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Assessment of the Carcinogenicity of Stevioside in F344 Rats

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Abstract—The carcinogenic potential of stevioside, a compound that is used as a sweetener for food and drink, was examined in F344 rats of both sexes. Stevioside was added to powdered diet at concentrations of 0 (control), 2.5 and 5%. The doses were selected on the basis of results from a 13-wk subchronic toxicity study and administered to groups of 50 male and 50 female rats *ad lib.* for 104 wk. All surviving rats were killed at wk 108. Body weight gains were slightly depressed in line with the dose of stevioside, in both sexes, and a significant decrease in the final survival rate was observed for the 5% treated males. Histopathologically, however, there was no significantly altered development of neoplastic or non-neoplastic lesions attributable to the stevioside 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. It is concluded that stevioside is not carcinogenic in F344 rats under the experimental conditions described. © *1997 Elsevier Science Ltd*

Abbreviation: LGL = large granular lymphocyte.

INTRODUCTION

Stevioside is the major sweet constituent found in the leaves of *Stevia rebaudiana* Bertoni (Compositae), a herb occurring wild in northern Paraguay and in parts of Brazil where its leaves have traditionally been used as a sweetener. Pure stevioside is about 250–300 times as sweet as sucrose, and is composed of steviol, a diterpenic carboxylic alcohol, and three D-glucose molecules, with a molecular weight of 804.90 (Fig. 1) (Hanson and De Oliveira, 1993). The leaves of *S. rebaudiana* also contain several structurally related sweet compounds such as rebaudiosides A–E, steviolbioside, and dulcoside A. Extracts of the leaves and its processed substances, including stevioside, have been used as a sugar substitute in Japan and other parts of the world, with particular advantages for those suffering from obesity, diabetes mellitus, heart disease and dental caries (Kinghorn and Soejarto, 1985).

Although toxicological studies of high purity stevioside have been limited, a considerable number of the reports have been published with regard to crude extracts of *S. rebaudiana* or mixtures of stevioside and other sweet components of *S. rebaudiana*. The oral LD_{50} of stevioside of 93.5% purity was found to be more than 15 g/kg for mice of both sexes (Akashi and Yokoyama, 1975). In our previous study in rats of both sexes there were no obvious toxicological findings on dietary administration of a 95.6% pure stevioside preparation at dose levels of 0.31, 0.62, 1.25, 2.5 and 5% for 13 wk (Aze *et al.*, 1991). According to Mori *et al.* (1981), administration of stevioside of 95.98% purity at dietary doses of 0.15, 0.75 or 3.00% to male rats for 60 days and to female rats for 14 days before mating had no adverse effect on the pregnancy rate or the development of foetuses.

No mutagenic activity was noted for stevioside on performance of the Ames test, forward mutation assay, *umu* test, *Rec*-assay and chromosomal aberration test (Ishidate *et al.*, 1984; Matsui *et al.*, 1989; Suttajit *et al.*, 1993). However, steviol, an aglycone of stevioside, was demonstrated to be mutagenic with metabolic activation in the forward mutation assay, *umu* test, chromosomal aberration test and gene mutation assay (Matsui *et al.*, 1989; Pezzuto *et al.*, 1985). Furthermore, it has been reported that stevioside is decomposed by intestinal microflora of rats to steviol and sugars, and these are then absorbed in the lower part of the intestine (Nakayama *et al.*, 1986; Wingard *et al.*, 1980).

From these observations, potential carcinogenicity of stevioside can be speculated through the mutagenicity of steviol when the compound is

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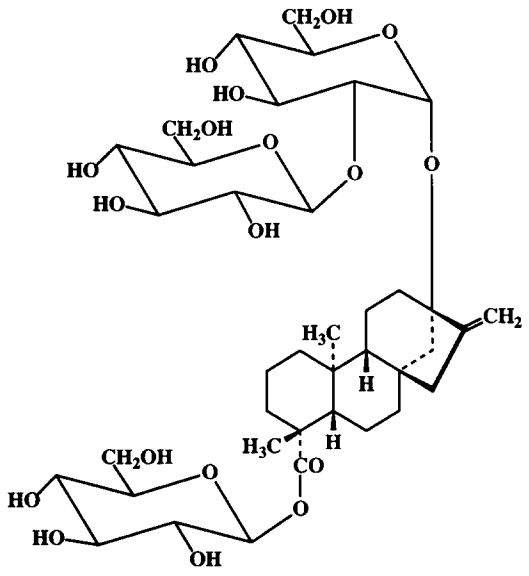

Fig. 1. Chemical structure of stevioside.

administered orally to rats. However, in previous studies conducted to evaluate the carcinogenic potential of stevioside there were problems regarding the purity or dose levels administered (Xili *et al.*, 1992; Yamada *et al.*, 1985). In the present study, we therefore investigated the long-term effects of stevioside administration to F344 rats at dietary dose levels of 2.5 and 5% using stevioside of high purity (95.6%).

