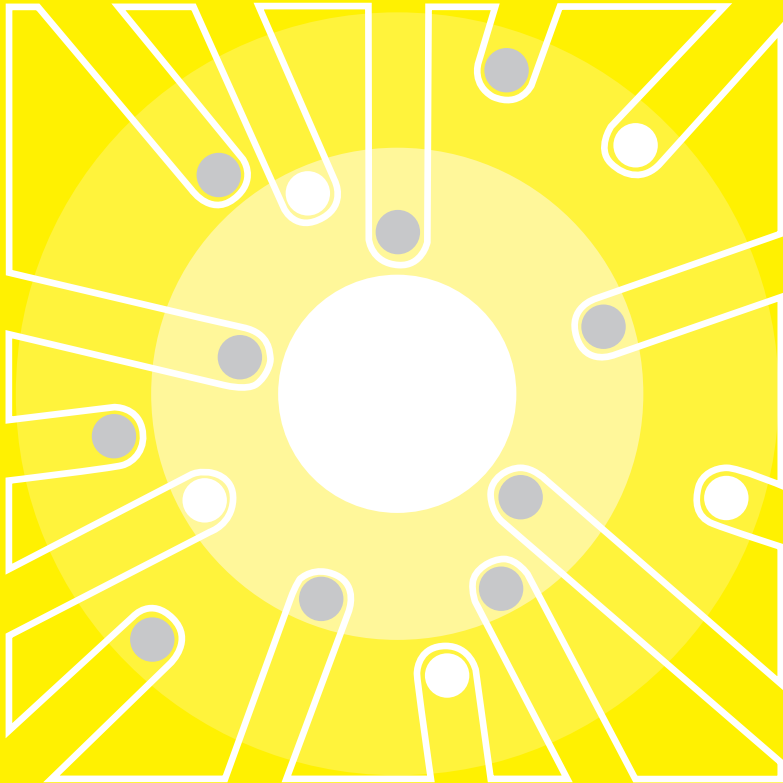

WHO FOOD
ADDITIVES
SERIES: 54

Safety evaluation of certain food additives

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



IPCS

International Programme on Chemical Safety
World Health Organization, Geneva

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PREFACE

The monographs contained in this volume were prepared at the sixty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO Headquarters in Geneva, Switzerland, 8–17 June 2004. These monographs summarize the safety data on selected food additives reviewed by the Committee.

The sixty-third report of JECFA has been published by the World Health Organization as WHO Technical Report No. 928. 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; a summary of the conclusions of the Committee is given in Annex 4. Some of the substances listed in Annex 4 were evaluated at the meeting only for specifications. Annex 5 contains a summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%.

Specifications that were developed at the sixty-third meeting of JECFA have been issued separately by FAO as Food and Nutrition Paper, No. 52, Addendum 12. The monographs in the present publication should be read in conjunction with the specifications and the report.

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 and Contaminants 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 toxicological monographs contained in the 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.

Many proprietary unpublished 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 developed on all the data that were submitted, and all of these reports were available to the Committee when it made its evaluation. The monographs were edited by H. Mattock, Illkirch-Graffenstaden, France.

The preparation and editing of the monographs included in this volume were made possible through the technical and financial contributions of the Participating Organizations of the International Programme on Chemical Safety (IPCS), which supports the activities of JECFA.

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 the IPCS 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, International Programme on Chemical Safety, World Health Organization, Avenue Appia, 1211 Geneva 27, Switzerland.

STEVIOIOL GLYCOSIDES

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1. EXPLANATION

Steviol glycosides are natural constituents of the plant *Stevia rebaudiana* Bertoni, a member of the *Compositae* family. The leaves of *S. rebaudiana* Bertoni contain at least ten different glycosides, the major constituents being stevioside and rebaudioside A. The material evaluated at the present meeting contains not less than 95% glycosylated derivatives of steviol, primarily stevioside, rebaudiosides A and C and dulcoside A (Figure 1), with minor amounts of rubusoside, steviolbioside, and rebaudiosides B, D, E and F (Figure 2).

At its fifty-first meeting (Annex 1, reference 149), the Committee evaluated toxicological data on stevioside and the aglycone steviol. The Committee noted several shortcomings in the available information and requested that specifications should be developed to ensure that the material tested is representative of the material of commerce. Further information was required on the nature of the substance tested, on the metabolism of stevioside in humans and on the activity of steviol in suitable studies of genotoxicity in vivo.

There is no single common or trivial name in common usage for the evaluated mixture of glycosylated derivatives of steviol. At its thirty-third meeting (Annex 1, reference 83), the Committee developed guidelines for designating titles for specification monographs. According to these guidelines, the title of a monograph should, in such circumstances, be selected from the available scientific, common and trivial names. The name chosen must be nonproprietary and should be a scientifically accurate description of the substance. In addition, the name should communicate to the consumer an accurate description of the substance, within the scope of existing names for food additives. At its present meeting, the Committee established that the evaluated material of commerce for which specifications were developed should be known as 'steviol glycosides'. The Committee reviewed additional biochemical and toxicological data on the major glycosylated derivatives of steviol and on the aglycone, steviol.

This monograph describes the new data on steviol glycosides discussed at the present meeting, together with summaries of the key toxicological data on stevioside evaluated by the Committee at its fifty-first meeting.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

At its fifty-first meeting (Annex 1, reference 149), the Committee noted that in rats treated orally stevioside is not readily absorbed from the upper small intestine but is hydrolysed to the aglycone, steviol, before absorption from the gut. New information on absorption in in-vitro models and in rats was available at the present meeting.

Intestinal transport of stevioside (1 mmol/l), rebaudioside A (1 mmol) and steviol (30 µmol/l – 1 mol/l) has been investigated in a Caco-2 cell monolayer model. The integrity of the monolayer was verified with fluorescein. Transport of stevioside and rebaudioside A was very low (apparent permeability coefficients, 0.16×10^{-6} and 0.11×10^{-6} cm/s, respectively). Steviol was transported more effectively, with a higher apparent permeability coefficient for absorptive transport (44.5×10^{-6} cm/s) than for secretory transport (7.93×10^{-6} cm/s) at a concentration of 100 µmol/l. At concentrations of 300 µmol/l and 1 mol/l, steviol slightly compromised the integrity of the monolayers during transport (Geuns et al., 2003a).

The intestinal absorption of a *Stevia* mixture and the aglycone steviol was investigated using everted gastrointestinal sacs from four male Sprague-Dawley

