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A 90-Day Oral (Dietary) Toxicity Study of Rebaudioside A in Sprague-Dawley Rats

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Rebaudioside A is one of several glycosides found in the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (Compositae) stevia that has been identified as a potential sweetener. The present study (initiated in April 2006 and completed in October 2006) evaluated the safety of this sweetener when administered as a dietary admix at target exposure levels of 500, 1000, and 2000 mg/kg/day to Sprague-Dawley rats for 90 days. There were no treatment-related effects on the general condition and behavior of the animals as determined by clinical observations, functional observational battery, and locomotor activity assessments. Evaluation of clinical pathology parameters revealed no toxicologically relevant, treatment-related effects on hematology, serum chemistry, or urinalysis. Macroscopic and microscopic findings revealed no treatment-related effects on any organ evaluated. Lower mean body weight gains were noted in males in the 2000 mg/kg/day group throughout the study, which was considered to be test article related; however, given the small magnitude of the difference as compared to controls, this effect was not considered to be adverse. Results of this study clearly demonstrate that dietary administration of high concentrations of rebaudioside A for 90 consecutive days to Sprague-Dawley rats was not associated with any signs of toxicity.

Keywords Rat, Rebaudioside A, Subchronic Toxicity, Sweetener

Rebaudioside A is a major constituent of the leaves of the plant, *Stevia rebaudiana* (Bertoni) Bertoni (Compositae), and possesses about 250 to 450 times the relative sweetness intensity of sucrose (Kinghorn 2002). Rebaudioside A is one of at least 11 glycosides of the diterpene derivative, steviol (ent-13-hydroxykaur-16-en-18-oic acid) naturally occurring in *S. rebaudiana* (Kennelly 2002). The chemical structure of rebaudioside A is provided in Figure 1. The two principle steviol glycosides in commercial products are stevioside and rebaudioside A. Stevioside is the most abundant of the steviol glycosides, with extraction yields from the dry leaves of *S. rebaudiana* reportedly varying from 5% to 22% depending on cultivar and growing conditions; whereas the yield from the dry leaves of *S. rebaudiana* for rebaudioside A is stated to range from 25% to 54% relative to stevioside levels (Kennelly 2002). In contrast, Abudula et al. (2004) report that stevioside and rebaudioside A account for 5% and 2%, respectively, of the mass from dry leaves of *S. rebaudiana*. *S. rebaudiana* extracts containing mostly steviol glycosides are authorized as food additives with a functional use as a sweetener in a number of South American and Asian countries (e.g., Brazil, Argentina, Paraguay, South Korea, and Japan). Although not currently authorized as food additives, steviol glycosides also are used in herbal preparations or dietary supplements in other countries, including the People's Republic of China, the United States, and certain countries in Western Europe (Kinghorn 2002).

During its 63rd meeting, the Joint Food and Agriculatural Organization of the United Nations/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) evaluated steviol glycosides and assigned a temporary acceptable daily intake (ADI) of 0–2 mg/kg body weight (bw) (expressed as steviol) and established tentative specifications. The temporary ADI was established on the basis of the no-observed-effect level (NOEL) for stevioside of 970 mg/kg bw/day (or 383 mg/kg bw/day, expressed as steviol) in a 2-year study of stevioside (purity, 95.6%) in rats and a safety factor of 200 (JECFA 2005). During this evaluation, JECFA assumed that all steviol glycosides hydrolyze upon ingestion to steviol. More recently, JECFA reevaluated steviol glycosides, revising the specifications to cover a range of compositions that could include product that is at least 95% stevioside or at least 95% rebaudioside A (JECFA 2007).

The present 90-day subchronic dietary toxicity study was conducted in rats based on the following rationale: (1) if

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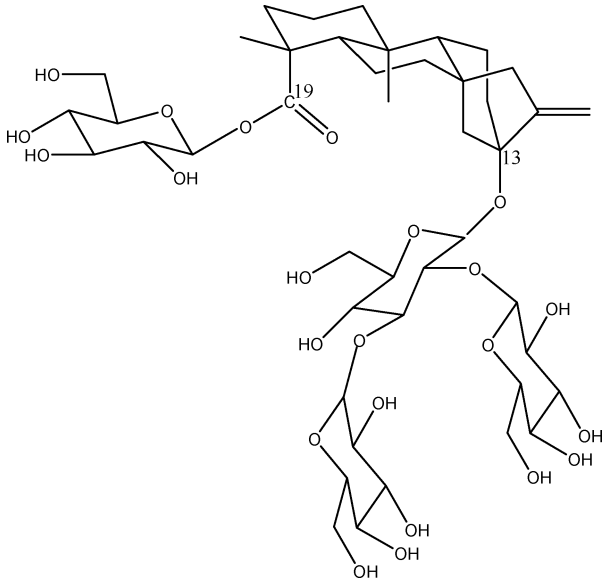