MATERIALS AND METHODS

Animals and chemical

Male and female 4 wk old F344/DuCrj rats were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were maintained on pelleted basal diet (CRF-1; Charles River Japan Inc.) and tap water until they were 5 wk old, when the study was started. The rats were housed in plastic cages with hardwood-chip bedding in an air-conditioned room at 24 ± 1°C, 55 ± 5% humidity with a 12 hr light/12 hr dark cycle. Stevioside (white powder of 95.6% purity) was supplied by the Stevia Industrial Association (Japan), and incorporated into the CRF-1 diet and pelleted every 3 months by Oriental Yeast Co., Ltd (Tokyo, Japan). The diet was stored in our cold room at 4°C until use. The concentration of stevioside in diet stored at 4°C for 3 months was analysed by gas chromatography and/or HPLC twice during the experimental period, and was shown to be unchanged from that at the time of the diet preparation.

Determination of dose levels

A complete report of the subchronic toxicity study was published in a Japanese journal (Aze *et al.*, 1991) but the main findings are briefly described here. Stevioside was incorporated into the powered diet (CRF-1) at concentrations of 0, 0.31, 0.62, 1.25, 2.5 and 5%, and given to 10 rats of each sex group *ad lib.* for 13 wk. No rats died and none of the treated groups exhibited more than a 10% reduction in body weight, compared with the control value. No toxicological changes related to the treatment were observed on histopathological examination. On the basis of these results, the maximum tolerated dose (MTD) of stevioside given in the diet was estimated to be higher than 5% for both sexes in F344 rats. The dose levels chosen for the present carcinogenicity study were therefore 2.5 and 5%.

Carcinogenicity study

Rats were randomly allocated to three groups, each consisting of 50 males and 50 females. Three or four males, or five females, were housed in each cage. Groups of rats were maintained on diet containing stevioside at concentrations of 0, 2.5 or 5%. After the 104-wk treatment, all surviving animals were placed on the basal diet for an additional 4-wk period.

Throughout the experiment, rats in all groups were given free access to both tap water and diet. They were observed daily and clinical signs and deaths were recorded. Body weights were measured once weekly for the first 8 wk of the study and then once every 4 wk. Food consumption was measured once every 4 wk. At wk 108, all surviving animals were killed by exsanguination under ether anaesthesia after overnight fasting, and autopsied. Blood samples were taken from the abdominal aorta, and examined for numbers of white blood cells, red blood cells and platelets, amounts of haemoglobin and levels of haematocrit, using a fully automated haematology analyser (Sysmex M-2000, Toa Medical Electronics Co., Ltd, Hyogo, Japan). Gross lesions were recorded and tissues were collected for microscopic study of neoplastic and non-neoplastic changes. The brain, salivary glands, lungs, heart, spleen, liver, kidneys, adrenal glands, testes and ovaries were weighed. All organs and tissues, as well as any gross lesions, were routinely fixed in 10% buffered formalin, processed for embedding in paraffin, sectioned, and stained with haematoxylin and eosin. The rats that died or were killed *in extremis* before the termination of the experiment also underwent complete gross and microscopic examination. Those for which histo-

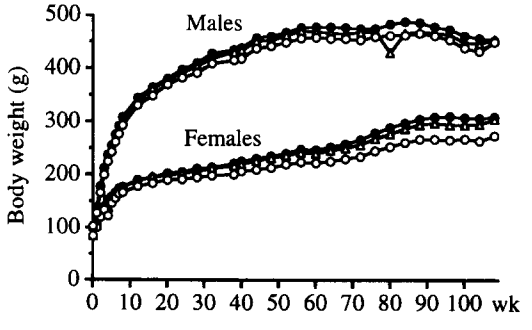

Fig. 2. Growth curves of male and female F344 rats given stevioside at 0% (●, control), 2.5% (△) or 5% (○) in the diet for 104 wk.

