

SAFETY EVALUATION OF *STEVIA* AND STEVIOSIDE.

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ABSTRACT: The literature about *Stevia* and stevioside used as a sweetener is discussed. Injection experiments or perfusion experiments of organs are considered as not relevant for the use of *Stevia* or stevioside as food, and therefore these studies are not included in this safety evaluation.

The metabolism of stevioside is discussed in relation with the possible formation of steviol. Different mutagenicity studies as well as studies on carcinogenicity are discussed. Acute and subacute toxicity studies revealed a very low toxicity of *Stevia* and stevioside. A survey is given of calculated ADI's. Fertility and teratogenicity studies are discussed as well as the effects on the bio-availability of other nutrients in the diet.

The conclusion is that *Stevia* and stevioside are safe when used as a sweetener. It is suited for both diabetics, and PKU patients, as well as for obese persons intending to lose weight by avoiding sugar supplements in the diet. No allergic reactions to it seem to exist.

INTRODUCTION: *STEVIA* and STEVIOSIDE

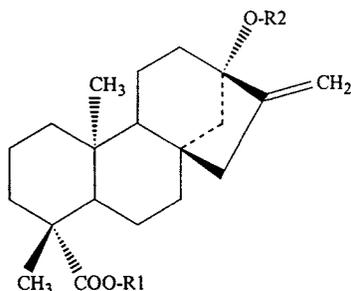
Stevia rebaudiana Bertoni is a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America (Paraguay and Brazil). It is known to the Guarany people, native to these regions since time immemorial, by several names all of which refer to the sweet taste of the leaf, and especially to its use in "mate" tea (*Ilex paraguariensis*). It is often referred to as "the sweet herb of Paraguay".

The Spanish Conquistadors of the Sixteenth Century sent back news to Spain that the indigenous population used *Stevia* to sweeten their herbal teas since ancient times, i.e. predating 1500 AD.

Stevia has been cultivated or is still cultivated in many countries: Paraguay, the USA, Mexico, Central America, Japan, China, Malaysia, South Korea, Spain, Italy, Belgium and the UK.

The main sweet component in the leaves of *Stevia rebaudiana* Bertoni is stevioside **3** (see Fig.(1)). Its content varies between 4 and 20 % of the

dry weight of the leaves, and around 11% in many economical crop productions. Other compounds present but in much lower concentration are: dulcoside A **9** (± 0.5 %), steviolbioside **2** (trace), rebaudioside A **4** (± 3 %), B **5** (trace), C **6** (± 1.5 %), D **7** and E **8** (traces) (See Fig.(1)).



	Compound name	R1	R2
1	steviol	H	H
2	steviolbioside	H	β Glc2-1 β Glc
3	stevioside	β Glc	β Glc2-1 β Glc
4	rebaudioside A	β Glc	β Glc2-1 β Glc 3-1 β Glc
5	rebaudioside B	H	β Glc2-1 β Glc 3-1 β Glc
6	rebaudioside C (dulcoside B)	β Glc	β Glc2-1 α Rha 3-1 β Glc
7	rebaudioside D	β Glc2-1 β Glc	β Glc2-1 β Glc 3-1 β Glc
8	rebaudioside E	β Glc2-1 β Glc	β Glc2-1 β Glc
9	dulcoside A	β Glc	β Glc2-1 α Rha

Fig. (1). Structures of stevioside and related compounds. In rebaudioside A, B, C, D and E an additional sugar moiety is added on carbon 3 of the first β Glc.

A typical composition on a dry weight basis of the most important components of the leaves is as follows:

- proteins: ± 6.2 %
- lipids: ± 5.6 %
- total carbohydrates (anthrone): ± 53 %
- stevioside: ± 11 %
- rebaudioside A: ± 2 %
- rebaudioside C: ± 2 %

Stevioside is a diterpene glycoside occurring in *Stevia rebaudiana* Bertoni leaves. It is a high intensity sweetener that is about 300 times

sweeter than sucrose. In many countries it is used as a low calorie sweetener in a wide range of food products and beverages. Both the plant, its extracts, and stevioside have been used for several years as a sweetener in South America, Asia, Japan, China and even in the USA it is used as a dietary supplement since 1995.

Remarks to toxicological studies: Neither those scientific studies where *Stevia* extract or solution of pure stevioside were injected in animals, nor those studies employing perfusion experiments of organs, are considered relevant for the use of *Stevia* or stevioside as food and are not discussed in this review.

METABOLISM OF STEVIOSIDE

Compounds used as or added to food must be absolutely safe. This means that not only the added parent compound, but also its possible metabolites must be safe, for the possibility exists that compounds that themselves are not harmful can be taken up by the human body and be metabolised into products that may have some harmful effects. Therefore, the parent compounds as well as their metabolites should be thoroughly tested in toxicological studies. In one such study, steviol, the aglycone of stevioside, showed a weak mutagenic activity [1] and, although in later experiments these results could not be reproduced [2], this has led to a whole controversy in scientific literature.