FIGURE 1
Chemical structure of rebaudioside A.

approved internationally by regulatory agencies, high-purity rebaudioside A may become an important food additive based on interest in natural noncaloric sweeteners; (2) the published toxicology database for rebaudioside A is limited to acute oral toxicity in mice ($LD_{50} > 2000$ mg/kg bw) and bacterial mutation potential (negative) (Medon et al. 1982); (3) an ADI for rebaudioside A does not exist; 4) the JECFA temporary ADI for steviol glycosides, including rebaudioside A, is based on a long-term dietary toxicity study with stevioside and assumes all steviol glycosides hydrolyze to steviol following ingestion; (5) hydrolysis of rebaudioside A to steviol is substantially slower than stevioside (Wingard et al. 1980), suggesting that a JECFA temporary ADI (expressed as steviol) based on the NOEL for stevioside and hydrolysis of all steviol glycosides, including rebaudioside A, to steviol may be overly conservative; and (6) a 90-day subchronic dietary toxicity study of rebaudioside A in rats conducted using appropriate U.S. Food and Drug Administration Redbook 2000 and Organization of Economic Cooperation and Development (OECD) testing guidelines and in compliance with U.S. Food and Drug Administration (FDA) Good Laboratory Practice Regulations and OECD Principles of Good Laboratory Practice would allow an ADI to be established for rebaudioside A.

MATERIALS AND METHODS

The safety of rebaudioside A was evaluated in a 90-day feeding study in Sprague-Dawley rats conducted at WIL Research Laboratories, Ashland, Ohio. This study (initiated in April 2006 and completed in October 2006) was conducted in accordance with the OECD 408 (OECD 1998a) and U.S. Food and Drug Administration (FDA) Redbook 2000 testing guidelines (US FDA 2003) and in compliance with the U.S. FDA Good Laboratory Practice Regulations (US FDA 1987) and the OECD Principles of Good Laboratory Practice (OECD 1998b).

Experimental Design Overview

Rebaudioside A was administered for a minimum of 90 consecutive days on a continuous basis in the diet to three groups (groups 2 to 4) of Crl:CD(SD) rats. Target dosage levels were 500, 1000, and 2000 mg/kg/day. Dietary concentrations were adjusted weekly based on expected average weight and current food consumption. A concurrent control group (group 1) received the basal diet, PMI Nutrition International Certified Rodent LabDiet 5002 (meal), on a comparable regimen. Each group consisted of 20 animals/sex. Following at least 90 days of dietary exposure, all animals were euthanized. Table 1 provides the animal allocation to groups 1 to 4 and the mean average rebaudioside A consumption for the 90-day period.

All animals, which were individually housed for the duration of the study, were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. Functional observational battery (parameters detailed in Table 2) and locomotor activity data were recorded for 10 animals/sex/group during study week 12. Ophthalmic examinations were performed during study weeks −1 and 12. Blood samples were collected for hematology and serum chemistry evaluations from 10 animals/sex/group during study weeks 2 and 5 and

TABLE 1
Experimental design and mean average rebaudioside A consumption

Group number	Test article	Number of animals		Target dosage level[a] (mg/kg/day)	Mean calculated test article consumption (mg/kg/day)	
		Males	Females		Males	Females
1	Basal Diet	20	20	0	0	0
2	Rebaudioside A	20	20	500	517	511
3	Rebaudioside A	20	20	1000	1035	1019
4	Rebaudioside A	20	20	2000	2055	2050

[a]Target dosage level is expressed as mg of test article per kg body weight of the animals.

TABLE 2
Functional observational (FOB) parameters evaluated in rats

Home cage observations	
Posture	Biting
Convulsions/tremors	Palpebral (eyelid) closure
Feces consistency	
Handling observations	
Ease of removal from cage	Ease of handling animal in hand
Lacrimation/chromodacryorrhea	Salivation
Piloerection	Fur appearance
Palpebral closure	Respiratory rate/character
Eye prominence	Mucous membranes/eye/skin color
Red/Crusty deposits	Muscle tone
Open field observations (performed over a 2-min period)	
Mobility	Gait
Rearing	Arousal
Convulsions/tremors	Urination/defecation
Grooming	Gait score
Bizarre/stereotypic behavior	Backing
Time to first step (seconds)	
Sensory observations	
Approach response	Touch response
Startle response	Tail pinch response
Pupil response	Eyeblink response
Forelimb extension	Hindlimb extension
Air righting reflex	Olfactory orientation
Neuromuscular observations	
Hindlimb extensor strength	Grip strengthhind and forelimb
Hindlimb foot splay	Rotarod performance
Physiological observations	
Catalepsy	Body weight
Body temperature	

clinical pathology evaluations (hematology, serum chemistry, and urinalysis) were performed on the same 10 animals/sex/group at the scheduled necropsy (study week 13). Complete necropsies were conducted on all animals, and selected organs were weighed at the scheduled necropsy. Selected tissues were examined microscopically from all animals in the control and 2000 mg/kg/day groups.