Figure 1. Structures of the major steviol glycosides

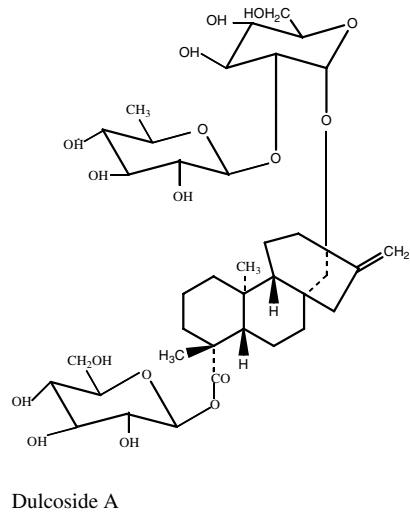
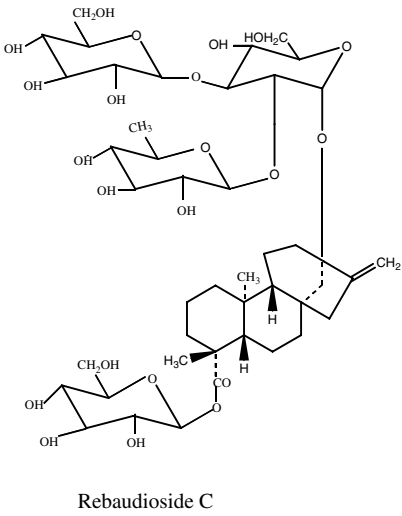
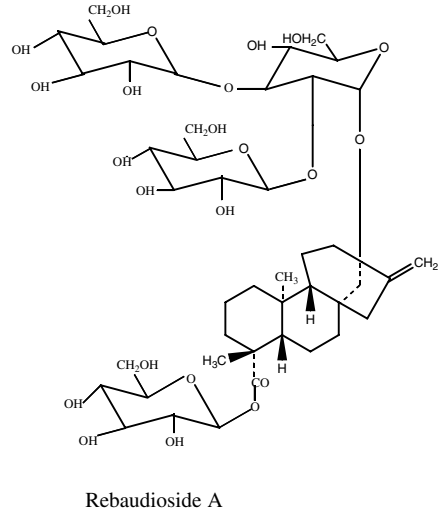
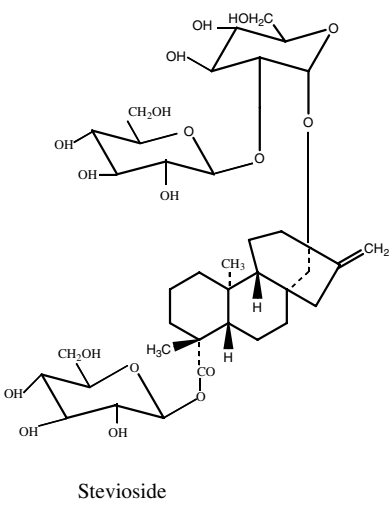
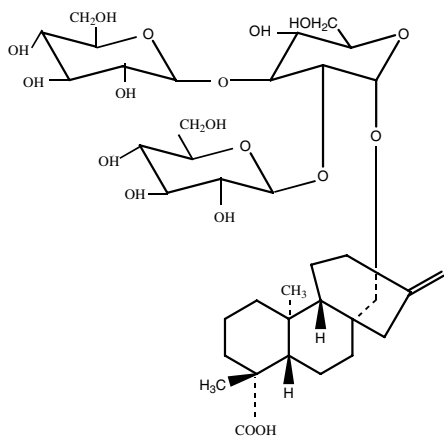
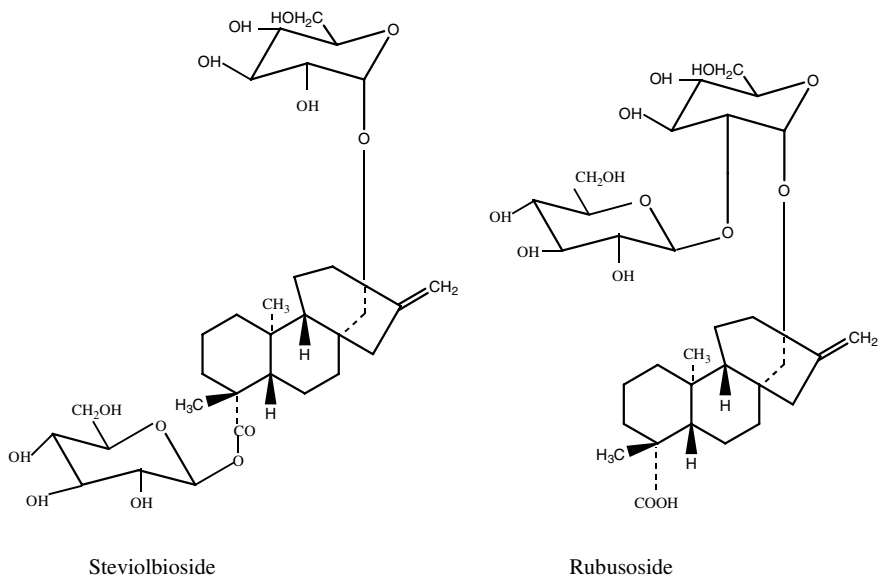


Figure 2. Structures of minor steviol glycosides

rats. The *Stevia* mixture contained rebaudioside A (28.8%), rebaudioside C (25.2%), stevioside (17.0%) and dulcoside A (10.2%). The everted sacs were incubated in *Stevia* mixture (0.5 mg/ml) or steviol (0.1 mg/ml) for 30 min. Transport of salicylic acid (10 µg/ml) was used to confirm that the sacs were functional. Steviol was transported in both the duodenum–jejunum and the ileum (76% and 95% of salicylic acid transport, respectively). The steviol glycosides were poorly absorbed from the *Stevia* mixture, with more than 93% remaining in the mucosal fluid (Koyama et al., 2003a).

Absorption of the *Stevia* mixture described above was also investigated in vivo in four male Sprague-Dawley rats. *Stevia* mixture (in 2% gum arabic) was administered at a dose of 125 mg/kg bw. Steviol was not detected in plasma at 1 h, but was detected at increasing concentrations between 2 h and 8 h, when the concentration reached a peak of about 5 µg/ml. In contrast, the peak plasma concentration of steviol (18.31 µg/ml) was observed 15 min after a single oral administration of steviol (45 mg/kg in corn oil). These doses were approximately equimolar for steviol (Koyama et al., 2003a).

Similarly, in male Sprague-Dawley rats given a single oral dose of stevioside (purity, 95%) at 0.5 g/kg bw, low concentrations of steviol were detected in plasma for the first 8 h, followed by a rapid increase to a concentration of about 1000 ng/ml at 24 h. This study used a highly sensitive method for detection of steviol, but did not examine levels of stevioside or other metabolites (Wang et al., 2004).

One study has reported detectable levels of stevioside, but not steviol, in plasma after administration of a *Stevia* product. Groups of male Sprague-Dawley rats were given T100 sunstevia 95% (containing 70% stevioside) at a dose of 0.5 or 2 g/kg bw by gavage. Stevioside was detected in plasma 5 min after dosing. There was considerable variation between animals, with the time to maximum plasma concentration varying from 10 to 300 min. Clearance did not differ significantly between the doses. Reported plasma half-lives were 10.6 ± 8.7 and 6.7 ± 3.7 h at 0.5 and 2.0 g/kg bw, respectively. At 48 h, 5.7–16.9% and 1–6.7% of the total administered dose of stevioside was recovered in the faeces and urine, respectively. Steviol was detected in faeces collected up to 48 h, but not in plasma sampled up to 24 h after dosing (limit of detection, 1 µg/ml) (Sung, 2002).

2.1.2. Biotransformation

Incubation of stevioside (purity, >96%; concentration, 50 mg/l) with chicken excreta under anaerobic conditions for 24 h resulted in a 20% conversion of stevioside into steviol (Geuns et al., 2003b).

Faecal bacterial suspensions from eleven healthy volunteers (six men and five women) were incubated under anaerobic conditions with 40 mg of stevioside (purity, 85%) and 40 mg of rebaudioside A (purity, 90%) for 72 h. Stevioside and rebaudioside A were completely hydrolysed to the aglycone steviol within 10 and 24 h, respectively. Among cultures of coliforms, bifidobacteria, enterococci and bacteroides, only the bacteroides were able to hydrolyse these compounds. The data indicated that both glycosides were initially hydrolysed to steviolbioside (this occurred more slowly with rebaudioside A), and the steviolbioside was then rapidly

metabolized to steviol. Steviol remained unchanged during the 72h incubation, indicating that bacterial enzymes are not able to cleave the steviol structure (Gardana et al., 2003).

