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Metabolically activated steviol, the aglycone of stevioside, is mutagenic

(activé mutagenic metabolite/chemical syntheses of *ent*-kaurene derivatives/mutagenic evaluation of sweet-tasting *ent*-kaurene glycosides/dietary mutagen)

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ABSTRACT **Stevioside, a constituent of *Stevia rebaudiana*, is commonly used as a noncaloric sugar substitute in Japan. Consistent with reports in the literature, we have found that stevioside is not mutagenic as judged by utilization of *Salmonella typhimurium* strain TM677, either in the presence or in the absence of a metabolic activating system. Similar negative results were obtained with several structurally related sweet-tasting glycosides. However, steviol, the aglycone of stevioside, was found to be highly mutagenic when evaluated in the presence of a 9000 × *g* supernatant fraction derived from the livers of Aroclor 1254-pretreated rats. Expression of mutagenic activity was dependent on both pretreatment of the rats with Aroclor 1254 and addition of NADPH; unmetabolized steviol was not active. The structurally related species, isosteviol, was not active regardless of metabolic activation. Similarly, chemical reduction of the unsaturated bond linking the carbon-16 and -17 positions of steviol resulted in the generation of two isomeric products, dihydrosteviol A and B, that were not mutagenic. In addition, *ent*-kaurenoic acid was found to be inactive. It is therefore clear that a metabolite of an integral component of stevioside is mutagenic; structural features of requisite importance for the expression of mutagenic activity include a hydroxy group at position 13 and an unsaturated bond joining the carbon atoms at positions 16 and 17. A potential metabolite of steviol, steviol-16α,17-epoxide, was synthesized chemically and found to be ineffective as a direct-acting mutagen. Thus, although stevioside itself appears innocuous, it would seem prudent to expeditiously and unequivocally establish the human metabolic disposition of this substance.**

Stevia rebaudiana Bertoni (Compositae) is a sweet herb indigenous to elevated terrain in the northeastern corner of Paraguay, adjacent to its frontier with Brazil (1). Extracts of this plant and stevioside, its major sweet constituent, are commercially available in Japan, where they have been used for nearly a decade to sweeten a variety of foods including sea foods, pickled vegetables, dessert items, soft drinks, and confectionery (2–4). Recently, *S. rebaudiana* products were cleared for sale in Brazil (5, 6), and they are also used in Paraguay for the treatment of hyperglycemia (1).

Stevioside is one of eight known sweet *ent*-kaurene glycoside constituents of *S. rebaudiana*, with the others being steviolbioside, rebaudiosides A–E, and dulcoside A (7). The results of several studies to assess the safety for human consumption of certain of these compounds and *S. rebaudiana* extracts have appeared in the literature. For example, neither stevioside nor two different *S. rebaudiana* extracts were found to be significantly active during acute toxicity tests in mice and subacute toxicity tests in rats (8). Also, no dose-related abnormalities in an extensive range of blood chemistry values were observed after subacute oral administration to rats of a *S. rebaudiana* extract containing 50% (wt/wt) stevioside, apart from a significant decrease in lactic acid dehydrogenase levels (9). Stevioside and crude extracts derived from *S. rebaudiana* have been determined to be nonmutagenic in several laboratories when tested against several strains of *Salmonella typhimurium, Escherichia coli*, and *Bacillus subtilis*, both in the presence and in the absence of a metabolic activating system derived from the liver of rats (2, 10, 11). In addition, no activity was demonstrated by these same test materials in a silkworm oocyte test system, and they did not enhance sister chromatid exchange with human fetal fibroblasts or induce chromosomal aberrations with these cells or rat medullary cells (11).

The present paper describes mutagenicity studies carried out with steviol (*ent*-13-hydroxykaur-16-en-19-oic acid) and several of its sweet glycosides and other derivatives. We report here that steviol is mutagenic toward *S. typhimurium* strain TM677, in the presence of a 9000 × *g* supernatant fraction obtained from the liver of Aroclor 1254-pretreated rats. The potential importance of the results in relation to the human ingestion of stevioside is discussed.*

MATERIALS AND METHODS

Isolation of Stevioside and Structurally Related Sweet Diterpene Glycosides. The steviol (**1**) glycosides† stevioside (**3**), rebaudioside A (**5**), rebaudioside C (**6**), and dulcoside A (**7**) were isolated from a 1-butanol-soluble extract of *S. rebaudiana* by absorption chromatography as described by Kinghorn *et al.* (13). Steviolbioside (**2**) and rebaudioside B (**5**) were prepared from stevioside and rebaudioside A, respectively, by alkaline hydrolysis (13).