Test Article and Control Diet

Test Article

The test article, rebaudioside A (CAS RN 58543–16-1), was received from Stevian Biotechnology, SDN BHD, Negeri Sembilan, Malaysia. The purity of the test article was 99.5%. The test article was stored at room temperature, protected from light, and was considered stable under these conditions.

Preparation of Diets

For the control group, based on the laboratory's experience, an appropriate amount of PMI Nutrition International, Certified Rodent LabDiet 5002 (meal) was weighed in a properly labeled storage bag on a weekly basis.

Diets containing the test article were prepared on a weight/weight basis as follows. A sufficient amount of the test article was weighed into tared weighing vessels and transferred to a Hobart mixer with one half of the total batch size of the basal diet and premixed for approximately 3 minutes. The remaining amount of the basal diet was added to the Hobart mixer and the diet was mixed for approximately 10 minutes to achieve a total batch of homogeneous diet at the appropriate concentration per test group. The diets containing test article were prepared approximately weekly and placed in properly labeled storage bags. The initial diet concentrations were based on average food consumption and body weights during the pretest period. Test article concentration in the diet was adjusted as necessary based on the mean body weight and food consumption for each group (by sex) to maintain the appropriate target dosage.

Test diets were prepared at the concentrations as indicated in Table 3.

Administration of Diets

The test article and control diets were offered ad libitum for 90, 91, 92, or 93 consecutive days, until the day prior to the scheduled necropsy. The weekly dietary inclusion rates (Table 3) at these dosage levels were expected to provide

TABLE 3
Concentration of rebaudioside A in the formulated test diet by study week (g/kg)

Target dosage level	Males			Females		
(mg/kg/day)	500	1000	2000	500	1000	2000
Study week 0	5.21	10.38	20.92	4.53	9.56	19.11
Study week 1	5.74	11.15	21.78	4.97	10.28	20.44
Study week 2	6.41	12.32	25.04	5.50	10.79	21.68
Study week 3	6.18	12.16	24.80	5.38	10.52	21.05
Study week 4	7.18	13.97	27.93	5.86	11.95	25.05
Study week 5	7.71	15.07	29.45	6.30	13.56	26.00
Study week 6	7.93	15.48	31.07	6.42	13.21	24.80
Study week 7	8.24	15.60	33.21	6.72	13.68	26.20
Study week 8	8.69	17.03	32.62	6.85	13.89	27.20
Study week 9	9.14	17.38	34.79	7.37	14.47	28.53
Study week 10	9.10	17.33	35.79	7.12	14.63	28.95
Study week 11	9.02	17.13	34.33	7.20	14.20	27.80
Study week 12	9.84	18.90	35.93	7.42	15.78	30.42

TABLE 4
Body weights ± *SD* (g) during selected intervals

	Target dietary levels (mg/kg/day) of rebaudioside A			
	0	500	1000	2000
Males				
Week −1	151 ± 9.9	152 ± 7.9	151 ± 10.6	151 ± 8.8
Week 0	211 ± 12.4	211 ± 11.9	210 ± 11.2	211 ± 11.5
	[34.7 ± 2.92]	[34.5 ± 3.03]	[35.1 ± 2.73]	[35.2 ± 2.77]
Week 1	273 ± 17.0	274 ± 14.0	268 ± 15.6	264 ± 17.3
	[32.6 ± 4.12]	[33.4 ± 2.72]	[30.7 ± 4.27]	[28.9 ± 3.22**]
Week 2	319 ± 23.2	320 ± 16.3	317 ± 19.5	311 ± 26.5
	[25.1 ± 6.60]	[24.4 ± 4.98]	[24.5 ± 4.89]	[24.5 ± 4.28]
Week 4	407 ± 33.3	405 ± 24.5	396 ± 29.9	385 ± 32.7
	[21.6 ± 4.85]	[21.4 ± 3.90]	[19.4 ± 3.22]	[17.2 ± 4.20**]
Week 8	507 ± 52.6	492 ± 35.5	484 ± 47.8	470 ± 51.4
	[10.7 ± 2.73]	[10.5 ± 4.42]	[9.4 ± 2.79]	[7.3 ± 3.00**]
Week 13	563 ± 63.8	536 ± 44.6	527 ± 56.0	512 ± 55.7*
	[NA]	[NA]	[NA]	[NA]
Females				
Week −1	117 ± 10.1	115 ± 10.3	114 ± 10.5	114 ± 10.4
Week 0	148 ± 12.5	149 ± 11.9	149 ± 10.8	149 ± 11.6
	[24.1 ± 4.89]	[25.6 ± 3.14]	[26.5 ± 3.81]	[26.8 ± 3.13]
Week 1	173 ± 14.3	174 ± 16.0	172 ± 15.9	171 ± 13.3
	[19.1 ± 3.77]	[18.7 ± 4.75]	[18.0 ± 4.41]	[17.3 ± 3.36]
Week 2	193 ± 14.4	196 ± 18.0	193 ± 17.3	193 ± 16.5
	[15.9 ± 4.98]	[17.0 ± 3.82]	[15.8 ± 5.03]	[17.2 ± 3.05]
Week 4	228 ± 19.3	235 ± 26.7	229 ± 22.6	228 ± 22.1
	[13.5 ± 5.28]	[13.8 ± 3.58]	[13.3 ± 3.29]	[12.6 ± 3.84]
Week 8	266 ± 24.2	271 ± 30.2	262 ± 28.1	266 ± 23.2
	[6.5 ± 5.92]	[5.8 ± 3.90]	[2.1 ± 8.50]	[7.1 ± 7.12]
Week 13	281 ± 25.0	284 ± 33.2	280 ± 31.5	276 ± 23.4
	[NA]	[NA]	[NA]	[NA]