Human faecal metabolism of *Stevia* compounds was investigated in pooled faecal homogenates obtained from five healthy Japanese male volunteers. The materials tested were *Stevia* mixture (main components: rebaudioside A, stevioside, rebaudioside C, dulcoside A), its α -glucose derivative, referred to as enzymatically modified *Stevia* (main components: α -glucosylrebaudioside A, α -glucosylstevioside, α -glucosylrebaudioside C, α -glucosyl dulcoside A), rebaudioside A, stevioside, steviol, rebaudioside C, dulcoside A, rebaudioside B, rubusoside, α -monoglucosylrebaudioside A and α -monoglucosylstevioside. After incubation of the faecal homogenates under anaerobic conditions for 24h, the *Stevia* mixture, glycosides and α -glucose derivatives were all rapidly degraded. Stevioside was hydrolysed, with successive removal of glucose units via rubusoside, to the aglycone steviol. The metabolism of α -monoglucosylstevioside was similar to that of stevioside after α -deglucosylation. For rebaudioside there were two pathways, a major pathway in which rebaudioside A was hydrolysed via stevioside to steviol, and a minor pathway that suggested that rebaudioside A is metabolized via rebaudioside B to steviol. The metabolism of α -monoglucosylrebaudioside A was similar to that of rebaudioside A after α -deglucosylation. No degradation of steviol was observed over the 24h incubation period. The authors concluded that steviol was the only final product of the metabolism of *Stevia*-related compounds, including enzymatically modified *Stevia* in human intestinal microflora, and that there were no apparent species differences in the intestinal metabolism of *Stevia* mixture between rats and humans (Koyama et al., 2003b).

Metabolism of steviol (purity not specified) in rats and humans has been investigated using pooled human liver microsomal preparations from five male and five female donors, and from rat liver microsomal preparations with the same protein content. Metabolite formation required a nicotinamide adenine dinucleotide phosphate, reduced (NADPH)-generating system, indicating cytochrome P450 (CYP)-dependent metabolism. The metabolic profile obtained with human liver microsomal fractions was similar to that obtained with rat liver microsomal preparations; mass spectrometric analysis indicated the presence of two dihydroxy metabolites and four monohydroxy metabolites. One additional monohydroxy metabolite was detected with the rat preparation. The liver microsomal clearance of steviol was approximately four times lower in humans than in rats (Koyama et al., 2003a).

Hamsters were given stevioside (purity not specified) at a dose of 1g/kgbw by gavage and metabolites were measured in the plasma, urine and faeces at 3, 24 and 24h, respectively. The samples were treated with glucuronidase/sulfatase to hydrolyse conjugated metabolites. Steviol-16,17 α -epoxide, stevioside, 15 α -hydroxysteviol and steviolbioside were detected in the plasma, urine and faeces. In addition, isosteviol was detected in the urine and faeces, and steviol was detected in the faeces (Hutapea et al., 1999).

Chickens were given stevioside (purity, >96%) at a dose of 643 or 1168 mg/kgbw by intubation. Most of the stevioside was recovered unchanged in excreta in the 24–48 h after administration, and only about 2% was converted to steviol. Neither stevioside nor steviol were detected in the blood. Sixteen broiler chickens and four laying hens were also given stevioside at a dose of 667 mg/kg of feed for 14 and 10 days, respectively. Most of the stevioside was untransformed in the excreta, with about 21.5% and 7.3% being converted to steviol by broiler chickens or laying hens, respectively. No stevioside or steviol was detected in the blood or in the eggs (Geuns et al., 2003b).

Six female pigswere given stevioside (purity, >96%) at a dose of 1.67 g/kg of feed for 14 days (equivalent to approximately 70 mg/kgbw per day). Steviol, but not stevioside, was detected in the faeces, indicating bacterial metabolism of stevioside to steviol. No stevioside or steviol was detected in the blood. The authors concluded that stevioside was completely converted to steviol and suggested that the possible uptake from the colon was very low (Geuns et al., 2003a).

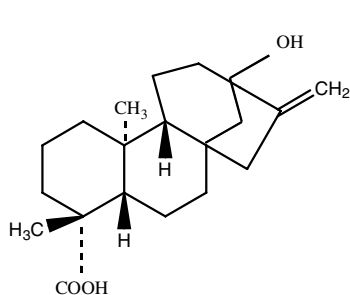
Metabolism of stevioside by human volunteers has been investigated in a collaborative study conducted in Belgium and Italy. In Italy, nine healthy men (aged 20–50 years) were given capsules containing 375 mg of stevioside (purity not specified) after an overnight fast. Low concentrations of stevioside were detected in the plasma of seven of the subjects, with a maximum of 0.1 µg/ml. Peak plasma concentrations occurred at 60 to 180 min after dosing. Steviol glucuronide was detected in five of the men. No free steviol, steviol-16,17 α -epoxide, 15 α -hydroxysteviol or 15-oxo-steviol was detected. Steviol glucuronide was detected in the urine of all men, and low concentrations of stevioside were also present in the urine of two men. Free steviol or its unconjugated metabolites were not detected. Only free steviol was detected in the faeces. In Belgium, five male and five female volunteers (aged 24 \pm 2 years) were each given nine doses of 250 mg of stevioside (purity, >97%; impurities being other *Stevia* glycosides) at 8 h intervals on three successive days. No stevioside or free steviol was detected in the blood. After hydrolysis with β -glucuronidase/sulfatase, steviol was detected at concentrations ranging from 0.7 to 21.3 µg/ml, with peak concentrations occurring at varying times up to 5 h. Similarly, stevioside and conjugated steviol were detected in the urine at 24 h. The only compound detected in the faeces was free steviol. The differences between the two studies were considered to be due to the different doses of stevioside administered and the different detection limits of the analytical method for stevioside. The total recovery of steviol metabolites varied between 22% and 86% of the administered daily dose of stevioside (mean total recovery, 52.1 \pm 27%) (Geuns & Pietta, 2004).

The major metabolites of steviol glycosides are shown in Figure 3.

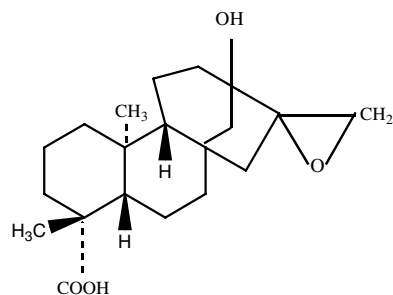
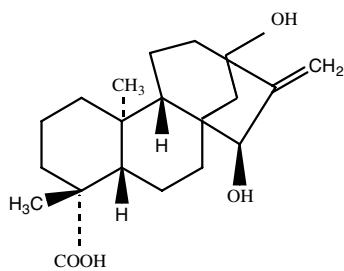
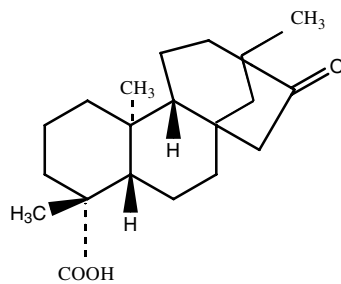
2.1.3 Effects on enzymes and other biochemical parameters in vitro

In isolated aortic rings from normal rats, stevioside (purity not stated) at a concentration of 10⁻⁸ to 10⁻⁵ mol/l caused a concentration-dependent relaxation of vasopressin-induced vasoconstriction when incubated in medium containing calcium, but not in calcium-free medium. The results of studies in a rat aortic

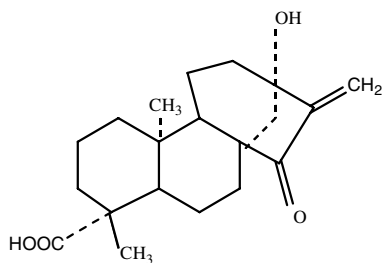
Figure 3. Metabolites of steviol glycosides



Steviol

Steviol-16,17 α -epoxide15 α -hydroxysteviol

Isosteviol



15-oxo-steviol

smooth muscle cell line (A7r5) indicated that this was due to inhibition of the stimulatory effects of vasopressin on intracellular calcium ions (Ca^{2+}). Stevioside did not inhibit calcium ionophore (A23187)-induced Ca^{2+} influx. The effects of stevioside were not inhibited by methylene blue. The authors concluded that the vasorelaxation effect of stevioside was mediated mainly through inhibition of Ca^{2+} influx and was not related to nitric oxide (Lee et al., 2001; Liu et al., 2003).