It has been shown that stevioside is not taken up by the human body and none of the digestive enzymes from the gastro-intestinal tract of different animals and man are able to degrade stevioside into steviol 1, the aglycone of stevioside [3]. The lack of metabolism is due to the fact that the bonds in stevioside are β -glycosidic and we do not have the enzymes to split these β -glycosidic bonds. Stevioside was incubated with salivary α -amylase, pancreatic α -amylase, saliva, pepsin, gastric secretion, pancreatin and intestinal brush border membrane enzymes of mice, rats and hamsters. None of these enzymes digested stevioside. Nevertheless, in feeding experiments with rats and hamsters stevioside was metabolised to steviol by the bacterial flora of the caecum. After several hours steviol was found in the blood of the animals, the maximum concentration occurring after 8 hours [4]. In rodents coprophagy occurs (this means that rodents eat their own faeces and in this way they reabsorb nutrients set free by the bacteria of the caecum). In the cited studies, it was not indicated that

coprophagy was prevented, so it is not clear if the steviol occurring in the blood was taken up directly from the colon or indirectly from the ingested faeces (after passing through the intestines again). Although bacteria isolated from the human colon are able to transform stevioside into steviol *in vitro* [3], it has never been proven that this is also the case *in vivo* nor that the steviol eventually formed is taken up directly from the colon. Moreover, studies with roosters [5] indicate that stevioside is rapidly eliminated from the body, largely untransformed. Roosters resemble humans as they too have a low functioning caecum.

Only the bacteria from the caecum or colon were able to degrade stevioside into steviol (caecum of mice, rats and hamsters; colon of man). The bacteria from the human colon also formed steviol epoxid *in vitro*, that was again metabolised to steviol. However, *in vivo* this epoxid formation probably will not occur due to the anaerobic conditions of the human colon. It was correctly concluded that steviol is the only possible metabolite [3]. Anyway, steviol epoxid has been tested in mutagenicity studies and showed to be inactive [1].

MUTAGENICITY STUDIES AND CARCINOGENICITY

Each new compound in the food chain has to be extensively tested to be sure that it is not carcinogenic. As studies with animals take several years and become expensive when many compounds need to be tested, so called mutagenicity tests were developed that are much faster and less expensive. In these tests compounds are evaluated to see if they provoke mutations or alterations of hereditary material. It should be emphasised that compounds giving a positive response in mutagenicity tests are not carcinogenic *in se*. Compounds having a positive score should be tested for carcinogenic activity, but not all mutations lead to cancer. This can be exemplified by the following examples. A point mutation in the gene that codes for the growth hormone receptor in chickens causes dwarf mutants (bantams). A dominant gene produces hairs on the mid-phalanx of the ring finger and its recessive mutation causes lack of hair. In a population about one fourth of the people have the recessive mutation (no hair) but this does not mean that they will have cancer because of this mutation.

Mutagenicity tests

In the *Ames test* or *bacterial reverse mutation test*, use is made of mutated bacteria that eg. cannot form an amino acid histidine (his⁻). Therefore, these bacteria are unable to grow on a medium lacking histidine. When these bacteria are grown on such a medium, most bacteria will die, but in some bacteria a natural reverse mutation occurs, i.e. the original mutation is repaired by a reverse mutation and these bacteria can again form histidine and can grow and form colonies on the medium without histidine. Mutagenic compounds will enhance the number of reverse mutations occurring. As many compounds are mutagenic only after metabolism in the body, the test is also done after adding a so-called metabolic activation system. This is a liver supernatant fraction (S9000g) of animals pretreated with known harmful substances (eg. polychlorinated biphenyls). This S9000g fraction will ensure the further metabolism of the compounds added in the Ames test.

In the *forward mutation test*, bacteria are added to a medium containing a harmful substance (eg. 8-azaguanine). Under these conditions most of the bacteria will die, but some will survive because they have adapted their genotype by mutation. In the forward mutation test the development of resistance (by mutation) above the control mutation frequency is studied in the presence of the test compound. This is also done in the presence and absence of the metabolic activation system.

In the *micronucleus test*, mice are given the test substance by intraperitoneal injection. Mice are killed 24 or 48 h after treatment and the femoral marrow cells are smeared on glass slides, fixed, stained and the numbers of micronucleated polychromatic erythrocytes and of micronucleated normochromatic erythrocytes are recorded. The numbers of micronucleated erythrocytes and the proportion of polychromatic erythrocytes relative to the total erythrocytes are evaluated by observing 1000 erythrocytes on the same slide.