Note. The values in brackets are body weight gained as percent ± *SD* of feed consumed.
*Significantly different from the control group at .05 using Dunnett's test.
**Significantly different from the control group at .01 using Dunnett's test.
NA = Not applicable; animals were fasted during this interval.

adequate exposure to the test article in all treated groups to achieve target dosages. The dose levels selected for this study were based on the published literature for related steviol glycosides and results of a previous 14-day range-finding study (Eapen 2007) and represent feasible dietary exposures that are palatable and do not replace a significant amount of nutrients and/or calories in the basal rodent diet. The impact of the high dietary inclusion rates on body weights was evaluated by using calculations of food efficiency (body weight gained as percent of feed consumed) for male and female control and treatment groups (Table 4). The selected route of administration for this study was oral (dietary) as the test article is a food ingredient and intended for human consumption.

Analysis of Rebaudioside A in Rat Feed

Previous analysis (Eapen 2007) showed that the test article is stable in the diet for up to 10 days following room temperature storage. Prior to the initiation of dietary exposure, samples (approximately 100 g each) for homogeneity determination were collected from the top, middle, and bottom strata of the first dietary formulations for groups 2 and 4. In addition, samples (approximately 100 g each) for concentration determinations were collected during study weeks 0, 3, 7, and 12 from the middle strata of the dietary formulations for groups 1 to 4. All analyses were conducted by the Analytical Chemistry Department, WIL Research Laboratories, using a validated analytical method.

Animal Receipt, Acclimation, and Husbandry

Ninety-five male and 95 female Crl:CD(SD) rats were received in good health on 4 April 2006 from Charles River Laboratories, Raleigh, North Carolina. The animals were approximately 29 days old at receipt. Females were nulliparous and non-pregnant. Each animal was examined by a qualified technician on the day of receipt and weighed 3 days later. Each animal was uniquely identified by a Monel metal eartag displaying the permanent identification number. All animals were housed for a 14-day acclimation/pretest period. During this period, each animal was observed twice daily for mortality and changes in general appearance or behavior. Individual body weights and food consumption were recorded and detailed physical examinations were performed during the acclimation/pretest period to ensure the use of healthy animals.

Upon arrival, all animals were housed three per cage for approximately 3 days. Thereafter, all animals were housed individually in clean, stainless steel, wire-mesh cages suspended above cage-board. All animals were housed throughout the acclimation period and during the study in an environmentally controlled room. Controls were set to maintain a temperature of $71^{\circ}F \pm 5^{\circ}F$ ($22^{\circ}C \pm 3^{o}C$) and a relative humidity of approximately 30% to 70%. Room temperature and relative humidity were recorded daily. Light timers were set to provide a 12 h light/12 h dark photoperiod. Reverse osmosis–treated drinking water, delivered by an automatic watering system, was provided ad libitum throughout the study period. The protocol was reviewed and approved by the WIL Research Institutional Animal Care and Use Committee (IACUC). Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996).

Assignment of Animals to Treatment Groups

On the day prior to the initiation of test diet administration, all available rats were weighed and examined in detail for physical abnormalities. These data were collected using the WIL Toxicology Data Management System (WTDMS) and reviewed by the Study Director. The animals judged suitable for assignment to the study were selected for use in a computerized randomization procedure. A printout containing the animal numbers, corresponding body weights, and individual group assignments was generated based on body weight stratification in a block design. The animals were then arranged into groups according to the printout. Individual body weights at randomization were within $\pm 20\%$ of the mean for each sex. Each group consisted of 20 males and 20 females. These animals were then randomized into four study replicates to allow for the reasonable conduct of the functional observational battery and locomotor activity assessments. Each dose group and sex was equally represented within each study replicate. The selected animals were approximately 6 weeks old at the initiation of dose administration; individual body weights ranged from 185 to 237 g for males and from 121 to 170 g for females.