The role of potassium channels in the vasodilator effect of isosteviol (purity not stated) was investigated in isolated aortic rings prepared from Wistar rats. Isosteviol at concentrations of 10^{-8} to 10^{-5} mol/l relaxed the vasopressin-induced vasoconstriction in a concentration-dependent manner. Potassium chloride, and inhibitors specific for the ATP-sensitive potassium channel, inhibited the vasodilator effect of isosteviol. Methylene blue failed to modify the vasodilation produced by isosteviol, suggesting that nitric oxide did not play a role. The authors concluded that vasodilation induced by isosteviol was related to the opening of the calcium-activated and ATP-sensitive potassium channels (Wong et al., 2004).

Stevioside (purity, 95%) and steviol (purity, 90%) at concentrations of 10^{-9} to 10^{-3} mol/l enhanced insulin secretion in isolated mouse pancreatic islets and in a pancreatic- β -cell line (INS-1). The maximal effect was observed with steviol at 10^{-6} mol/l and with stevioside at 10^{-3} mol/l. The insulinotropic effect was dependent on the concentration of glucose (Jeppesen et al., 2000).

Subsequent studies indicated that stevioside at 10^{-3} mol/l enhanced the insulin content of the INS-1 cells, partly by induction of genes involved in glycolysis. Stevioside upregulated the expression of the liver-type pyruvate and acetyl-coenzyme A (CoA)-carboxylase and downregulated the expression of carnitine palmitoyl-transferase 1 (CPT-1), long-chain acyl-CoA dehydrogenase, cytosolic epoxide hydrolase and 3-oxoacyl-CoA thiolase. In addition, stevioside improved nutrient sensing mechanisms, increased cytosolic long-chain fatty acyl-CoA and downregulated phosphodiesterase 1 (PDE1). Steviol showed similar effects (Jeppesen et al., 2003).

The effect of stevioside (purity, 95%) on the transepithelial transport of *p*-aminohippurate was investigated in isolated S_2 segments of the rabbit proximal renal tubules. Stevioside (0.70 mol/l) in the tubular lumen had no effect on the transport of *p*-aminohippurate transport, but when present in the bathing medium it inhibited transport by 25–35%; the inhibitory effect was gradually abolished after stevioside was removed. Stevioside had no effect on Na^+/K^+ -activated ATPase activity or cell ATP content. The authors concluded that stevioside at a pharmacological concentration of 0.7 mol/l inhibits transepithelial transport of *p*-aminohippurate by interfering with the basolateral entry step, the rate-limiting step for transepithelial transport. The lack of effect of stevioside on transepithelial transport of *p*-aminohippurate on the luminal side and the reversible inhibitory effect on the basolateral side indicated that stevioside did not permanently change *p*-aminohippurate transport and would not be expected to harm renal tubular function at normal levels of intake in humans (Jutabha et al., 2000).

Rats

Groups of normotensive Wistar-Kyoto rats, spontaneously hypertensive rats, deoxycorticosterone acetate-salt sensitive rats and renal hypertensive rats were given stevioside (purity not stated) at a dose of 50, 100, 200 or 400 mg/kg bw per day by intraperitoneal injection for 1 to 10 days. Treatment with stevioside resulted in significantly decreased blood pressure in all strains of rat, and the effect persisted throughout the 10 days of treatment. Decreased blood pressure was also observed in mature, spontaneously hypertensive rats given drinking-water containing 0.1% stevioside. Administration of drinking-water containing 0.1% stevioside also slowed the age-related progressive increase in blood pressure that occurs in rats of this strain (Hsu et al., 2002).

In Goto-Kakizaki rats (which are used as a non-obese animal model of type-2 diabetes), the intravenous administration of stevioside (purity, 96%) at a dose of 200 mg/kg bw resulted in suppressed plasma glucagon, increased insulin response and suppressed the response to a glucose tolerance test (incremental area-under-the-curve: stevioside, 648 ± 50 mol/l \times 120 min; control, 958 ± 85 mol/l \times 120 min). In normal Wistar rats, insulin concentrations were increased without altering the blood glucose response or glucagon concentrations (Jeppesen, 2002).

In Goto-Kakizaki rats given drinking-water containing stevioside (purity, >99.6%) at a dose of 25 mg/kg bw per day for 6 weeks, an antihyperglycaemic effect was observed, with enhanced insulin response and suppressed glucagon concentrations, and a pronounced suppression of systolic and diastolic blood pressure (Jeppesen, 2003).

Insulin-sensitive lean Zucker rats and insulin-resistant obese Zucker rats were given stevioside (purity not stated) at a dose of 200 or 500 mg/kg bw by oral gavage, 2 h before an oral test for glucose tolerance. There was no effect on plasma glucose, insulin or free fatty acid concentrations in either the lean or obese groups. At the higher dose, stevioside enhanced whole-body sensitivity to insulin in the lean and obese rats, as shown by a decreased insulin incremental area under the curve and glucose–insulin index. No effect was observed after administration of stevioside at 200 mg/kg bw.

In vitro, stevioside at concentrations of 0.01–0.1 mol/l was found to enhance insulin-stimulated glucose transport in type 1 soleus and type IIb epitrochlearis muscle of both lean and obese Zucker rats. Higher concentrations of stevioside inhibited the insulin-stimulated transport of glucose. The authors concluded that one potential site of action of stevioside was the skeletal muscle glucose transport system (Lailerd et al., 2004).

Dogs

In healthy mongrel dogs, nasogastric administration of stevioside (purity not stated) at a dose of 200 mg/kg bw resulted in a lowering of blood pressure that was maximal at 90 min, returning to baseline by 180 min. A more rapid decrease in blood pressure was observed after intravenous injection of stevioside at 50 mg/kg bw, with the maximum decrease at 5–10 min. In dogs with renal hypertension

induced by ligation of the left renal artery, intravenous administration of stevioside at 20–160 mg/kg bw resulted in a dose-dependent decrease in systolic and diastolic blood pressure. No effect was observed at 10 mg/kg bw (Liu et al., 2003).

2.2 Toxicological studies

2.2.1 Short-term studies of toxicity

Chickens

Sixteen broiler chickens and four laying hens were given diets containing stevioside (purity, >96%) at a concentration of 667 mg/kg of feed for 14 and 10 days, respectively. No significant differences were found in feed intake, body-weight gain and feed conversion (Geuns et al., 2003b).