In the *chromosome mutation test* Chinese hamster lung fibroblast cell line (CHL) can be used. The cells are treated for 24 or 48 hours with a test compound at different dose levels. Both with and without the metabolic activation system, the cells are treated with colcemid (0.2 mg/ml) for 2 h to arrest cell divisions and chromosome preparations are made. The chromosome aberrations are recorded in at least 100 metaphases of each treatment. Five groups of structural chromosome aberrations can be found:

chromatid and chromosome gaps, chromatid breaks, chromatid exchanges, chromosome breaks and chromosome exchanges.

In the *mammalian cell mutation test* CHL cells are exposed to the test compound for 3 h with and without the metabolic activation system. After 7 days of culture, diphtheria toxin is added and the cells are left again to culture for another 7 days until the number of diphtheria toxin resistant colonies are scored.

In 1985 it was published [1] that stevioside was completely safe but that metabolically activated steviol was mutagenic in a "Forward Mutation Test". Steviol had to be applied together with the microsomal fraction of liver of animals treated with carcinogenic compounds (polychlorinated biphenyl or phenobarbital plus 5,6-benzoflavone). This publication has led to a confusing discussion between advocates and opponents of the use of *Stevia* or stevioside. To unravel the problem we must consider the opinion of authoritative international organisations such as OECD (Organisation for Economic Co-Operation and Development) and ICH (International Council of Harmonisation). To accept new substances as food three different mutagenesis tests are accepted and required by the OECD as well as by the ICH. These can be seen in Table 1.

Table 1: Mutagenesis tests required by the OECD and ICH.

Test Type	Recommended Test
1 Bacteria <i>in vitro</i> test (so-called Ames-Test)	"Bacterial reverse mutation" Test with <i>Salmonella typhimurium</i> TA 1535, TA1537 or TA97 or TA97a, TA98, TA100, <i>E coli</i> WP2uvrA or <i>E coli</i> WP2uvrA/pKM101 or TA102
2 Non bacterial <i>in vitro</i> tests	"Mammalian cell mutation" Test with the following Test-System: "L5178Y mouse lymphoma gene and chromosomal mutation test" or "Chromosomal aberration" Test with e.g. "human lymphocytes"
3 <i>In vivo</i> Tests	"Micronucleus" with bone marrow of mice

During the last years several mutagenicity tests were done with steviol. Steviol did not show any mutagenic activity in the standard tests executed following the prescribed protocol:

- 1) It was inactive in the Ames test with or without metabolic activation [2, 6, 7],
- 2) It did not show any mutagenic action on chromosome aberrations of human lymphocytes [6],
- 3) It did not induce micronuclei in bone marrow of mice [8].

The other tests systems used were not standard prescribed tests. The positive mutagenic results for steviol shown by [1] were contradicted by [2] who were unable to reproduce Pezzuto's results. The positive results by [1] were shown to be due to a wrong experimental setup (a too low number of bacteria used : 10^6 instead of the prescribed 10^8) and to a misinterpretation of the results. It was shown that so far unknown steviol metabolites in *Salmonella typhimurium* TM677 after metabolic activation caused mutations, i.e. transitions, transversions, duplications and deletions at the guanine phosphoribosyltransferase (gpt) gene [9]. However, steviol was completely negative in the reverse mutation assays using *Escherichia coli* WP2uvrA/pKM101 or using different *S. typhimurium* TA strains even when activation S9 mix was present [9].

After metabolic activation of steviol some gene mutation and chromosomal aberration was found in Chinese hamster lung (CHL) fibroblasts [8]. It has to be said that of all animals tested hamsters had the most sensitive response. Moreover, in hamster several metabolites of stevioside were found that are not formed in rats or humans. Therefore, the relevance of experiments with hamsters should be questioned.

It has been shown that steviol does not bind to DNA, as claimed by the authors an absolute prerequisite to have any mutagenic activity [10]. Anyway, it would be very peculiar that steviol would have a mutagenic activity, while it only differs from its direct precursor *ent*-kaurenate by a hydroxyl at Carbon-13 and that itself is not mutagenic. All plants contain the precursor *ent*-kaurenate and other very similar compounds, including the gibberellins, a group of natural plant hormones, that are not mutagenic and of which many contain also a 13-hydroxyl function. These compounds are daily consumed by the whole world population as they are present in all vegetables.

Male and female F344 rats were daily fed with a ration containing 0.1, 0.3 or 1 % of stevioside and rebaudioside for a period of 22 (males) or 24 (female rats) months [11]. The animals were then killed, and the researchers conducted biochemical, anatomic, patho-logical and carcinogenic tests on 41 organs following autopsy. In addition they performed ongoing hematologic and urine tests on the same animals. Each of the animals was matched to a control animal that experienced exactly the same treatment, except for the stevioside. No significant dose-related changes were found in the growth, general appearance, hematological and blood biochemical findings, organ weights, and macroscopic or microscopic observations (41 organs were analysed). It was also

concluded that any neoplasms that occurred were not attributable to the administration of stevioside. Even at the highest dose of 1% no significant effects were found. This high dose is equivalent to 125 times the average daily dose of sweeteners that a normal human would require.