Parameters Evaluated

Clinical Observations and Survival

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity. All animals received a clinical examination daily. Detailed physical examinations were conducted on all animals weekly, beginning at least one week prior to test article administration and prior to the scheduled necropsy.

Body Weights

Individual body weights were recorded at least weekly, beginning approximately 2 weeks prior to test article administration (study week -2). Mean body weights and mean body weight changes were calculated for the corresponding intervals. Final body weights (fasted) were recorded prior to the scheduled necropsy.

Food Consumption

Individual food consumption was recorded weekly, beginning approximately 1 week prior to test article administration (study weeks -1 to 0). Food intake was calculated as g/animal/day for the corresponding body weight intervals. The mean amounts of rebaudioside A consumed (mg/kg/day) by each sex per dose group were calculated from the mean food consumed (g/kg/day) and the appropriate target concentration of test article in the food (mg/kg).

Functional Observational Battery (FOB)

Functional observational battery (FOB) assessments were recorded for 10 animals/sex/group during study week 12. Testing was performed by trained technicians without knowledge of the animals' group assignments. The FOB was performed in a sound-attenuated room equipped with a whitenoise generator set to operate at 70 ± 10 dB. Animals were observed for the parameters listed in Table 2, which are based on previously developed protocols (Gad 1982; Haggerty 1989; Irwin 1968; Moser et al. 1988; Moser, McDaniel, and Phillips 1991; O'Donoghue 1989).

Locomotor Activity

Locomotor activity was assessed for 10 animals/sex/group during study week 12. Locomotor activity, recorded after the completion of the FOB, was measured automatically using the SDI Photobeam Activity System (San Diego Instruments, San Diego, California). This fully validated, personal computer-controlled system utilized a series of infrared photobeams surrounding an amber plastic, rectangular cage to quantify the motor activity of each animal. Four-sided black plastic enclosures were used to surround the amber plastic boxes and decrease the potential for distraction from extraneous environmental stimuli or activity by technicians or adjacent animals. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. The locomotor activity assessment was performed in a sound-attenuated room

equipped with a whitenoise generator set to operate at 70 ± 10 dB. The testing of treatment groups was conducted according to replicate sequence. Each animal was tested separately. Data were collected in 5-min epochs and the test session duration was 60 minutes. These data were compiled as four 15-min subsessions for tabulation.

Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills (i.e., grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Clinical Pathology

Blood samples for hematology and serum chemistry evaluations were collected from 10 animals/sex/group during study weeks 2 and 5, and blood and urine samples for clinical pathology evaluations (serum chemistry, hematology, and urinalysis) were collected from the same 10 animals/sex/group at the scheduled necropsy (study week 13). The animals were fasted overnight prior to each blood collection. Urine was collected overnight prior to necropsy from animals housed in metabolism cages. Blood for hematology and serum chemistry evaluations was collected from a retroorbital sinus from animals anesthetized with isoflurane and from the vena cava at the time of necropsy for coagulation evaluations from animals euthanized by carbon dioxide inhalation. Blood was collected into tubes containing potassium EDTA (hematology), sodium citrate (clotting determinations), or no anticoagulant (serum chemistry).

Serum chemistry parameters (albumin, total protein, globulin, A/G ratio, total bilirubin, urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, glucose, total cholesterol, calcium, chloride, phosphorus, potassium, sodium, triglycerides, and sorbitol dehydrogenase) were determined using a Hitachi Model 912 chemistry analyzer using application codes provided by the manufacturer and reagents provided by Boehringer Mannheim (Indianapolis, IN). Additional serum chemistry parameters (total bile acids and glutamate dehydrogenase) were determined using a Hitachi Model 717 chemistry analyzer. Insulin was measured using a Gamma-C12 counter with Biotrak rat insulin ^{125}I assay system. Hematological parameters (total leukocyte count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], platelet count, reticulocyte count [percent and absolute], percent, and absolute leukocyte count [neutrophil, lymphocyte, monocyte, eosinophil and basophil]) were determined using a Bayer ADVIA 120 according to the manufacturer's operator's manual. Coagulation parameters (prothrombin time and activated partial thromboplastin time) were determined using an AMAX Destiny Amelung Coagulation analyzer according to the manufacturer's operator's manual. Standard urinalysis parameters (volume, urobilinogen, color, appearance, pH, protein, glucose, ketones and bilirubin, occult blood, leukocytes, and nitrates) were determined using Bayer reagent strips that were read using a CLINITEK 500+ Urine Chemistry Analyzer. Specific gravity was measured using an ATAGO Urine Specific Gravity Refractometer manufactured by NSG Pression Cells, Inc. Urinary sediment was separated by centrifugation and examined microscopically using routine methods.