2.2.2 Long-term studies of carcinogenicity

In a study discussed by the Committee at its fifty-first meeting, groups of 50 male and 50 female Fischer 344.DuCrj rats were given access ad libitum to diets containing stevioside (purity, 95.6%; stevioside was added to the powdered diet, which was then pelleted) at a concentration of 0, 2.5 or 5% (equal to doses of 0, 970 and 2000 mg/kg bw per day for males, and 0, 1100 and 2400 mg/kg bw per day for females) for 104 weeks. The doses were selected on the basis of the results of a 13-week study. Thereafter, all of the groups were maintained on basal (0% stevioside) diet for 4 weeks. All surviving rats were killed in week 108. The body-weight gain of the treated animals was slightly depressed, and a dose–response relationship was seen in males (2.3% and 4.4%) and females (2.4% and 9.2%) at the lowest and highest doses, respectively. Food consumption did not differ between the groups. The final survival rate of males receiving diets containing 5% stevioside was significantly decreased (60%) compared with that of the controls (78%). Absolute weights of the kidney were decreased in males and females at the highest dose; however, there was no significant histopathological evidence of neoplastic or non-neoplastic lesions attributable to treatment in any organ or tissue, except for a decreased incidence of mammary adenomas in females and a reduced severity of chronic nephropathy in males. The authors concluded that stevioside was not carcinogenic in Fischer 344 rats under the experimental conditions used (Toyoda et al., 1995, 1997).

The effects of stevioside (purity not stated) have been investigated in models of two-stage skin carcinogenesis in mice. Groups of 15 male ICR mice were initiated by topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA; 100 µg). Promotion treatment commenced 1 week later, and involved topical administration of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 1 µg) twice per week for 20 weeks. Topical administration of stevioside (68 µg) 1 h before the TPA resulted in a significant decrease in the percentage of animals with papillomas at 10 and 15 weeks, and in the number of papillomas per mouse at 15 and 20 weeks. In a similar study, groups of 15 female SENCAR mice were initiated by administration of peroxyinitrite (33.1 µg) followed by promotion with TPA, twice per week for 20 weeks. Administration of drinking-water containing 0.0025% stevioside from 1 week before to 1 week

after initiation inhibited tumour formation. There was a statistically significant decrease in the percentage of animals with papillomas at 10 and 15 weeks, and in the number of papillomas per mouse at 10, 15 and 20 weeks (Konoshima & Takasaki, 2002).

2.2.3 Genotoxicity

Studies of genotoxicity with purified *Stevia* extract and its major components, stevioside and rebaudioside A, reviewed by the Committee at its fifty-first and present meetings, are summarized in Table 1. These compounds gave negative results *in vitro* and *in vivo*. Studies of genotoxicity with steviol and other *Stevia*-derived compounds are summarized in Table 2.

Steviol and its oxidative derivatives steviol-16,17-epoxide, 15-oxo-steviol, steviol methylester and 13,16-*seco*-13-oxo-steviol methylester induced forward mutations in *S. typhimurium* TM677 in the presence, but not in the absence, of a metabolic activation system. The metabolizing system decreased the mutagenicity of steviol methylester 8,13-lactone. The results for 15 α -hydroxy-steviol, steviol methylester and 13,16-*seco*-13 α -hydroxy-steviol methylester were negative in this assay (Terai et al., 2002).

Steviol gave negative results in assays for cell mutation and DNA damage in cultured cells (Oh et al., 1999; Sekihashi et al., 2002).

Steviol (purity, >99%) has been investigated in two independent studies of DNA damage using the comet assay. In one study, groups of four male BDF1 mice were given steviol at a dose of 0, 250, 500 or 2000 mg/kg bw and the liver, stomach and colon were examined for the presence of comets. In the second study, groups of four male CRJ:CD-1 mice were given steviol at a dose of 0, 500, 1000 or 2000 mg/kg bw and the liver, kidney, colon and testes were examined for the presence of comets. In both studies, groups of animals were sacrificed at 3 h and 24 h after dosing and methylmethanesulfonate (MMS) was used as a positive control. There were no significant differences in DNA migration distance in any of the organs examined. MMS induced a positive response in all organs examined in both studies (Sekihashi et al., 2002).

Steviol (purity, about 90%) has also been tested in assays for induction of micronuclei formation in the bone marrow of Syrian golden hamsters, Wistar rats and Swiss albino mice. Groups of 20 male and 20 female animals were given steviol at a dose of 4000 mg/kg bw (hamsters) or 8000 mg/kg bw (rats and mice) by gavage. Five animals in each group were killed 24, 30, 48 and 72 h after dosing. An additional group, which served as a positive control, was treated with cyclophosphamide and sacrificed at 30 h. There were no significant increases in the frequencies of micronucleated polychromatic erythrocytes (PCEs) in any of the groups treated with stevioside. The ratio of PCEs to normochromatic erythrocytes (NCEs) was significantly reduced in the female hamsters at 72 h after treatment, and in female rats and mice at 48 h and 72 h. The PCE:NCE ratio did not change in male animals. Cyclophosphamide induced a positive response (Temcharoen et al., 2000).

Table 1. Studies of genotoxicity with purified Stevia extract and its major components, stevioside and rebaudioside A

End-point	Test system	Material	Purity (%)	Concentration or dose	Result	Reference
<i>In vitro</i>						
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Stevioside	99	50 mg/plate	Negative ^a	Suttajit et al. (1993)
Reverse mutation	<i>S. typhimurium</i> TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Stevioside	83	5 mg/plate ^e 1 mg/plate ^f	Negative	Matsui et al. (1996a)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	83	10 mg/plate	Negative ^a	Matsui et al. (1996)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	NS	Not specified	Negative ^a	Medon et al. (1982)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	NS	10 mg/plate	Negative ^a	Pezzuto et al. (1985)
Gene mutation (<i>umu</i>)	<i>S. typhimurium</i> TA1535/ pSK1002	Stevioside	83	5 mg/plate	Negative ^a	Matsui et al. (1996)
Gene mutation	<i>B. subtilis</i> H17 <i>rec+</i> , M45 <i>rec-</i>	Stevioside	83	10 mg/disk	Negative ^a	Matsui et al. (1996)
Gene mutation	Mouse lymphoma L5178Y cells, <i>Tk</i> ^{-/-} locus	Stevioside	NS	5 mg/m	Negative ^{a,b}	Oh et al. (1999)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	83	8 mg/ml ^e 12 mg/ml ^f	Negative	Matsui et al. (1996)
Chromosomal aberration	Human lymphocytes	Stevioside	NS	10 mg/ml	Negative	Suttajit et al. (1993)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	85	12 mg/ml	Negative ^e	Ishidate et al. (1984)
Chromosome aberrations	CHL/IU Chinese hamster lung fibroblasts	Rebaudioside A	NS	1.2–55 mg/ml	Negative ^a	Nakajima (2000a)

Table 1. (contd)

End-point	Test system	Material	Purity (%)	Concentration or dose	Result	Reference
<i>In vivo</i> Mutation DNA damage (comet assay)	<i>D. melanogaster</i> Muller 5 strain Male BDF1 mouse stomach, colon, liver	Stevioside Stevia extract	NS Stevioside, 52; rebaudioside A, 22	2% in feed 250–2000 mg/kg	Negative ^b Negative ^c	Kerr et al. (1983) Sekihashi et al. (2002)
DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	Stevia	NS	2000 mg/kg	Negative ^c	Sasaki et al. (2002)
Micronucleus formation	ddY mouse bone marrow and regenerating liver	Stevioside	NS	62.5–250 mg/kg	Negative ^b	Oh et al. (1999)
Micronucleus formation	BDF1 mouse bone marrow	Rebaudioside A	NS	500–2000 mg/kg bw per day for 2 days	Negative ^d	Nakajima (2000b)

NS, not specified.

^a With and without metabolic activation (source not specified in original monograph).