Preparation of Steviol from Stevioside. Stevioside (11 g) was treated with sodium periodate and sodium hydroxide as described by Ogawa *et al.* (14). The residue obtained after workup of the reaction mixture was chromatographed over a 5.5 × 100 cm column containing silica gel (particle size, 63–200 μm; Merck, Darmstadt, F.R.G.), eluting with 2 liters of chloroform-methanol, 49:1 (vol/vol). Removal of solvent and crystallization from methanol gave pure steviol (1.9 g), mp 211–213°C, $[\alpha]_D^{25}$ −65.2° (*c* 0.35, methanol) [lit. mp 206–208°C, $[\alpha]_D^{25}$ −74.0°, 95% ethanol] (15). The elemental compo-

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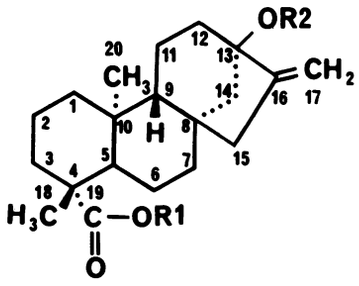
*Some of the data in this manuscript were presented at the 74th Annual Meeting of the American Association for Cancer Research, San Diego, CA, May 25–28, 1983 (12).

†For the steviol glycosides described here in the *O*-glycoside linkages from carbons-13 and 19 are connected, in each case, to carbon-1 of all sugar moieties.

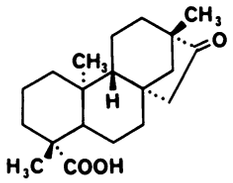


Compound	R1	R2	Trivial name
1	H	H	Steviol
2	H	β-Glc-β-Glc(2 → 1)	Steviolbioside
3	β-Glc	β-Glc-β-Glc(2 → 1)	Stevioside
4	β-Glc	β-Glc-α-Rha(2 → 1)	Dulcoside A
5	H	β-Glc-β-Glc(2 → 1) | β-Glc(3 → 1)	Rebaudioside B
6	β-Glc	β-Glc-β-Glc(2 → 1) | β-Glc(3 → 1)	Rebaudioside A
7	β-Glc	β-Glc-α-Rha(2 → 1) | β-Glc(3 → 1)	Rebaudioside C

sition of this compound was determined to be $C_{20}H_{30}O_3$ by high-resolution mass spectrometry. Other spectral values (IR, UV, PMR) were measured, and data appropriate to structure **1** were obtained.

Steviol was also obtained from stevioside enzymatically, by incubation of 10 g of the glycoside with commercial pectinase (Pectinol 50L, Corning Biosystems), according to the procedure of Ruddat *et al.* (16). The pure aglycone (3.1 g) obtained after workup exhibited physical and spectroscopic data identical to those of steviol produced from stevioside by the procedure described above.

Preparation of Isosteviol from Stevioside. Isosteviol (**8**) was prepared from stevioside (25 g) by reaction with 20% aqueous H_2SO_4 (750 ml) on a steam bath for 5 hr.



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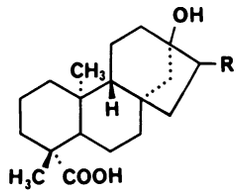
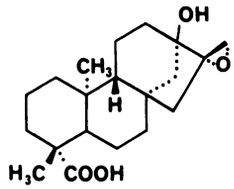
The reaction mixture was cooled and extracted with chloroform (5 × 300 ml), and the pooled chloroform layers were washed with water and allowed to evaporate under reduced pressure to yield a residue. This residue was chromatographed over 200 g silica gel, eluting with chloroform (2 liters). Removal of solvent and crystallization from methanol gave pure isosteviol (4.5 g), which exhibited physical and spectroscopic data closely comparable with previously published values (17).

Preparation of Dihydrosteviol A and Dihydrosteviol B by Hydrogenation of Steviol. Steviol (1 g) was dissolved in ethanol (75 ml) and hydrogenated by passing hydrogen gas in the presence of 5% platinum on activated carbon catalyst (Aldrich) through the solution overnight at room temperature. On workup, the solid residue was purified by column chromatography over silica gel. Elution with petroleum ether/acetone, 23:2 (vol/vol), yielded dihydrosteviol A (**9**) [100 mg; mp 190–192°C, $[\alpha]_D^{25}$ −28.2° (*c* 0.43, $CHCl_3$)], and further elution with petroleum ether/acetone (9:1) afforded dihydrosteviol B (**10**) [100 mg; mp 216–218°C, $[\alpha]_D^{25}$ −110.9° (*c* 0.33, $CHCl_3$)]. The methyl esters of **9** and **10** were found to exhibit physical data comparable with literature values (18).