Macroscopic Examination

A complete necropsy was conducted on all animals. Animals were euthanized by carbon dioxide inhalation followed by exsanguination. The necropsies included examination of the external surface, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities including viscera. At the time of necropsy, the following tissues and organs were collected and placed in 10% neutral-buffered formalin: adrenal glands, aorta, bone with marrow (femur and sternum), brain (cerebrum level 1, cerebrum level 2, and cerebellum with medulla/pons), gastrointestinal tract (esophagus, stomach, duodenum, jejunum, ileum [including Peyer's patch], cecum, colon, and rectum]), harderian gland, heart, kidneys, liver, lungs (including bronchi), lymph nodes (mandibular and mesenteric), mammary gland (females only), nasal cavity, ovaries with oviducts, pancreas, peripheral nerve (sciatic), pituitary gland, prostate, salivary glands (mandibular), seminal vesicles, skeletal muscle (rectus femoris), skin, spinal cord (cervical, midthoracic, and lumbar), spleen, thymus, thyroids/parathyroids, trachea, urinary bladder, uterus with vagina and cervix, and gross lesions. Bone marrow smears were collected at necropsy, but not placed in formalin; slides were examined only if scientifically warranted. The epididymides and testes were preserved in Bouin's solution, whereas the eyes with optic nerves were preserved in Davidson's solution. Sectioning of the nasal cavity was done in accordance with the method of Young et al. (1981). The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries with oviducts, pituitary, prostate, spleen, testes, thymus, thyroids with parathyroids, and uterus. Paired organs were weighed together. Organ-to–final body weight and organ-to-brain weight ratios were calculated.

Histopathologic Procedures and Microscopic Examination

After fixation, the collected tissues were trimmed as described by Thompson et al. (1966). Trimmed specimens were placed in appropriately labeled and numbered cassettes. The fixed tissue samples were processed into paraffin blocks. The labeled paraffin blocks were sectioned at 4 to 8 μm and the paraffin ribbons of the sectioned tissue were placed on clean glass microscope slides, labeled with the appropriate study, animal, group, and cassette numbers. Upon completion of staining with hematoxylin and eosin (Luna 1968), cover slips were placed on the slides. Microscopic examinations were performed on all tissues listed in *Macroscopic Examination* from all animals in the control and 2000 mg/kg/day groups. Gross lesions were examined from all animals in the 500 and 1000 mg/kg/day groups as well.

Statistical Methods

Analyses were conducted using two-tailed tests for significance levels of 5% and 1%, comparing each test article-treated group to the control group by sex. Body weight, body weight change, food consumption, locomotor activity data, clinical pathology parameters, and absolute and relative organ weight values were subjected to a parametric one-way analysis of variance (ANOVA) (Snedecor and Cochran 1980), followed by Dunnett's Test (Dunnett 1964). Functional observational battery parameters that yielded scalar or descriptive data were analyzed using Fisher's Exact Test (Steel and Torrie 1980).

RESULTS

Analyses of Test Diet Formulations

The analyses of the test article dietary formulations were found to contain 90.0% to 105% of the protocol specified concentration of test article throughout the study and were homogeneous (data not shown).

Survival and Clinical Observations

All animals survived to the scheduled necropsy. There were no test article–related clinical observations. All clinical findings in the test article–treated groups were noted with similar incidence in the control group, were limited to single animals, were not noted in a dose-related manner and/or were common findings for laboratory rats of this age and strain.

Body Weights, Food Consumption, and Rebaudioside A Intake

Test article–related lower mean body weight gains were noted in the 2000 mg/kg/day group males generally throughout the study (Table 4). These lower mean body weight gains resulted in statistically significant lower cumulative body weight gains and a statistically significant mean body weight difference that was 9.1% lower compared to the control group at the end of the dosing period (study week 13). The lower mean body weights were not considered to be adverse due to the small magnitude of difference from the control group value. Because of the proportion of basal diet that was replaced with the test article containing little caloric value, the lower mean body weight gains may have been the result of the animals not consuming an equivalent number of calories as the concentration of the test article in the diet increased over the course of the study. A similar trend in mean body weights did not occur in the female test article–treated groups; however, this may be explained by the fact that the overall inclusion rates of test article in the diet were lower for the females as compared to the males throughout the study (Table 3). The impact of the lower caloric content of the treatment diets may be assessed by examining the food efficiency data. The food efficiency data for males demonstrate that body weight gained as a percent of feed consumed is generally decreased as compared to the control group for all test article–treatment groups and statistically significantly decreased at 2000 mg/kg/day as compared to the control group for the following intervals: weeks 0 to 1, 3 to 4, and 7 to 8 (Table 4). In contrast, the food efficiency data for females demonstrate that body weight gained as a percent of feed consumed is generally similar for test article–treatment groups as compared to the control group (Table 4).