^b Inadequate detail available.

^c Killed at 3 h and 24 h.

^d Killed 30 h after second administration.

^e Without metabolic activation.

^f With metabolic activation.

Table 2. Studies of genotoxicity with steviol and other Stevia-derived compounds

End-point	Test system	Material	Purity (%)	Concentration/dose	Result	Reference
<i>In vitro</i>						
Reverse mutation	<i>S. typhimurium</i> TA98 and TA100	Steviol	NS	20mg/plate	Negative ^a	Suttajit et al. (1993)
Reverse mutation	<i>S. typhimurium</i> TA97, TA98, TA100, TA102, TA104, TA1535 and TA1537	Steviol	99	5mg/plate	Negative ^a	Matsui et al. (1996)
Forward mutation	<i>S. typhimurium</i> TM677	Steviol	NS	10 mg/plate ^h	Negative	Matsui et al. (1996)
Forward mutation	<i>S. typhimurium</i> TM677	Steviol	NS	0.5–10 mg/plate ^l	Positive	
Forward mutation	<i>S. typhimurium</i> TM677	Steviol	NS	10 mg/plate ^h	Negative	Pezzuto et al. (1985)
Forward mutation	<i>S. typhimurium</i> TM677	Steviol	NS	10 mg/plate ^l	Positive	
Forward mutation	<i>S. typhimurium</i> TM677	Steviol	NS	NS	Positive ^l	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	Steviol-16,17-epoxide	NS	NS	Positive ^l	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	15 α -hydroxysteviol	NS	NS	Negative ^a	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	15-oxo-steviol	NS	NS	Positive ^l	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	Steviol methylester	NS	NS	Positive ^l	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	16-oxo-steviol methylester	NS	NS	Negative ^a	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	13,16- <i>seco</i> -13-oxo-steviol methylester	NS	NS	Positive ^l	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	13,16- <i>seco</i> -13 α -hydroxy-steviol methylester	NS	NS	Negative ^a	Terai et al. (2002)
Forward mutation	<i>S. typhimurium</i> TM677	Steviol methylester 8,13-lactone	NS	NS	Positive ^o	Terai et al. (2002)
Gene mutation (<i>umu</i>)	<i>S. typhimurium</i> TA1535/pSK1002	Steviol	99	625–1250 μ g/ plate ^h	Positive	Matsui et al. (1996)
Gene mutation	<i>B. subtilis</i> H17 <i>rec</i> ⁺ , M45 <i>rec</i> ⁻	Steviol	99	1259–2500 μ g/plate ^l 10 mg/disk	Positive Negative ^a	Matsui et al. (1996)
Gene mutation	Chinese hamster lung fibroblasts	Steviol	99	400 μ g/ml ^l	Positive	Matsui et al. (1996)

Table 2. (contd)

End-point	Test system	Material	Purity (%)	Concentration/dose	Result	Reference
Gene mutation	Mouse lymphoma L5178Y cells, <i>TK</i> ^{-/-} locus	Steviol	NS	340 µg/ml	Negative ^{a,b}	Oh et al. (1999)
Chromosomal aberration	Chinese hamster lung fibroblasts	Steviol	NS	0.5 g/ml ^b 1–1.5 mg/ml ^f	Negative Positive	Matsui et al. (1996)
Chromosomal aberration	Human lymphocytes	Steviol	NS	0.2 mg/ml	Negative	Suttajit et al. (1993)
DNA damage (comet assay)	TK6 and WTK1 cells	Steviol	NS	62.5–500 µg/ml	Negative ^a	Sekihashi et al. (2002)
<i>In vivo</i> DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver; male CRJ: CD1 mouse liver kidney, colon and testes	Steviol	>99	250–2000 mg/kg	Negative ^c	Sekihashi et al. (2002)
Micronucleus formation	MS/Ae mice	Steviol	99	1000 mg/kg bw	Negative	Matsui et al. (1996)
Micronucleus formation	Swiss mouse bone marrow	Steviol	About 90	8000 mg/kg	Negative ^g	Temcharoen et al. (2000)
Micronucleus formation	Wistar rat bone marrow	Steviol	About 90	8000 mg/kg	Negative ^g	Temcharoen et al. (2000)
Micronucleus formation	Syrian golden hamster bone marrow	Steviol	About 90	4000 mg/kg	Negative ^g	Temcharoen et al. (2000)
Micronucleus formation	ddY Mouse regenerating liver	Steviol	NS	50–200 mg/kg	Negative ^b	Oh et al. (1999)

NS, not specified.

^a With and without metabolic activation (source not specified in original monograph).

^b Inadequate detail available.

^c Killed at 3 and 24 h.

^e The presence of metabolic activation decreased the mutagenicity.

^f With metabolic activation.

^g Killed at 24, 30, 48 and 72 h. Ratio of polychromatic to normochromatic erythrocytes was decreased at later time-point(s) in females.

^h Without metabolic activation.

In a study with limited reporting, available in Korean, groups of five partially hepatectomized ddY mice were given steviol (purity not stated) at an oral dose of 0, 50, 100 or 200 mg/kg bw. Steviol had no significant effect on the numbers of micronucleated hepatocytes. A group of mice treated with mitomycin C, the positive control, did show a positive response (Oh et al., 1999).

2.2.4 Reproductive toxicity

At its fifty-first meeting, the Committee reviewed a number of studies of reproductive and developmental toxicity with stevioside and *Stevia* extracts and noted that administration of stevioside (purity, 90–96%) at doses of up to 2500 mg/kg bw per day in hamsters and 3000 mg/kg bw per day in rats had no effect. The Committee also noted that, although an aqueous infusion of *S. rebaudiana* administered orally to female rats was reported to cause a severe, long-lasting reduction in fertility, the contraceptive effect of *Stevia* was probably not due to stevioside. Stevioside (purity, 95.6%) had neither teratogenic nor embryotoxic effects at doses of up to 1000 mg/kg bw per day in rats treated by gavage. At its present meeting, the Committee reviewed two additional studies.

Rats

Ten male Wistar rats (aged 25–30 days) were each given 2 ml of a crude aqueous extract of *S. rebaudiana* (corresponding to 0.67 g of dried leaves per ml), by gastric intubation, daily for 60 days. Ten control animals received saline only. There were no significant effects on food consumption or body-weight gain. Animals treated with *Stevia* extract showed decreased relative weights of the cauda epididymides, seminal vesicles and testes, accompanied by a reduction in plasma concentration of testosterone and in numbers of spermatazoa in the cauda epididymidis. The fructose content of the prostate and seminal vesicle was also decreased, which was considered by the author to be caused at least in part by a deficiency in testosterone stimulation (Melis, 1999).

Chickens

On day 7 of incubation, fertile broiler eggs were injected with 0.08–4.00 mg of stevioside (purity, >96%) or 0.025–1.25 mg of steviol (purity, >98%). The chicks were examined at hatching and 1 week later. There were no effects on embryonic mortality, body weight, malformations or body-weight gain during the first week after hatching. No stevioside or steviol was detected in the blood of the hatchlings sacrificed at age 1 day (Guens et al., 2003c).

2.2.5 Special study: effects on human microflora

Forty mg of stevioside (purity, 85%) and 40 mg of rebaudioside A (purity, 90%) were incubated for 72 h under anaerobic conditions with 40 ml of faecal bacterial suspensions from eleven healthy volunteers (six men and five women). Stevioside and rebaudioside A did not significantly influence the composition of faecal cultures. However, stevioside caused a weak inhibition of the growth of anaerobic

bacteria, while rebaudioside A caused a weak inhibition of the growth of aerobic bacteria, particularly coliforms (Gardana et al., 2003).