The effects of a chronic oral feeding of stevioside were studied in rats during 24 months [12]. The concentrations used were: 0, 0.2, 0.6 and 1.2%. Growth, food utilisation and consumption, general appearance and mortality were similar in treated and control groups. The mean lifespan of rats given stevioside was not significantly different from that of the controls. No treatment-related changes were observed in haematological, urinary or clinical biochemical values at any stage of the study. The incidence and severity of non-neoplastic and neoplastic changes were unrelated to the level of stevioside in the diet. The maximum no-observed-effect level of stevioside was 1.2%, and this was the highest concentration tested by the authors. The authors suggested an acceptable daily intake (ADI) of stevioside for humans of 7.938 mg/kg body wt/day (safety factor 100). For a person of 70 kg this is about 555 mg pure stevioside. However, no higher concentrations were tested and therefore the suggested acceptable daily intake does not mean that a higher intake would be harmful! Therefore, this ADI has to be considered as a minimum ADI.

In a chronic toxicity study with F344 rats [13] it was concluded that there were no significant increases in the incidence of neoplastic lesions in any organ of tissue in the stevioside treated groups (2.5 and 5 %, i.e. daily dose of 385 and 775 mg per rat, i.e. 1 and 2 g/kg body weight/day, which is a very high dose not far from the LD50!). In male animals the number of testicular tumours had the tendency to decrease. Moreover, the incidence of adenomas of the mammary gland in the stevioside-treated females was significantly lower than that in the controls. The severity of chronic nephropathy in males was also clearly reduced by both stevioside concentrations.

The JECFA clearly stated [14]:" Stevioside has a very low acute oral toxicity. Oral administration of stevioside at a dietary concentration of 2,5% to rats for two years, equal to 970 and 1100 mg/kg body weight (BW) per day in males and females, respectively, had no significant effect. Reduced body-weight gain and survival rate were observed at a dietary concentration of 5% stevioside. There was no indication of carcinogenic potential in a long-term study..."

Also in favour of the use of stevioside is a study by [14] who found that stevioside did not promote urinary bladder carcinogenesis in rats.

There have never appeared reports proving that the use of *Stevia* or stevioside enhances the number of cancers in populations, even after a very long time of use (eg. Paraguay: more than 500 years, Japan: more than 25 years, South-Korea: 15 years, Brasil: 12 years, China: 11 years or the USA: since 1995 admitted as a dietary supplement). Thus the general conclusion is that *Stevia* and its sweeteners stevioside and rebaudioside A are completely safe.

ACUTE TOXICITY

Dead is an unambiguous criterion. In acute toxicity tests the dose is determined at which half of the animals die (so-called LD50 or lethal dose 50%).

Stevioside and steviol have a very low acute oral toxicity in the mouse, rat and hamster, meaning that the LD50 value is large [16, 17]. Stevioside at a dose as high as 15g/kg BW was not lethal to either mice, rats or hamsters. Hamsters were found to be more susceptible to steviol, the aglycone of stevioside, than rats or mice. LD50 values of steviol in hamsters were 5.2 and 6.1 g/kg BW for males and females, respectively. In rats and mice, LD50 values of steviol were higher than 15g/kg BW in both sexes [17].

An oral LD50 of 17 g/kg body weight was shown for the *Stevia* extract (20 % of stevioside) and 15 g/kg for purified stevioside (purity of 93.5%). With rats and mice a LD50 of 8.2 g/kg was found for stevioside by oral intake, and given intraperitoneally, a LD50 of 2.99 g/kg of body weight [18].

As stevioside is 300 times sweeter than sugar, a LD50 of 8.2 g/kg corresponds to about 2.5 kg sugar/kg body weight!

SUBACUTE TOXICITY, CHRONIC TOXICITY

In subacute toxicity studies lower concentrations are given to determine the so-called NOEL (No Effect Level), i.e. the maximum concentration at which no effect of the applied substance can be seen. This NOEL is important to determine the ADI (acceptable daily intake, see Table 2). This is done by dividing the NOEL by 100 (a factor of 10 to pass from animals to man and a safety factor of 10x). In the subacute toxicity studies

the test compounds are given during long time periods, hence "chronic toxicity studies".

Subacute toxicity studies were performed with purified stevioside supplied to rats' rations in concentrations up to 7% daily during 3 months [19]. In other studies rats were fed with 2.5g/kg/day of purified stevioside, also by adding it to their daily ration during 3 months [18]. Both studies did not produce any effect related with the stevioside doses on the animals tested. This means that the NOEL is higher than 2.5g/kg/day and the ADI can be calculated to be at least 25 mg/kg body weight.