9 (R = β-CH_3)
10 (R = α-CH_3)

Preparation of Steviol-16α,17-epoxide. Steviol (0.64 g), in 15 ml of methylene chloride, was treated for 6 hr at room temperature with 0.42 g of *m*-chloroperbenzoic acid in 10 ml of methylene chloride. On completion of the reaction, 100 ml of water was added, and the products were extracted with chloroform (3 × 50 ml). This organic layer was washed with water (4 × 200 ml), dried over anhydrous sodium sulfate, and allowed to evaporate to yield a white residue. Repeated crystallization of this residue from methanol gave pure steviol-16α,17-epoxide (**11**) [300 mg; mp 211–213°C, $[\alpha]_D^{25}$ −138.4° (*c* 0.26, $CHCl_3$)].



11

Spectral data measured for **11** were consistent with the proposed structure of this compound; its stereochemistry was assigned on the basis of expected attack by the epoxidizing agent on the less hindered side of the steviol molecule (19, 20). When **11** was methylated with diazomethane, the product exhibited physical and spectroscopic data identical to those of the known compound steviol-16α,17-epoxide methyl ester (21).

Treatment of Animals and Isolation of the 9000 × *g* Liver Supernatant. Male Sprague–Dawley rats (body weight, 100–120 g; King Animal Laboratories, Oregon, WI) were housed in air-conditioned quarters with a 12-hr/12-hr light–dark cycle and given food and water ad lib. The rats (generally groups of 10) were given (*i*) no treatment, (*ii*) a single i.p. injection of Aroclor 1254 (400 mg/kg of body weight in corn oil), (*iii*) four consecutive daily i.p. injections of 3-methylcholanthrene (25 mg/kg of body weight in corn oil), or (*iv*) four consecutive daily i.p. injections of phenobarbital (75 mg/kg of body weight in H_2O).

Either 4 days after treatment with Aroclor 1254 or the day after the final treatment with 3-methylcholanthrene or phenobarbital, the rats were sacrificed by decapitation. The liver was immediately excised, rinsed several times with cold 0.14 M NaCl, minced, and homogenized with 3 vol (vol/wt) of 50 mM Tris·HCl, pH 7.5/0.14 M KCl using a glass homogenizing vessel and a motor-driven Teflon pestle. The homogenate was filtered through cheesecloth and centrifuged at 800 × *g* for 10 min. The resulting supernatant was then centrifuged at 9000 × *g* for 20 min to yield the S-9 fraction. Protein was estimated by the method of Lowry *et al.* (22) using bovine serum albumin as the standard, and the isolate was stored as small aliquots in liquid nitrogen. All of the isolation procedures described above were conducted at 0–4°C.

Bactericidal and Bacterial Mutagenesis Assays. *S. typhimurium* strain TM677 (carrying the "R-factor" plasmid pKM101) was used for the assessment of mutagenic activity. The forward mutation assay procedures originally described by Thilly and his co-workers (23–25) were essentially followed. Briefly, duplicate 1.0-ml reaction mixtures containing 1.0 mg $NADP^+$, 1.0 mg of glucose 6-phosphate, 0.8 unit of glucose-6-phosphate dehydrogenase, 0.67 mg of $MgCl_2$, the S-9 fraction, and $\approx 7 \times 10^6$ bacteria (in logarithmic phase) were prepared in minimal essential medium. When metabolic activation was not required, only bacteria and minimal essential medium were mixed (unless otherwise indicated).

After addition of the test substance (dissolved in 20 μl of dimethyl sulfoxide), the mixtures were slowly rotated at 37°C for 2 hr. The reaction was then quenched by the addition of 4 ml of phosphate-buffered saline. The bacteria were recovered by centrifugation, resuspended, diluted as appropriate, and plated (in triplicate) in the presence and absence of 8-azaguanine. Plates were scored after a 36- to 40-hr growth period at 37°C and the mutant fraction was expressed as the average number of colonies observed on plates containing 8-azaguanine divided by the average number of colonies observed on plates not containing 8-azaguanine. The latter value was used to define the percentage of bacteria surviving the treatment, relative to a control in which only an equivalent amount of solvent was added.