There were no test article–related effects on food consumption. Although some intervals in the female test article–treated groups were statistically significantly increased compared to the control group, these differences were not considered to be test article-related as the mean food consumption for those groups was unchanged from the preceding intervals and there was no dose- or time-dependent trend noted. There were no other statistically significant differences when the control and test article-treated groups were compared (data not shown).

Average rebaudioside A consumption (mg/kg/day), was based on nominal dietary levels of the test article and is presented in Table 1.

Functional Observational Battery (FOB) and Motor Activity

No test article–related effects were observed during the FOB (data not presented) on home cage, handling, open field and sensory, neuromuscular, or physiological parameters. Ambulatory and total motor activity (data not presented) were unaffected by dietary administration of rebaudioside A.

Hematology, Serum Chemistry, and Urinalysis

There were no test article–related effects on week 13 hematology parameters (Tables 5 and 6). However, some statistically significant differences were observed when the control and test article–treated groups were compared. At various intervals (week 2 and 5 data not presented), increased mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration and decreased % basophil counts were noted in test article–treated male groups. In females treated with 500 mg/kg/day, lower red cell count and hemoglobin values were noted at study week 2. These group mean differences were within ± 2 standard deviations of historical control range values, did not occur in a clear concentration-dependent manner, and were not observed in a time-dependent manner and were therefore not regarded as test article dependent.

There were no test article-related effects on serum chemistry parameters (Tables 7 to 9). However, at various intervals (week 2 and 5 data not presented), some statistically significant differences were observed when the control and test article–treated groups were compared (i.e., decreased total protein, cholesterol, calcium, phosphorous, and triglyceride values and/or increased chloride and sodium values in one or more test article–treated male groups). These group mean differences were not considered to be test article-related because the values did not show a dose- or time-related response.

There were no test article-related effects on urinalysis parameters (Table 10).

TABLE 5
Hematology values (RBC and coagulation parameters ± *SD*) at week 13

	RBC ($10^6/\mu$l)	HB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelet ($10^3/\mu$l)	PT (s)	APTT (s)
					Target dietary levels (mg/kg/day) of rebaudioside A				
Males	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0	9.33 ± 0.491	16.1 ± 0.63	48.1 ± 2.44	51.5 ± 1.84	17.3 ± 0.58	33.6 ± 0.64	866 ± 276.2	14.3 ± 1.33	21.5 ± 2.09
500	9.06 ± 0.317	16.3 ± 0.47	48.5 ± 1.63	53.5 ± 1.35*	18.0 ± 0.44*	33.6 ± 0.40	963 ± 146.6	15.0 ± 0.90	24.0 ± 2.06
1000	9.09 ± 0.438	16.3 ± 0.59	48.5 ± 2.42	53.4 ± 1.45*	18.0 ± 0.48*	33.6 ± 0.54	823 ± 214.5	15.1 ± 1.60	22.4 ± 2.06
2000	9.08 ± 0.536	16.4 ± 0.56	48.6 ± 2.28	53.5 ± 1.09*	18.0 ± 0.54**	33.7 ± 0.74	845 ± 105.2	15.6 ± 1.70	23.3 ± 2.49
Females									
0	8.34 ± 0.347	15.5 ± 0.76	45.6 ± 2.80	54.6 ± 2.15	18.6 ± 0.57	34.1 ± 0.59	889 ± 188.5	12.9 ± 0.76	18.5 ± 3.50
500	8.33 ± 0.429	15.5 ± 0.75	46.0 ± 2.92	55.2 ± 1.55	18.6 ± 0.41	33.7 ± 0.70	883 ± 183.5	12.6 ± 1.07	18.6 ± 1.97
1000	8.32 ± 0.472	15.4 ± 0.58	45.4 ± 2.08	54.6 ± 1.62	18.5 ± 0.47	33.9 ± 0.56	910 ± 166.3	12.7 ± 0.81	19.4 ± 2.66
2000	8.26 ± 0.239	15.2 ± 0.35	45.3 ± 1.58	54.8 ± 0.84	18.4 ± 0.32	33.6 ± 0.57	918 ± 226.8	12.8 ± 0.71	18.7 ± 2.05

Note. RBC = red blood cell count; HB = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PT = prothrombin time; APTT = activated partial thromboplastin time.

N = number of rats/sex/group

*Significantly different from the control group at .05 using Dunnett's test.