2.3 Observations in humans

In a multicentre randomized, double-blind, placebo-controlled trial of hypertensive Chinese men and women (aged 28–75 years), 60 patients were given capsules containing 250 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 750 mg of stevioside per day (equivalent to 12.5 mg/kg bw per day, assuming an average body weight of 60 kg) and followed up at monthly intervals for one year. Forty-six patients were given a placebo. After 3 months, systolic and diastolic blood pressure in men and women receiving stevioside decreased significantly and the effect persisted over the year. Blood biochemistry parameters, including lipids and glucose, showed no significant changes. Three patients receiving stevioside and one receiving the placebo withdrew from the study as a result of side-effects (nausea, abdominal fullness, dizziness). In addition, four patients receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Chan et al., 2000).

A follow-up multicentre randomized, double-blind placebo-controlled trial was conducted in hypertensive Chinese men and women (aged 20–75 years). Eighty-five patients were given capsules containing 500 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 1500 mg of stevioside per day (equivalent to 25 mg/kg bw per day, assuming an average body-weight of 60 kg). Eighty-nine patients were given a placebo. Three patients in each group withdrew during the course of the study. There were no significant changes in body mass index or blood biochemistry parameters throughout the study. In the group receiving stevioside, mean systolic and diastolic blood pressure was significantly decreased compared with the baseline, commencing from about 1 week after the start of treatment. After 2 years, 6 out of 52 patients (11.5%) in the group receiving stevioside had left ventricular hypertrophy compared with 17 of 50 patients (34%) in the group receiving the placebo ($p < 0.001$). Eight patients in each group reported minor side-effects (nausea, dizziness and asthenia), which led two patients in each group to withdraw from the study. Four patients in the group receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Hsieh et al., 2003).

In a paired cross-over study, 12 patients with type-2 diabetes were given either 1 g of stevioside (stevioside, 91%; other *Stevia* glycosides, 9%) or 1 g of maize starch (control group), which was taken with a standard carbohydrate-rich test meal. Blood samples were drawn at 30 min before and for 240 min after ingestion of the test meal. Stevioside reduced postprandial blood glucose concentrations by an average of 18% and increased the insulinogenic index by an average of 40%, indicating beneficial effects on glucose metabolism. Insulin secretion was not significantly increased. No hypoglycaemic or adverse effects were reported by the

patients or observed by the investigators. Systolic and diastolic blood pressure was not altered by stevioside administration (Gregersen et al., 2004).

Forty-eight hyperlipidaemic volunteers were recruited to a randomized, double-blind trial designed to investigate the hypolipidaemic and hepatotoxic potential of steviol glycoside extract. The extract used in this study was a product containing stevioside ($73 \pm 2\%$), rebaudioside A ($24 \pm 2\%$) and other plant polysaccharides (3%). The subjects were given two capsules, each containing 50 mg of steviol glycoside extract or placebo, twice daily (i.e. 200 mg/day, equivalent to 3.3 mg/kgbw per day assuming an average body weight of 60 kg), for 3 months. One volunteer receiving placebo, and three volunteers receiving steviol glycoside failed to complete the study for personal reasons, not related to adverse reactions. At the end of the study, both groups showed decreased serum concentrations of total cholesterol and of low density lipoproteins. Analyses of serum concentrations of triglycerides, liver-derived enzymes and glucose indicated no adverse effects. The authors questioned the subjects' compliance with the dosing regime, in view of the similarity of effect between treatment and placebo (Anonymous, 2004a). In a follow-up study, 12 patients were given steviol glycoside extract in incremental doses of 3.25, 7.5 and 15 mg/kgbw per day, for 30 days per dose. Preliminary results indicated no adverse responses in blood and urine biochemical parameters (Anonymous, 2004b).

3. INTAKE

3.1 Introduction

The Committee evaluated information on exposure to steviol glycosides submitted by Japan and China. Additional information was taken from a report on *S. rebaudiana* Bertoni plants and leaves that was prepared for the European Commission by the Scientific Committee on Food (European Commission, 1999). All of the intake results are presented in terms of equivalents of steviol, based on a conversion of 40% from steviol glycosides.

3.2 Use in foods

Steviol glycosides are used to sweeten a number of foods in China, Japan, and South America. Table 3 summarizes the information submitted to the Committee.

It is also known that *Stevia* leaves are used to prepare a sweetened tea in a number of countries throughout the world. The concentrations of steviol glycosides in these teas are likely to be lower than those reported in Table 3.

3.3 International estimates of intake

The WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme (GEMS/Food) database was used by the Committee to prepare international estimates of intake of steviol glycosides

Table 3. Food use levels of steviol glycosides reported to the Committee

Food type	Maximum use level reported (mg/kg)
Beverages	500
Desserts	500
Yogurt	500
Cold confectionery	500
Sauces	1000
Pickles	1000
Delicacies	1000
Sweetcorn	200
Bread	160
Biscuits	300

(as steviol). It was assumed that steviol glycosides would replace all sweeteners used in or as food, reflecting the minimum reported relative sweetness of steviol glycosides and sucrose of 200:1. The estimates are shown in Table 4.

These estimates are conservative in that it is very unlikely that a user of steviol glycosides would replace all commodity sweeteners found in their diets (WHO, 2003).

3.4 National estimates of intake of steviol glycosides

Japan submitted an estimate of intake of steviol glycosides per capita based on the total demand for steviol glycosides in Japan, estimated at 200 tonnes per year. The estimate assumed a population of 120 million persons and an average body weight of 50kg. The resulting estimate of intake of steviol glycosides (as steviol) was 0.04 mg/kgbw per day.

Additionally, the Japanese submission included two 'maximum' consumption estimates for steviol glycosides. These assumed that 10% of all added sugar in the diets of Japan or the USA would be replaced by steviol glycosides, at a ratio of 200:1, based on sweetness. The consumption of sugar in Japan was taken as 25kg/person per year, while that in the USA was 125 pounds/person per year (57kg/person per year). The average body weight for both Japan and the USA was assumed to be 50kg. The resulting estimates of maximum consumption of steviol glycosides (as steviol) were 0.3mg/kgbw per day for Japan and 0.6mg/kgbw per day for the USA. The Committee concluded that there was no evidence to suggest that only 10% of sugar consumed would be replaced. Therefore, the Committee calculated 'maximum' intakes based on the replacement of all sugar in diets in Japan and the USA, resulting in estimates of 3mg/kgbw per day for a 50kg consumer in Japan and 5mg/kgbw per day for a 60kg consumer in the USA.

Table 4. International estimates of intakes of steviol glycosides as steviol

Food code	Food type (CM)	GEMS/Food diet														
		Middle Eastern			Far Eastern			African			Latin American			European		
		CM	SG		CM	SG		CM	SG		CM	SG		CM	SG	
GS659	Refined sugar		73 (g/person per day)	2.4	43	1.4	25.5	0.8	97.3	3.3	96.8	3.3				
GS659	Total sugar and honey		95.8 (g/person per day)	3.2	50.5	1.6	42.7	1.3	104.3	3.5	107.3	3.5				

CM, commodity sweetener (refined sugar or total sugar and honey); SG, steviol glycosides. CM intakes are given in grams per person per day, while SG intakes are given in mg/kgbw per day, using a factor of 200 for the relative sweetness and assuming a body weight of 60 kg.

Table 5. Summary of estimates of intakes of steviol glycosides (as steviol)

Estimate	Intake (mg/kgbw per day)
GEMS/Food (international) ^a	1.3–3.5 (60 kg person)
Japan, per capita	0.04
Japan, maximum consumption ^b	3
USA, maximum consumption ^b	5

GEMS/Food, WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.