Stevioside was fed to F344 rats in doses of 0.625, 1.25 and 2.5 g/kg body weight during 13 weeks [20]. The authors concluded that there was a lack of any clear response and concluded that 2.5 g stevioside/kg body weight was a suitable maximum tolerable dose. From these results an ADI of 25 mg/kg can be calculated.

Male and female hamsters were daily force-fed with stevioside (0, 0.5, 1 and 2.5 g/kg body wt./day respectively) [21]. This was done over several generations. The authors concluded that stevioside at a dose as high as 2.5 g/kg body weight/day did not affect growth nor reproduction in hamsters. Also from these results an ADI of 25 mg/kg can be calculated.

Table 2: Survey of ADI's for stevioside calculated from published NOELs.

ADI (mg/kg body weight/day)	Animal	NOEL g/kg BW/d	Reference
7.938*	Wistar rat	0.794*	[12]
25	rat	2.5	[19]
25	rat	2.5	[18]
25	rat F344	2.5	[20]
25	hamster	2.5	[21]
21 (males)	Wistar rat	2.1	[22]
24 (females)	Wistar rat	2.4	[22]
6.25**	hamster	**	[23]

* The ADI calculated by [12] is a minimum value as the authors did not test higher concentrations of stevioside.

** This ADI is calculated from the NOEL of steviol (250 mg/kg BW/day) under very unfavourable conditions as steviol is easily taken up by the intestines and metabolised to various unknown compounds, whereas stevioside is not. Moreover, hamsters are known to be very sensitive animals towards steviol and stevioside.

FERTILITY and TERATOGENICITY

Although it was reported [24] that a water decoction of *Stevia* leaves was used as an oral contraceptive by Paraguayan Matto Grosso Indian tribes and that these decoctions reduced the fertility in adult female rats of proven fertility, subsequent studies could not reproduce their results. Moreover, [25] reported that the use of *Stevia* as a contraceptive has never been confirmed [19, 26] and in a field study made throughout Paraguay not any local use of *Stevia* as a contraceptive could be found. Inquiries were made in Asunción, Concepción, Pedro Juan Caballero, and in Cerro Kuatiá. In the last mentioned location, interviews were conducted with members of the native Indian group, Pa'y Tav'y Terá, as well as other populations that have recently settled in the area.

The fact that *Stevia* and stevioside do not have any effect on fertility or reproduction is illustrated by the work of the following authors.

High doses of 525 mg/kg/day of stevioside equivalent aqueous *Stevia* extract were administered to male and female mice during copulation and pregnancy periods [19]. The authors could not observe any difference in the copulation and conception averages and any change in the fetus and broods when compared with control groups.

Male and female Wistar rats received feed containing 0.15 %, 0.75% and 3% stevioside before and at the start of pregnancy [22]. Consumption of stevioside in the 3 treated groups was 100, 480 and 2100 mg/kg/day in males and 120, 530 and 2400 mg/kg/day in females (i.e. about 20x, 100x and 400x times the ADI). Males received stevioside from 60 days before mating and females from 14 days before mating to day 7 of pregnancy. No differences could be found between test and control groups regarding sexual cycle, mating or pregnancy, nor was there any abnormality in the test groups regarding implantation and survival of the fetus. There were no abnormalities found in the growth, general appearance, viscera or skeleton of the fetus. The final body weight showed no differences between the groups, although a small reduction in body weight was observed at first in the 3% group in both sexes. This might have been due simply to an adaptation of the rats to the extreme sweetness of the feed, avoiding it in the beginning. The authors conclude that the addition of stevioside to feed up to a concentration of 3%, which is 400 times the ADI, has no effect on mating, fertility or the development and the state of fetuses in rats given the diet prior to and during the early stage of pregnancy.

No effects of concentrated leaf extracts (0.667 g dried leaves/ml, 2 ml/rat twice a day) were found on male fertility nor on the growth of prepubertal male rats [27].

The effects were studied of 0, 5, 25 and 100 % of *Stevia* extracts (= 2.6 % stevioside) (10 ml extract/kg BW per day for 31 days) on male Wistar rats that weighed 270 g at the start of the experiments [28]. The amounts of stevioside given were 0, 13, 65 and 260 mg/kg BW per day. Clinical examinations revealed that there was a total absence of any signs of intolerance. Not any influence could be found on the weight of the animals. No differences could be found in the weights of the male organs (seminal vesicles, prostate, hypo-physis, testicles). The anatomic-pathological examination of the testicles disclosed no evidence of any effect due to the treatment. No atrophy of the seminal tubes was found and the generation of semen was present in all the samples studied. The Leydig interstitial cells were not hyperplastic and did not show degenerative changes. No evidence of inflammatory infiltration was found.