RESULTS

As shown in Table 1, no significant mutagenic activity was found when *ent*-kaurene glycosides **2–7** were assayed in the presence of the S-9 fraction derived from Aroclor 1254-pretreated rats. Mutagenic activity was also not significant when the bacteria were treated with the test substances in the absence of the S-9 fraction (data not shown) and, under either of the test conditions, no bactericidal activity has been noted. Similar negative results have been reported on assessing stevioside for mutagenic activity with other *Salmonella* strains (2, 10, 11).

At the highest concentration tested, however, steviol (**1**) was found to have substantial mutagenic activity (Table 1). To verify and further characterize the mutagenic potential of this substance, the bacteria were treated with it at various concentrations. The data summarized in Table 2 illustrate a clear dose–response relationship in terms of both mutagenic and bactericidal activity. [It should be noted that, with the exceptions of **3**, **4**, **6**, and **7**, the test compounds are not highly soluble in H_2O. Thus, the indicated concentrations represent the quantities added to the reaction mixtures and not the true quantities in solution. Also, at the concentration required to elicit a mutagenic response, **1** is significantly toxic. Although this may yield a select population of viable bacteria, the procedures described herein (i.e., expression of results as a mutant fraction) permit quantitative interpretation of the resulting experimental data.] When evaluated at steviol concentrations as high as 10 mg/ml in the absence of the S-9 fraction, however, no significant activity was detected (data not shown).

Table 1. Evaluation of steviol (**1**) and several related glycosides for mutagenic activity

Conc., mg/ml	Activity of compound, mutant fraction × 10^5						
	1	**2**	**3**	**4**	**5**	**6**	**7**
0.1	6.8	12.3	6.7	3.4	2.5	11.7	2.6
1.0	12.4	12.4	6.6	4.3	2.5	9.8	3.1
10.0	94.9	6.2	5.8	3.2	2.4	11.6	3.2

Each incubation mixture contained the S-9 fraction derived from Aroclor 1254-pretreated rats (≈2.5 mg of protein) and the indicated concentration of test substance.

Table 2. Concentration-dependent mutagenic and bactericidal activity of steviol

Conc., mg/ml	Mutant fraction × 10^5	Survival, %
0	5.2	100
0.1	10.9	95.0
0.5	9.2	63.9
1.0	28.2	40.3
2.5	94.2	13.2
5.0	136	11.6

Steviol was added to the reaction mixtures at the indicated concentration. Other conditions were as described for Table 1.

The steviol used for the studies described above was obtained by treatment of stevioside with sodium periodate and potassium hydroxide followed by chromatographic purification. Although unlikely, it was of concern that a small quantity of a chemical contaminant might have copurified with the steviol and elicited the mutagenic/bactericidal response. Thus, to negate this possibility, steviol was also prepared by an enzymatic procedure (16). Comparison of results obtained with steviol derived from nonmutagenic stevioside by either of these procedures showed no significant differences in activity.

Studies were then carried out to examine the metabolic conditions required to facilitate the mutagenic potential of steviol. As shown in Table 3, slight activity was observed when steviol was incubated with NADPH or the Aroclor 1254 S-9 fraction in the presence of bacteria, but the greatest mutagenic and bactericidal response required admixture of these substances. Further, under the reaction conditions used, the S-9 fraction derived from control or 3-methylcholanthrene-pretreated rats was unable to effectively catalyze steviol-mediated mutagenesis whereas the preparation obtained from phenobarbital pretreated rats was effective in this process. Thus, the required pretreatment (with Aroclor 1254 or phenobarbital) and addition of NADPH strongly suggests a cytochrome P-450-mediated metabolic activation of steviol to a mutagenic species.