^a 'International' refers to the international estimates presented in Table 4.

^b These estimates were prepared in parallel to those for the international estimates: it was assumed that all dietary sugars in diets in Japan and the USA would be replaced by steviol glycosides, at a ratio of 200:1.

The submission from China contained information on the annual production of steviol glycosides. It was reported that up to 1000 tonnes were produced each year, with 200 tonnes retained for domestic consumption. In view of the larger population in China than in Japan or the USA, the Committee noted that any estimates prepared using these data would result in lower exposures than those reported above.

3.5 Summary of intakes

Table 5 contains a summary of the intakes of steviol glycosides evaluated or derived by the Committee.

The Committee concluded that the replacement estimates were highly conservative and that intake of steviol glycosides (as steviol) would be likely to be 20–30% of these values.

4. COMMENTS

After oral administration, steviol glycosides are poorly absorbed in experimental animals and in humans.

Intestinal microflora metabolize steviol glycosides to the aglycone, steviol, by successive hydrolytic removal of glucose units. Data reviewed by the Committee at its current and fifty-first meetings (Annex 1, reference 149) indicated that this process is similar in rats and humans. The hydrolysis of rebaudioside A to steviol was slower than that of stevioside. In humans treated orally with stevioside, small amounts of steviol were detected in the plasma, with considerable interindividual variability. The major route by which steviol is metabolized in humans *in vivo* appears to be via conjugation with glucuronide and/or sulfate. Studies with liver microsomal preparations indicated that steviol is also metabolized to a number of hydroxy and dihydroxy derivatives via CYP-dependent pathways.

Stevioside and/or steviol affected a variety of biochemical parameters in models *in vitro*, indicating possible mechanisms of antihypertensive and antiglycaemic effects that involve modulation of ion channels. High concentrations (e.g. 1 mmol/l) of stevioside were required to produce a maximal increase in insulin secretion, while steviol was effective at a concentration that was about three orders of magnitude lower. Stevioside also affected a variety of biochemical parameters in different animal species *in vivo*, mostly with parenteral administration; these studies were considered by the Committee to be of limited relevance to dietary exposure.

No new long-term studies of toxicity or carcinogenicity were available at the present meeting. At its fifth-first meeting, the Committee noted that oral administration of stevioside (purity, 95.6%) at a dietary concentration of 2.5%, equal to 970 and 1100 mg/kgbw per day in male and female rats, respectively, for 2 years was not associated with toxicity. Reduced body-weight gain and survival rate were observed with stevioside at a dietary concentration of 5%. In a new study, stevioside was found to inhibit the promotion of skin tumours by TPA in a model of skin carcinogenesis in mice.

The Committee reviewed new data on genotoxicity that, considered together with data reviewed by the Committee at its fifth-first meeting, allowed a number of conclusions to be drawn. Stevioside and rebaudioside A have not shown evidence of genotoxicity *in vitro* or *in vivo*. Steviol and some of its oxidative derivatives show clear evidence of genotoxicity *in vitro*, particularly in the presence of a metabolic activation system. However, studies of DNA damage and micronucleus formation in rats, mice and hamsters *in vivo* indicate that the genotoxicity of steviol is not expressed at doses of up to 8000 mg/kgbw.

One new study of developmental toxicity was available at the present meeting. Adverse effects on the reproductive apparatus, which could be associated with impaired fertility, were observed in male rats given a crude extract of *S. rebaudiana*, at a dose corresponding to 1.34 g of dried leaves. However, at its fifth-first meeting, the Committee reviewed a number of studies of reproductive and developmental toxicity with stevioside (purity, 90% or 96.5%). Doses of up to 2500 mg/kgbw per day in hamsters and 3000 mg/kgbw per day in rats had no effect in studies of reproductive toxicity. No teratogenic or embryotoxic effects were observed in rats given stevioside at a dose of up to 1000 mg/kgbw per day by gavage. The Committee considered that the adverse reproductive effects associated with administration of aqueous extracts of *S. rebaudiana*, noted at the present and fifty-first meeting, were unlikely to be caused by steviol glycosides.

Stevioside is being investigated as a potential treatment for hypertension and diabetes. Administration of stevioside at a dose of 750 or 1500 mg per day for 3–24 months resulted in decreased blood pressure in hypertensive patients, with no adverse effects. These studies, in a limited number of subjects, provided some reassurance that stevioside at a dose of up to 25 mg/kgbw per day (equivalent to 10 mg/kgbw per day expressed as steviol) for up to 2 years shows no evidence of significant adverse effects in these individuals. There is no information on the effects of repeated administration of stevioside on blood pressure in normotensive individuals. A small study in 12 patients with type-2 diabetes showed that a single

dose of 1 g of stevioside reduced postprandial glucose concentrations and had no effect on blood pressure.

The Committee evaluated information on intake of steviol glycosides, submitted by Japan and China. Additional information was available from a report on *S. rebaudiana* Bertoni plants and leaves that was prepared for the European Commission by the Scientific Committee on Food. All the intake results are presented in terms of equivalents of steviol, based on a conversion of 40% from the steviol glycoside, stevioside (relative molecular mass: steviol, 318, stevioside, 805).

The Committee used the GEMS/Food database to prepare international estimates of intake of steviol glycosides (as steviol). It was assumed that steviol glycosides would replace all dietary sugars, at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, 200:1. The intakes ranged from 1.3 mg/kgbw per day (African diet) to 3.5 mg/kgbw per day (European diet).

The Committee evaluated estimates of per capita intake derived from disappearance (poundage) data supplied by Japan and China. The Committee also evaluated estimates of intake of steviol glycosides based on the replacement of all dietary sugars in the diets for Japan and the USA. These results are summarized in Table 5.

The Committee concluded that the replacement estimates were highly conservative and that intake of steviol glycosides (as steviol) would be likely to be 20–30% of these values.

5. EVALUATION

The Committee noted that most of the data requested at its fifty-first meeting, e.g. data on the metabolism of stevioside in humans, and on the activity of steviol in suitable studies of genotoxicity *in vivo*, had been made available.

The Committee concluded that stevioside and rebaudioside A are not genotoxic *in vitro* or *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*. The no-observed-effect level (NOEL) for stevioside was 970 mg/kgbw per day in a long-term study evaluated by the Committee at its fifty-first meeting.

The Committee noted that stevioside has shown some evidence of pharmacological effects in patients with hypertension or with type-2 diabetes at doses corresponding to about 12.5–25 mg/kgbw per day (equivalent to 5–10 mg/kgbw per day expressed as steviol). The evidence available at present was inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g. those with hypotension or diabetes). The Committee therefore decided to allocate a temporary acceptable daily intake (ADI), pending submission of further data on the pharmacological effects of steviol glycosides in humans.

A temporary ADI of 0–2 mg/kgbw was established for steviol glycosides, expressed as steviol, on the basis of the NOEL for stevioside of 970 mg/kgbw per day (or 383 mg/kgbw per day expressed as steviol) in the 2-year study in rats and

a safety factor of 200. This safety factor incorporates a factor of 100 for inter- and intraspecies differences and an additional factor of 2 because of the need for further information. The Committee noted that this temporary ADI only applies to products complying with the specifications.

The Committee required additional information, to be provided by 2007, on the pharmacological effects of steviol glycosides in humans. These studies should involve repeated exposure to dietary and therapeutic doses, in normotensive and hypotensive individuals and in insulin-dependent and insulin-independent diabetics.

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