One month old male and female hamsters were daily force-fed with stevioside (0, 0.5, 1 and 2.5 g/kg body wt./day respectively) [21]. No abnormalities were found in growth and fertility in both sexes. All males mated with females efficiently and successfully. Females showed normal 4-day oestrus cycles and became pregnant after mating. Each female was mated and allowed to bear three litters during the period of the experiment. The duration of pregnancy, number of fetuses, as well as number of young delivered each time from females in the experimental groups were not significantly different from those in the control group. The young F1 and F2 hamsters continuously receiving stevioside via drinking water until one month old and daily force-fed afterwards at the same doses as their parents showed normal growth and fertility. Histological examination of reproductive tissues from all three generations revealed no evidence of abnormality which could be linked to the effects of consuming stevioside. It was concluded that stevioside at a dose as high as 2.5 g/kg body weight/day affects neither growth nor reproduction in hamsters.

Toxicity to reproductive organs

Not any significant effect was found on spermatogenesis, nor on the interstitial cell proliferation and tumor formation in the testes [11].

Male rats were force fed (25-30-day-old) for 60 days with aqueous *S. rebaudiana* extracts corresponding to 0.667 g dried leaves/ml, 2 ml/rat twice a day [27]. This way each rat received the equivalent of about 2.668 g of dry *Stevia* leaves per day, i.e. 5.34 % of their body weight! (stevioside content not known). This is a very high amount: 53.4 g *Stevia* leaves/kg BW at the start of the experiments (rats weighing about 50 g) and about 13.75 g/kg BW at the end (rats weighing about 194 g). If we assume a stevioside content of 12% this means that the young animals received 6.4 g stevioside/kg BW and the older ones about 1.65 g/kg BW. The authors observed a decreased seminal vesicle weight by about 60 % but they concluded that if *Stevia* extract does have some potential to decrease rat fertility at all, this effect is almost certainly not exerted on the male. Unfortunately, only one high concentration was tested and no dose-effect studies were undertaken. In contrast to these results, it was reported [29] that concentrated *Stevia* extracts similar to those of [27] fed for 60 days to prepubertal male rats produced a decrease in final weight of testes, seminal vesicle and cauda epididymidis. In addition the fructose content of the accessory sex glands and the epididymal sperm concentration were decreased. *Stevia* extract treatment tended to decrease the testosterone level, probably by a putative affinity of glycosides of the extract for a certain androgen receptor. No alteration occurred in luteinizing hormone level. Whereas [29] suggested a possible decrease of the fertility of male rats, his results are in contradiction with those of [27] who applied extracts with similar stevioside content and these authors stated that there is certainly not an effect on male fertility. The difference between the extracts of [27] and [29] is that the last author completely dried the *Stevia* water extracts, and it is known that various chemical alterations may occur in the extract during drying, that probably were responsible for the observed effects. So it is not sure that the observed effects were indeed due to the stevioside present in the extract. It should also be mentioned that the used extract concentrations were extremely high, at the start of the experiments even 5.34 % of the body weight (or around 6.4 g stevioside/kg BW, which is near to the LD50)! For an adult person of 65 kg this means 3.47 kg of dry *Stevia* leaves or about 34.7 kg fresh leaves/day, i.e. more than 50% of his body weight! The significance of

such experiments where only one extremely high concentration was tested, should be questioned. Even the question may be posed if it is still ethical to force feed animals with such high concentrations. Melis' results are also in contradiction with the above and below cited studies that could not reveal any effect on fertility of male or female animals. Also in populations using stevioside as sweetener (Japan, Paraguay, China, South-Korea, USA) no adverse effects on fertility were reported.

Applied stevioside has no effect on fertility, mating performance, pregnancy, number of fetuses, nor on the growth and fertility of the offspring [21, 22, 27, 28, 30]. However, when steviol (the aglucone of stevioside) was given to hamsters on day 6-10 of pregnancy at doses of 500-1000 mg/kg body weight/day it induced toxicity [23]. The number of live foetuses per litter and mean foetal weight decreased. The maternal kidneys showed a dose-dependent increase in severity of convoluted tubules in the kidneys. The no-effect level (NOEL) for maternal and foetal toxicity was 250 mg/kg BW/day.

This study with steviol has nothing to do with the use of stevioside as a sweetener. When stevioside is fed to hamsters, no toxic effects were found even not in 3 successive generations [21]. The problem is that when steviol is given in the feed, it can be resorbed directly by the intestines, whereas stevioside is not. Stevioside is transformed only by the bacteria of the caecum or the colon from which steviol eventually may be resorbed, or taken up by coprophagy (see above). Moreover, hamsters are known to be very sensitive to steviol and stevioside [17], this is the reason that hamsters were chosen in this study. The NOEL of steviol was 250 mg/kg BW [23], which corresponds to 625 mg stevioside/kg BW. Even under these very unfavourable conditions an ADI of 2.5 mg steviol/kg BW, which corresponds to 6.25 mg stevioside/kg BW can be calculated, which is close to 7.9 mg/kg BW obtained by [12] (see also Table 2).