The mixed-function oxidase system, of course, is known to catalyze a variety of metabolic conversions (26, 27). Considering the structure of steviol, however, attention immediately focuses on the double bond between carbon atoms 16 and 17. To explore the functional importance of this region of the molecule, a group of closely related substances was assessed for mutagenic activity. Isosteviol (**8**), a known decomposition product of stevioside (18, 28), had no mutagenic or bactericidal activity in the presence (Table 4) or absence

Table 3. Effect of metabolic activating systems on the mutagenic and bactericidal activity of steviol

S-9 fraction added	NADPH	Mutant fraction × 10^5	Survival, %
None	−	8.9	100
	+	16.0	65.2
Pretreatment			
Aroclor 1254	−	21.3	78.2
	+	131.2	13.2
Control	+	10.4	100
Phenobarbital	+	50.3	36.7
3-Methylcholanthrene	+	11.0	97.1

Each incubation mixture contained steviol at 2.5 mg/ml; $NADP^+$ and a NADPH-regenerating system were present or absent as indicated. The average mutant fraction obtained on addition of solvent only was 11.6×10^{-5}. S-9 fractions obtained from pretreated rats contained protein as follows: Aroclor 1254, 2.9 mg; control, 1.6 mg; phenobarbital, 2.7 mg; 3-methylcholanthrene, 2.5 mg.

Table 4. Evaluation of isosteviol (**9**) and synthetic steviol derivatives for mutagenic activity

Conc., mg/ml	Activity of compound, mutant fraction × 10^5			
	8	**9**	**10**	**11**
1.0	7.3	ND	ND	ND
2.5	ND	9.2	8.5	6.5
5.0	ND	8.1	8.3	8.4
7.5	ND	ND	ND	7.1
10.0	7.8	11.4	8.7	8.7

Each compound was added to the incubation mixture at the indicated concentration. Compounds **8–10** were tested in the presence of the S-9 fraction derived from Aroclor 1254-pretreated rats and a NADPH-regenerating system, whereas **11** was assayed directly. No significant bactericidal activity was found. ND, not determined.

of the S-9 fraction (data not shown). Moreover, the results obtained with the two isomeric species obtained on catalytic reduction of the double bond (**9** and **10**) strongly support the requirement of this functional group since neither of these compounds was mutagenic in the presence of a metabolic activating system (Table 4). Similarly, no activity was detected with **9** or **10** in the absence of a metabolic activating system (data not shown).

Thus, analogous to compounds such as aflatoxin B_1 and benzo[*a*]pyrene, steviol requires both metabolic activation and the presence of a double bond to function as a mutagen. A unique structural feature of steviol, however, is an adjacent hydroxyl group located at position 13. The importance of this functional group was investigated by evaluating the mutagenic potential of *ent*-kaurenoic acid (**12**). When tested at concentrations as high as 5 mg/ml, this substance had neither toxic nor mutagenic activity in the presence or absence of a metabolic activating system (data not shown).

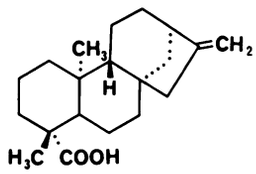
Finally, since several carcinogens are well-known to function as active mutagens subsequent to the metabolic generation of an epoxide or arene oxide (29–32), an epoxide of steviol (**11**) was synthesized chemically and tested directly for mutagenic activity. In the absence of a metabolic activating system, **11** did not serve as a mutagen (Table 4). It also was not bactericidal. In addition, no significant activity was found in the presence of a metabolic activating system derived from rat liver (data not shown).‡

DISCUSSION

Stevioside and the crude product used as a sweetening agent that is prepared from *S. rebaudiana* have been subjected to many standard genetic toxicological evaluations (2, 10, 11). Consistent with the results reported in the present study, no mutagenic activity has been found with stevioside on treatment of a variety of bacterial strains (2, 10, 11). Also reported here is the fact that an additional group of sweet-tasting glycosides structurally related to stevioside (**4–7**) are not mutagenic. Thus, the lack of mutagenic or genotoxic activity observed in these assays attests to the safety of stevioside.

Importantly, however, the aglycone of stevioside, steviol, is highly mutagenic. Metabolic activation is required to potentiate this activity. Under the assay conditions used for the metabolic activation and assessment of steviol, the mutagenic response at a concentration of 2.5 mg/ml was similar to that obtained with 80 μM benzo[*a*]pyrene or 150 μM 2-acetylaminofluorene (data not shown). For activity of this magnitude, it is likely that covalent interaction with bacterial DNA occurs (33, 34).

In further characterizing steviol-mediated mutagenicity, the following has been established: (*i*) Both NADPH and the rat liver S-9 fraction are required for activity, (*ii*) pretreatment of the rats with an inducer of cytochrome P-450 (Aroclor 1254 or phenobarbital) is necessary, (*iii*) structural congeners of steviol in which the carbon-16/carbon-17 double bond is sterically hindered (**2–7**) or reduced (**9** and **10**) are not active as mutagens, (*iv*) an analog of steviol (**12**) that is devoid of a hydroxy group at position 13 is not active as a mutagen, and (*v*) a synthetic steviol epoxide (**11**) does not mediate bacterial mutagenesis.



