not indicate a need to revise the Committee's previous evaluations of these ions. The Committee concluded that ferrous ammonium phosphate is acceptable for use as a source of iron for dietary fortification, provided that the total intake of iron does not exceed the PMTDI. Products, including ferrous ammonium phosphate, that are intended to provide a source of additional iron should not be consumed by individuals with any type of iron storage disease, except under medical supervision.
Glycerol ester of gum rosin (GEGR)	N, T	The Committee decided to include GEGR in the ADI for glycerol esters of wood rosin (GEWR) of 0–25 mg/kg bw, thereby establishing a group ADI of 0–25 mg/kg bw for GEWR and GEGR. The specifications for GEGR were made tentative pending the submission of infrared spectra that correspond to the commercially available products, data on the resin acid composition obtained with updated chromatographic techniques and additional information on methods that enable the identification of the individual glycerol esters of rosins and their differentiation. This information should be submitted by the end of 2010.
Glycerol ester of tall oil rosin (GETOR)	N, T	The Committee concluded in principle that the data from GEWR could be used in the evaluation of GETOR; however, the Committee did not have adequate information on the composition of GETOR, considering that the source material and production processes are different, which may result in different by-products. The Committee decided that it could not evaluate GETOR without additional information on its composition in order to clarify the extent and significance of any differences relative to other glycerol esters of rosins. The specifications for GETOR were made tentative pending the submission of infrared spectra that correspond to the commercially

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
		available products, data on the resin acid composition obtained with updated chromatographic techniques and additional information on methods that enable the identification of the individual glycerol esters of rosins and their differentiation. The Committee also requested information on the identity of the sulfur compounds in the commercial product. This information should be submitted by the end of 2010.
Lycopene from all sources		The Committee decided to revise the group ADI established at the sixty-seventh meeting and replace it with a group ADI "not specified" for lycopene from all sources when used as food colour. Hence, the previous group ADI of 0–0.5 mg/kg bw for lycopene has been withdrawn. The group ADI "not specified" applies to synthetic lycopene, lycopene derived from the fungus *Blakeslea trispora* and lycopene extract from tomato that comply with the specifications, when used in accordance with Good Manufacturing Practice.
Lycopene extract from tomato	N	The Committee established a group ADI "not specified" for synthetic lycopene, lycopene derived from the fungus *Blakeslea trispora* and lycopene extract from tomato, when used as food colour, that comply with the specifications, and when used in accordance with Good Manufacturing Practice.
Mineral oil (low and medium viscosity) class II and class III		The Committee was informed that finalization of the requested studies has been delayed. The Committee decided to further extend the temporary group ADI, but noted that the temporary group ADI will be withdrawn at the end of 2011 if the data are not submitted by that time.
Octenyl succinic acid (OSA) modified gum arabic	N	The Committee decided to allocate a temporary ADI "not specified" for OSA modified gum arabic used in the applications specified and in accordance with Good Manufacturing Practice. The ADI is temporary pending submission of data by the end of 2011 showing hydrolysis of OSA modified gum arabic to confirm the validity of using gum arabic data in the evaluation of OSA modified gum arabic.
Sodium hydrogen sulfate	R	The Committee allocated an ADI "not specified" for sodium hydrogen sulfate, in line with the principles established for ionizable

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
		salts at its twenty-ninth meeting, when used in the applications specified and in accordance with Good Manufacturing Practice. Specifications were revised to include a new technological use.
Sucrose oligoesters (SOE) type I and type II	N	The Committee considered it appropriate to include SOE type I and type II in a group ADI of 0–30 mg/kg bw for sucrose esters of fatty acids, sucroglycerides and SOE type I and type II. The Committee emphasized that this evaluation is valid only for the material as specified.

[a] N, new specifications prepared; R, existing specifications revised; T, tentative specifications.

[b] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of Good Manufacturing Practice, i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

2. FOOD ADDITIVES CONSIDERED FOR SPECIFICATIONS ONLY

Food additive	Specifications[a]
Diacetyltartaric and fatty acid esters of glycerol	R
Ethyl lauroyl arginate	R
Glycerol ester of wood rosin	R, T
Nisin preparation	R
Nitrous oxide	R, T
Pectins	R
Starch sodium octenyl succinate	R
Tannic acid	R
Titanium dioxide	R
Triethyl citrate	R

[a] R, existing specifications revised; T, tentative specifications.

This volume contains monographs prepared at the seventy-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 16 to 24 June 2009.

The toxicological monographs in this volume summarize the safety data on a number of food additives: branching glycosyltransferase from *Rhodothermus obamensis* expressed in *Bacillus subtilis*, cassia gum, ferrous ammonium phosphate, glycerol ester of gum rosin, glycerol ester of tall oil rosin, lycopene from all sources, octenyl succinic acid modified gum arabic, sodium hydrogen sulfate and sucrose oligoesters type I and type II. A monograph on the assessment of dietary exposure to cyclamic acid and its salts is also included.

This volume and others in the WHO Food Additives series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

ISBN 978 92 4 166062 4