Besides the lack of effects of *Stevia* extracts or stevioside in experiments on fertility, no negative reports on human fertility have appeared since the use of *Stevia* and stevioside in Japan (since more than 25 years), the USA (since 1995) or in other countries.

BIO-AVAILABILITY OF NUTRIENTS FROM THE DIET

Some compounds added to the food are able to influence the uptake of other essential elements. This way growth curves may be disturbed or

other adverse physiological effects may show up, e.g. natural oils of plant origin contain a lot of lipid soluble vitamins. Replacement of natural oils by synthetic fats exempt of vitamins may influence the uptake of other lipids and lead to a plentitude of physiological disturbances due to lack of essential lipids and vitamins.

Stevia used in small amounts has no negative consequences for the bioavailability of nutrients from the diet and it does not have adverse physiological effects. This can be deduced from the data used to determine the ADI (see above) and from the use of *Stevia* in countries as Paraguay (used since more than 500 years), Brasil, China, Japan (more than 25 years) and even in the USA (*Stevia* allowed as dietary supplement).

Studies in which *Stevia* extracts or solutions of pure stevioside have been injected in the animals or were used in perfusion experiments of organs, are considered as not relevant for the use of *Stevia* or stevioside as food. As indicated above the sweet components of *Stevia* used in low concentrations, are not taken up in the human body and are not metabolised by the digestive enzymes from the gastro-intestinal tract.

From the work of the authors cited in the chapter on subacute toxicity and the ADI, it can be deduced that *Stevia* had no effects on the bioavailability of nutrients, as no effects could be found either on the growth or the weight of the animals, nor on other physiological processes. *Stevia* extracts were extensively tested (low and high concentrations) in rats during 22 months (male rats) and 24 months (females) [11]. No differences were found with the control animals, implicating that all nutrients were taken up and metabolised in the same way. The same can be concluded from the experiments of [21] who force-fed one month old male and female hamsters with high and low concentrations of *stevioside* in their food. This was done over 3 generations. This means also that pregnant hamsters as well as their young litters were forced to eat stevioside. No differences in growth or in other parameters were found. This again proves that no effects on food uptake or metabolism are involved even at relatively high stevioside concentrations. The same conclusions are true for the work of [31] done with Wistar rats fed during 30 days with high amounts of *Stevia* extract. In chronic experiments with rats during 24 months [12] and in experiments with 1000 broiler chickens [32] no effects were found on food consumption or on the weight gain or loss of these animals.

Aqueous extracts of *Stevia* were chronically administered to prepubertal male rats [27]. The concentrated extract of leaves (66.7 g dried leaves/100

ml final solution) was given twice a day by means of gastric tubing (2ml/rat) during 60 days. In order to improve conditions for gastrointestinal absorption the animals were deprived of food and water 1 h before and 30 min after drug administration. No effects on body weight gain curves were found during the duration of the experiment. No differences were found in the results of glycemia and thyroid hormone determinations. Neither the serum levels of triiodothyronine (T3) and of thyroxine (T4) nor the available binding sites in thyroid-hormone carrier proteins were altered by the treatment. This is relevant in view of the very important relationships of thyroid function with body growth and with the endocrine function of the pancreas.

In experiments 60 human volunteers received 27.7 mg or 110.8 mg of pure stevioside per day [33]. The author concluded that concentrated *Stevia* extracts in normal doses to sweeten could be used without restriction by normal persons as well as by diabetics.

Pure stevioside and rebaudioside were tested in Sprague-Dawley rats in a concentration of 0.5%, which is still far above the concentrations needed as a sweetener [34]. No differences were found in food and water intake and weight gain.

In all of the above cited experiments, no indications of any influence on the bio-availability of nutrients, nor on physiological effects were found. The animal feed used in the experiments did not contain sugar supplements. Therefore, no reduction of weight gain was observed in the experiments as *Stevia* or stevioside did not substitute for added sugar.

Moreover, *Stevia* and stevioside are used extensively in countries like Paraguay, Brasil, Japan, China, USA etc. and no indications were found of effects on the bioavailability of other nutrients or on other physiological functions of the human body. *Stevia* and stevioside have been consumed by hundreds of millions of people and this during a very long period of time, both by adults and by children without giving the smallest indication of any harmful effect.