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On the basis of these data, it is probable that steviol is metabolized to active mutagenic species by the cytochrome P-450 system. However, since the synthetic steviol epoxide did not generate a mutagenic response, and rat liver fractions have been shown to produce several steviol metabolites,§ the chemical structure of the mutagenic species remains to be elucidated.

Because of a variety of factors, the commercial availability of a noncariogenic and/or noncaloric sugar substitute is highly desirable. Stevioside, which is 300-fold sweeter than sucrose (35), is currently used as such an agent in Japan and Brazil. For example, it was estimated in 1979 that 700–1000 tons of *S. rebaudiana* leaves were cultivated or imported for use in Japan (2), and one company is reported to be producing 2–3 tons of stevioside per month (35). The resulting material is either used directly or added to a variety of foods (2–4) and, therefore, it must be assumed that several tons of stevioside are ingested by the Japanese population on an annual basis. Similarly, *S. rebaudiana* tea and capsules were officially approved to be offered for sale in Brazil in 1980 (5, 6). In Paraguay, reports have indicated that *S. rebaudiana* was used to sweeten various beverages even before colonization by the Spaniards in the 16th century (36). Modern use, however, relates primarily to reports in the literature that have indicated a hypoglycemic effect of the substance (1). For example, one product sold for this purpose recommends an initial dose of *S. rebaudiana* leaves of up to 5 g/day for patients with high blood sugar, followed by a maintenance dose of 1 g/day.

It is therefore clear that human consumption of stevioside is fairly widespread. In the context of results presented in this communication, the questions of imminent importance are the chemical stability and metabolic disposition of this substance. Considering the former, a variety of chemical reaction conditions have been shown to result in the production of isosteviol (18, 28). Refluxing stevioside for 5 hr in aqueous methanol/1.5% HCl gives isosteviol in 78% yield (28). In a comprehensive study in which stevioside was incubated for up to 3 months at pH values ranging from 2 to 8 and

‡We have thus far been unable to efficiently synthesize and evaluate the isomeric counterpart of **11** as a direct acting mutagen. Although the species tested does not appear analogous to a mutagenic metabolite of steviol, the lack of activity in the presence of the rat liver S-9 fraction provides additional support for the functional importance of the carbon-16/carbon-17 region of the molecule.

§In separate experiments using incubation conditions known to mediate bacterial mutagenesis, the enzymic generation of approximately 10 steviol metabolites has been demonstrated by GC/MS (unpublished work).

temperatures ranging from 5 to 90°C, no discernible steviol was generated (unpublished observations). Further, by using a combination of HPLC and TLC techniques, stevioside, rebaudioside A, and rebaudioside C have been identified in a leaf herbarium sample of *S. rebaudiana* collected in Paraguay in 1919 (37). Thus, it seems unlikely that stevioside would be degraded to steviol when subjected to typical methods of cooking, storing, or processing.

Various enzymes, however, such as hesperidinase (38), pectinase (16), and hepatic pancreatic juice obtained from the vineyard snail *Helix pomata* (39, 40) are known to convert stevioside to steviol efficiently. Moreover, Wingard *et al.* (41) have reported 100% conversion of stevioside to steviol on incubation with isolated rat intestinal microflora and that oral administration of steviol to the rats led to nearly complete absorption. As concluded by Wingard *et al.* on the basis of these results, it would be anticipated that the organs of rats given stevioside in the diet would be exposed to steviol. It remains to be shown, however, that the microbial flora obtained from the rats used in these studies corresponds to that found in the human alimentary tract.

Finally, it should be emphasized that no reports have thus far appeared indicating that adverse effects have resulted from human use of *Stevia* products. Other substances found in the diet are known to mediate mutagenic responses with no apparent impact on health (42). Based on the results described herein, to potentiate the mutagenic effect, it would first be necessary to produce the aglycone of stevioside and then metabolically activate this species, although fewer required steps are possible. Nonetheless, complete metabolic conversion of stevioside to an active mutagenic species by human enzymic systems involved in the biotransformation of endogenous substrates or xenobiotics is possible. It therefore appears that adequate information is currently not available to condone widespread human consumption of stevioside. Additional studies relevant to safety assessment are required.

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