Table 1. Body weights, daily intakes of food and stevioside, final survival rates and survival times for F344 rats given stevioside in the diet for 104 wk

Group			Daily intake of			
Sex	Treatment	Body weight at wk 104 (g)	Food (g/rat)	Stevioside (mg/kg body weight)	Final survival rate (%)	Survival time (wk)
Male	Control	455 ± 34	15.2 ± 0.6	0	78	105.8 ± 5.1
	2.5%	445 ± 54	15.4 ± 0.7	969 ± 308	72	104.3 ± 9.2
	5%	431 ± 31*	15.5 ± 0.7	1997 ± 617	60‡	102.8 ± 9.5
Female	Control	305 ± 38	10.0 ± 0.8	0	78	105.4 ± 6.3
	2.5%	294 ± 30	10.1 ± 0.9	1120 ± 285	70	103.2 ± 10.4
	5%	263 ± 29†	10.0 ± 0.7	2387 ± 508	78	105.5 ± 7.5

Values are means ± SD.
*Significant difference from controls ($P < 0.05$; Scheffe's test).
†Significant difference from controls ($P < 0.01$; Scheffe's test).
‡Significant difference from controls ($P < 0.05$; Fisher's exact probability test).

pathological examinations could not be performed owing to advanced autolysis were excluded from the effective numbers.

Statistical analysis

Data for body and organ weights, survival times and daily food intake were analysed statistically using analysis of variance followed by Dunnett's test or Scheffe's test. Data for mortality rates and incidences of neoplastic and non-neoplastic lesions were analysed with the Fisher's exact probability test, and severity of chronic nephropathy was analysed with the cumulative chi-square test.

RESULTS

General condition

Rats of both sexes given the 5% stevioside diet had soft faeces in the first 2–3 wk of the experiment but not subsequently. No other noteworthy changes in the animals' general condition resulted from administration of stevioside.

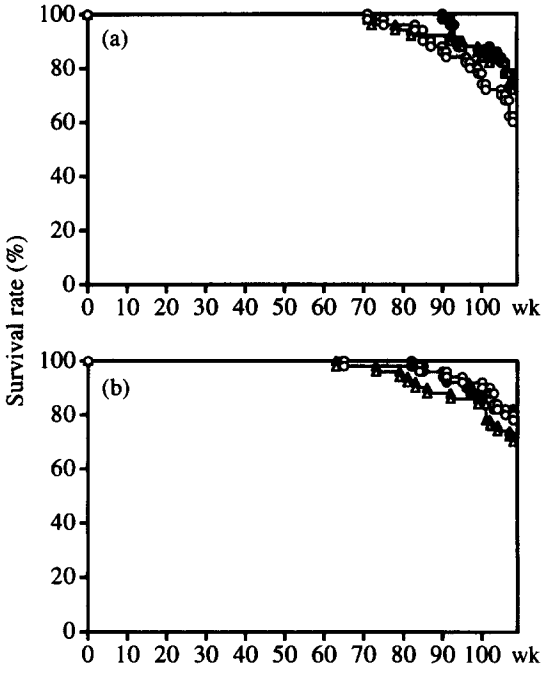

Fig. 3. Survival curves of (a) male and (b) female F344 rats given stevioside at 0% (●, control), 2.5% (△) or 5% (○) in the diet for 104 wk.

Table 2. Absolute body and organ weights, and relative organ weights (organ/body) for F344 rats given stevioside in the diet for 104 wk

	Males			Females		
	Control (49)‡	2.5% (48)	5% (47)	Control (49)	2.5% (46)	5% (47)
Absolute weight (g)						
Body	421.7 ± 55.6	429.9 ± 90.8	409.1 ± 44.2	280.3 ± 41.4	270.2 ± 40.4	251.4 ± 35.8†
Brain	2.142 ± 0.079	2.161 ± 0.071	2.139 ± 0.089	1.935 ± 0.100	1.927 ± 0.063	1.938 ± 0.073
Kidney—right	1.470 ± 0.228	1.405 ± 0.174	1.351 ± 0.186*	0.926 ± 0.228	0.890 ± 0.079	0.841 ± 0.076*
Kidney—left	1.463 ± 0.232	1.419 ± 0.161	1.359 ± 0.185*	0.895 ± 0.119	0.899 ± 0.082	0.843 ± 0.073*
Ovary—right	—	—	—	0.033 ± 0.009	0.042 ± 0.054	0.030 ± 0.024
Ovary—left	—	—	—	0.035 ± 0.011	0.037 ± 0.014	0.028 ± 0.006*
Relative weight (%)						
Brain	0.517 ± 0.077	0.522 ± 0.102	0.529 ± 0.064	0.706 ± 0.126	0.728 ± 0.115	0.788 ± 0.126†
Kidney—right	0.357 ± 0.093	0.339 ± 0.081	0.333 ± 0.053	0.342 ± 0.142	0.335 ± 0.049	0.341 ± 0.059
Kidney—left	0.355 ± 0.092	0.343 ± 0.078	0.335 ± 0.051	0.324 ± 0.053	0.338 ± 0.048	0.341 ± 0.052
Ovary—right	—	—	—	0.012 ± 0.003	0.015 ± 0.018	0.012 ± 0.011
Ovary—left	—	—	—	0.013 ± 0.004	0.014 ± 0.005	0.011 ± 0.003