STEVIA, STEVIOSIDE AND SPECIAL GROUPS OF THE POPULATION: NUTRITIONAL SIGNIFICANCE

Stevia and stevioside are absolutely safe for diabetics since it is used in minute amounts. Neither stevioside is taken up by our body, nor, due to the β -glycosidic bonds, is stevioside metabolised by the digestive enzymes

from the gastro-intestinal tract. The omission of excessively added sugar in the food is beneficial to diabetics by lowering the blood sugar content [33].

Stevia and stevioside are also safe for phenylketonuria (PKU) patients as no aromatic amino acids are involved.

Obese persons might lose weight by the fact that excessive sugar in the food is replaced by *Stevia* or stevioside. Omitting added sucrose in foods increases the relative proportion of polymeric carbohydrates. This has a beneficial effect for a balanced food intake and for human health [35].

NUTRITIONAL, MICROBIOLOGICAL, TOXICOLOGICAL, ALLERGENICITY PROBLEMS?

Stevia and stevioside are unlikely to give rise to nutritional, microbiological, toxicological and/or allergenicity problems. The use of *Stevia* by eg. Paraguayan, Japanese, Brazilian, American and other people has never led to the demonstration of problems of this kind. In the literature no reports on detrimental effects of either the living plants, or the dried leaves or stevioside can be found ([6]; see also all the references cited above).

A literature search could not reveal any publications about a possible allergenic activity of *Stevia* plants, dried leaves or stevioside itself. Kinghorn came to the same conclusions: no published reports have appeared that would suggest that extracts of *Stevia* leaves are immunologically active when taken internally [36]. Similarly there is no evidence that any of the constituents of *Stevia* caused allergic contact dermatitis.

More information on the use of *Stevia* in different countries can be found in the List of Food Additives excluding chemical synthetics (Japanese Ministry of Health and Welfare). The consumption of *Stevia* leaves in 1989 is given for different countries: Japan (2000 tons/year), Brazil (600 tons/year), China (400 tons/year), South Korea (300-400 tons/year), Thailand (100 tons/year), Taiwan (Formosa) (small quantities), Paraguay (150 tons/year) and Argentina (60 tons/year). Even in Belgium, England and The Netherlands several tons of dried leaves have been consumed. In 1989 the (known) world production was estimated at 4100 tons/year. No problems have been published in scientific literature

concerning nutritional, micro-biological, toxicological and/or allergenicity problems.

STEVIOSIDE AND CARIES

Stevioside and rebaudioside A were tested for cariogenicity in albino Sprague-Dawley rats [34]. Sixty rat pups colonised with *Streptococcus sobrinus* were divided into 4 groups and fed stevioside, rebaudioside A or sucrose added to the cariogenic diet 2000 as follows: group 1: 30% sucrose; group 2: 0.5% stevioside; group 3: 0.5% rebaudioside A and group 4 no addition. All pups received fresh cultures of *S. sobrinus* in the drinking water on days 18, 19 and 20. All 4 groups were sacrificed after 5 weeks. *S. sobrinus* counts were made on plaque samples collected from all the molars and cavities were evaluated. There were no differences in food and water intake and weight gains between the 4 groups. Group 1 (30% sucrose) had a very significant higher caries score and *S. sobrinus* counts than the other 3 groups. There were no significant differences between the stevioside, rebaudioside A and no-addition groups. It was concluded that neither stevioside nor rebaudioside A is cariogenic.

Although rather high concentrations of stevioside and *Stevia* extracts were shown to reduce the growth of some bacteria, the concentrations used for sweetening purposes are rather low. Therefore, the beneficial effect of the use of stevioside would rather be due to the substitution of sucrose in the food by a non-cariogenic substance.

PURITY OF STEVIOSIDE

The purity of the stevioside is not always the same in the various experiments or unknown in some experiments. However, in most experiments the purity of the stevioside used is over 90 % and often over 95%. The impurities occurring in the different stevioside preparations are due to compounds extracted from the *Stevia* leaves that themselves have been shown to be completely safe. Therefore, the problem of the purity of the stevioside is more a point for academic debate than a matter of concern of human health. Stevioside is safe as well as *Stevia* leaves and their crude extracts. Therefore, the occurrence of a slightly varying percentage (5-10%) of residual leaf extract in the stevioside preparation will not be

detrimental to human health, and will influence NOEL and ADI only by a few percent.

ABBREVIATIONS

ADI	=	Allowable daily intake
BW	=	Body weight
CHL	=	Chinese hamster lung fibroblast cell line
Glc	=	Glucose
ICH	=	International Council of Harmonisation
JECFA	=	Joint FAO/WHO Expert Committee on Food Additives
LD50	=	Lethal dose at which 50% of the animal die
OECD	=	Organisation for economic co-operation and development
NOEL	=	No effect level
PKU	=	Phenylketonuria
Rha	=	Rhamnose

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