‡Numbers in parentheses represent the numbers of rats examined.
Values are means ± SD. Asterisks indicate significant difference from controls (*$P < 0.05$; Scheffe's test). Daggers indicate significant difference from controls (†$P < 0.01$; Scheffe's test).

Table 3. Incidences of tumours in F344 rats given stevioside in the diet for 104 wk

Site	Histological type of tumour	No. of male rats with tumours			No. of female rats with tumours		
		Control (49)†	2.5% (48)	5% (47)	Control (49)	2.5% (46)	5% (47)
Brain	Glioma	0	0	0	1	0	0
Trigeminus	Schwannoma	0	0	0	1	0	0
Pituitary gland	Adenoma	13	5	8	11	14	9
	Carcinoma	0	0	1	1	0	1
Thyroid gland	C-cell adenoma	5	10	10	4	4	6
	C-cell carcinoma	5	2	4	1	0	1
	Follicular cell adenoma	0	0	1	0	0	0
	Follicular cell carcinoma	0	0	1	0	0	0
Adrenal gland	Cortical adenoma	1	1	0	0	0	1
	Phaeochromocytoma	15	10	7	3	1	7
	Ganglioneuroma	0	1	1	0	0	0
Pancreas	Islet cell adenoma	2	5	3	3	0	0
	Islet cell carcinoma	1	1	1	0	1	0
	Mixed acinar-islet cell adenoma	1	0	0	0	0	0
Haematopoietic system	Large granular lymphocyte leukaemia	6	9	11	7	9	10
	Malignant lymphoma	1	1	2	1	4	3
	Thymoma	1	0	0	0	1	0
Lung	Bronchiolar-alveolar adenoma	6	5	2	1	1	1
	Bronchiolar-alveolar carcinoma	2	0	3	0	0	1
Tongue	Squamous cell papilloma	1	1	0	0	0	0
Oral cavity	Squamous cell papilloma	0	0	0	0	0	1
Forestomach	Squamous cell papilloma	0	1	0	0	0	0
Small intestine	Adenocarcinoma	1	0	0	0	0	0
	Leiomyosarcoma	1	0	0	0	0	0
Large intestine	Adenoma	1	1	0	0	0	0
	Adenocarcinoma	0	0	0	0	1	1
	Malignant fibrous histiocytoma	0	0	1	0	0	0
Liver	Hepatocellular adenoma	5	3	3	0	0	0
	Hepatocellular carcinoma	0	1	0	0	0	0
Kidney	Renal cell adenoma	0	1	0	0	0	0
	Renal cell carcinoma	0	0	0	1	0	0
Testis	Interstitial cell tumour	47	44	40	—	—	—
Prostate	Adenoma	3	2	0	—	—	—
Ovary	Granulosa cell tumour	—	—	—	0	1	0
Uterus	Endometrial adenoma	—	—	—	0	1	0
	Endometrial adenocarcinoma	—	—	—	1	1	0
	Endometrial stromal polyp	—	—	—	7	8	9
	Endometrial stromal sarcoma	—	—	—	0	2	0
Mammary gland	Fibroma	5	8	2	2	1	0
	Fibroadenoma	5	4	1	5	6	6
	Adenoma	2	0	0	6	0*	0*
	Adenocarcinoma	0	0	1	0	0	1
Skin	Squamous cell carcinoma	2	4	0	0	0	0
	Keratoacanthoma	1	0	2	0	1	0
	Basal cell adenoma	1	0	0	0	0	1
	Trichoepithelioma	0	1	0	0	0	0
	Tricholemmoma	0	1	0	0	0	0
Subcutis	Fibroma	1	1	1	0	0	0
	Lipoma	1	0	2	0	0	0
	Haemangioma	0	0	0	0	0	1
	Malignant fibrous histiocytoma	0	0	1	1	0	0
	Fibrosarcoma	1	0	0	0	0	0
	Leiomyosarcoma	0	0	1	0	0	0
	Sarcoma (not otherwise specified)	0	0	0	1	0	0
Bone	Osteosarcoma	0	1	0	0	0	0
Pinna	Neural crest neoplasm	1	0	0	0	0	0
Zymbal's gland	Squamous cell carcinoma	0	0	1	0	0	0
Preputial/clitoral gland	Adenoma	6	9	3	6	3	2
Abdominal cavity	Mesothelioma	3	2	0	0	0	0
	Fibrosarcoma	0	0	0	0	1	0
	Malignant fibrous histiocytoma	0	0	1	0	0	0
	Chordoma	0	1	0	0	0	0
All sites		49	47	47	37	37	36

†Numbers in parentheses represent the numbers of rats examined.
Asterisks indicate significant differences from controls (*$P < 0.05$; Fisher's exact probability test).

Table 4. Incidence and severity of chronic nephropathy in F344 rats given stevioside in the diet for 104 wk

Group		No. of rats examined	No. of rats with chronic nephropathy	Severity of chronic nephropathy			
Sex	Treatment			Normal	Mild	Moderate	Severe
Male	Control	49	46	3	16	24	6
	2.5%	48	38	10	19	17	2*
	5%	47	40	7	27	9	4**
Female	Control	49	17	32	12	5	0
	2.5%	46	23	23	16	7	0
	5%	47	9	38	8	1	0

Asterisks indicate significant difference from control cases (*$P < 0.05$; **$P < 0.01$; cumulative chi-square test).

Body weights

Figure 2 shows the growth curves for each group. Body weight gains were depressed in line with the dose of stevioside, with the mean depression rates for males and females during the experimental period being, respectively, 4.4 and 9.2% in the 5% treated groups, and 2.3 and 2.4% in the 2.5% treated groups, in comparison with the control group. The body weights of males and females in the 5% treated groups at wk 104 were significantly lower than the control group values (Table 1). At wk 80, decrease of body weight, resulting from an accidental stoppage of the drinking water supply, was observed in the 2.5% treated males. The body weights of these rats had recovered by wk 84.

Food intake

Daily food intake in the stevioside-treated groups of both sexes did not differ from that of the control group and therefore a good correlation between the dose of stevioside and the daily intake was observed throughout (Table 1).

Survival rates and survival times

No significant differences in mean survival times were observed among the groups during the treatment period, although the final survival rate for the 5% stevioside-treated males was significantly lower than that of the controls (Table 1). Figure 3 shows the survival curves of each group in both sexes.

Haematological findings

Haematological examination of rats at wk 108 did not reveal any statistically significant variation in the numbers of white blood cells, red blood cells or platelets, amounts of haemoglobin, or levels of haematocrit between the stevioside-treated groups and the control group in either sex.

Organ weights

Table 2 summarizes data for the organ weights. The absolute kidney weights were significantly decreased in the 5% treated males, and the absolute weights of the kidneys and the left ovaries were reduced and the relative brain weights were increased in the 5% treated females, compared with the control values.

Histopathological findings

Table 3 shows the incidences of tumours developing in the rats in the present study, lesions being observed in many organs or tissues of all groups, including the controls. In males, tumours of the testes were the most frequent, followed by tumours of the thyroid, adrenal glands, haematopoietic system, mammary glands and pituitary; in females, tumours of the pituitary, haematopoietic system, uterus and mammary glands were common. However, there were no significant differences in the incidences between the stevioside-treated and control groups for both sexes, except for a decrease in the incidence of mammary adenomas in the 5% and 2.5% treated females.

In addition to the tumours, many kinds of non-neoplastic lesions were found in rats of all groups. The severity of chronic nephropathy in the 5% and 2.5% treated males was significantly lower than that in the control males (Table 4). In females, the severity of chronic nephropathy in the 5% treated group was also lower than that in the controls, although this was not significant. However, there were no significant differences in the incidences of any non-neoplastic lesions, including nephropathy, between the stevioside-treated and control groups for either sex.

DISCUSSION

In the present carcinogenicity study of stevioside, although a variety of tumours were detected in all groups, including the controls, their organ distributions and histological types were essentially similar to those of the spontaneous tumours described previously (Haseman *et al.*, 1990). There were no significant increases in the incidence of neoplastic lesions in any organ or tissue in the stevioside-treated groups. However, we did observe some changes that could be attributed to the stevioside treatment.

There have been many reports demonstrating a relationship between energy (or caloric) restriction and inhibition of spontaneous or induced mammary tumour development (Carroll, 1975; Klurfeld *et al.*, 1989), progression of age-related chronic nephropathy (Masoro *et al.*, 1989; Tapp *et al.*, 1989) and body weight gain. In the present study, the incidence of adenomas of the mammary gland in the

stevioside-treated females was significantly lower than that of the controls. The incidences of all tumours of the mammary glands in the 5% treated males (8.5%) and the 5% and 2.5% treated females (14.9% and 15.2%) were also lower than in the respective controls (24.5% in males and 26.5% in females), albeit without significance. The severity of chronic nephropathy in males was also clearly reduced by both concentrations of stevioside. These effects were presumably linked to the dose-dependent depression of body weight gain in males and females, without decrease in food intake or any specific histopathological findings indicative of toxicity. The observations were thus in close agreement with the phenomena expected from energy restriction. Stevioside itself is a non-caloric sweetener, and the aglycone steviol produced by the intestinal microflora of rats inhibits glucose absorption in the hamster intestine (Toskulkao *et al.*, 1995) and glucose generation and oxygen uptake in rat renal tubules *in vitro* (Yamamoto *et al.*, 1985). Stevioside and its derivatives also inhibit glucose and fructose transport across cell membranes in the isolated rat liver (Ishii *et al.*, 1987). Our knowledge of physiological effects therefore suggests that administration of stevioside evokes a status equivalent to that attained with energy restriction.

The main reason for the decrease in the final survival rate of the 5% treated males was rapid development of large granular lymphocyte (LGL) type leukaemia in the final weeks of the study. More animals died or were killed *in extremis* before the termination of the experiment owing to LGL leukaemia in the 5% treated males (nine rats) than in the controls (three rats). The final incidences of LGL leukaemia in the stevioside-treated males also tended to be higher than in the control males (23.4% in the 5% treated group, 18.8% in the 2.5% treated group and 12.2% in the control group), although this was not statistically significant. A total of 225 untreated control F344 males used in five 2-yr carcinogenicity studies performed recently in our laboratory showed a mean incidence of LGL leukaemia of 24.0% (range 16.0–33.3%). The incidences observed here were, therefore, in line with our background data and must be considered incidental.

As the histopathological examination revealed no specific toxicological changes in the kidneys of males, or in the kidneys, ovaries and brains of females in the 5% treated groups, the significant differences in the absolute or relative weights of these organs, in comparison with the control group values, were concluded to be a corollary of the observed inhibition of body weight gain.

It has been reported that stevioside itself shows no mutagenic potential (Ishidate *et al.*, 1984; Matsui *et al.*, 1989), whereas the aglycone steviol causes mutations after metabolic activation in the forward mutation assay, *umu* test, chromosomal aberration test and gene mutation assay (Matsui *et al.*, 1989; Pezzuto *et al.*, 1985) but not in the reverse mutation test (Ames test) using *Salmonella typhimurium* TA100, TA98, TA102 or TA97. The information with regard to mutagenic potential is intriguing, given the lack of carcinogenicity found in the present study. In our earlier experiment, male F344 rats were sequentially killed after treatment for 2 days, 1 wk, 2 wk and 6 wk with a 10% stevioside diet (five rats each): the large intestine was removed and the intestinal contents collected in a 50% methanol solution. Quantitative HPLC analysis of steviol in the contents of the large intestine revealed amounts of 5.1 ± 0.4 mg per rat at 2 days, 2.8 ± 1.5 at 1 wk, 2.8 ± 0.8 at 2 wk and 1.1 ± 0.7 at 6 wk. These unpublished data provide strong evidence that steviol is indeed produced in rats treated with stevioside, as in the present carcinogenicity study. Therefore, the conclusion that stevioside exerts no carcinogenic activity in F344 rats when administered continuously in the diet at concentrations of 2.5 or 5% for up to 104 wk, presumably also means that any mutagenicity exerted by steviol is not of significance for neoplasia under these circumstances.

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