**Significantly different from the control group at .01 using Dunnett's test.

TABLE 6
Hematology values (total and differential white blood cell counts ± *SD*) at week 13

Target Dietary Levels (mg/kg/day) of Rebaudioside A	WBC ($10^3/\mu$l)	Neutrophil ($10^3/\mu$l)	(%)	Lymphocyte ($10^3/\mu$l)	(%)	Monocyte ($10^3/\mu$l)	(%)	Eosinophil ($10^3/\mu$l)	(%)
Males	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0	10.47 ± 2.55	1.45 ± 0.52	13.9 ± 3.80	8.60 ± 2.16	82.1 ± 4.01	0.19 ± 0.09	1.8 ± 0.95	0.12 ± 0.07	1.2 ± 0.67
500	10.65 ± 1.97	1.27 ± 0.57	11.9 ± 4.08	8.96 ± 1.71	84.1 ± 4.10	0.15 ± 0.03	1.4 ± 0.23	0.14 ± 0.06	1.4 ± 0.60
1000	9.07 ± 1.41	1.43 ± 0.29	15.8 ± 1.43	7.27 ± 1.07	80.2 ± 1.55	0.16 ± 0.06	1.7 ± 0.44	0.12 ± 0.07	1.3 ± 0.68
2000	9.79 ± 2.40	1.71 ± 0.74	16.9 ± 4.26	7.70 ± 1.71	79.1 ± 4.64	0.16 ± 0.05	1.7 ± 0.43	0.13 ± 0.05	1.4 ± 0.48
Females									
0	6.41 ± 1.39	1.17 ± 0.58	18.5 ± 8.73	5.00 ± 1.27	77.6 ± 8.94	0.10 ± 0.03	1.5 ± 0.40	0.10 ± 0.04	1.5 ± 0.44
500	7.28 ± 2.65	0.96 ± 0.43	13.2 ± 3.82	6.03 ± 2.25	82.8 ± 4.32	0.11 ± 0.06	1.5 ± 0.58	0.10 ± 0.03	1.5 ± 0.47
1000	6.92 ± 2.16	0.95 ± 0.40	13.6 ± 3.85	5.68 ± 1.80	82.2 ± 4.92	0.10 ± 0.04	1.4 ± 0.58	0.13 ± 0.06	1.9 ± 0.93
2000	6.15 ± 1.21	0.99 ± 0.52	15.6 ± 6.12	4.89 ± 0.87	80.1 ± 6.71	0.11 ± 0.05	1.7 ± 0.56	0.11 ± 0.06	1.7 ± 0.99

Note. WBC = white blood cell count;N = number of rats/sex/group.

Macroscopic Examination and Organ Weights

There were no test article–related alterations noted during the gross necropsy examinations. Uterine clear fluid was more frequently noted in test article–treated females; however, the histologic alterations in these tissues were consistent with normal estrus cycle-related physiologic changes.

There were no test article–related changes in organ weights (Tables 11 and 12). In a few instances for test article treatment groups, some relative (to body weight) organ weights were statistically significantly different from the control group, but with the exception of reduced absolute liver weight in the 2000 mg/kg/day males, there were no statistically significant differences in mean absolute organ weights or organ-to-brain weight ratios (data not presented).

Microscopic Examination

No test article–related alterations were noted.

Dilatation of the uterus was more frequent in the 2000 mg/kg/day group females than in the control group. The uterine dilatations noted were considered to represent physiologic changes related to the estrous cycle and unrelated to test article administration.

Remaining histologic changes were considered to be incidental findings, manifestations of spontaneous diseases, or related to some aspect of experimental manipulation other than administration of the test article. There was no test article–related alteration in the incidence, severity, or histologic character of those incidental and spontaneous tissue alterations.

DISCUSSION

The present study was conducted to assess the question of safety with repeated exposure that must be addressed before any food additive can receive regulatory approvals. The results of this study verify the safe use of rebaudioside A for human dietary use in foods.

Dietary administration of rebaudioside A to Crl:CD(SD) rats for 90 days at average dosage levels of 517, 1035, and 2055 mg/kg/day to males and 511, 1019, and 2050 mg/kg/day to females resulted in mildly lower mean body weights in the high-dose group males. This finding was not considered to be adverse due to the magnitude of change and may have been the result of the amount of basal diet that was replaced with the test article containing little caloric value rather than a direct action of the test article itself. The lower caloric content of the treatment diets because of high dietary inclusion rates, food efficiency data, and body weight data are concordant for males and females, respectively. The food efficiency data for males demonstrate that body weight gained as a percent of feed consumed is generally decreased as compared to the control group for all test article treatment groups and statistically significantly decreased at 2000 mg/kg/day as compared to the control group for the following intervals: weeks 0 to 1, 3 to 4, and 7 to 8 (Table 4). In contrast, the food efficiency data for females demonstrate that body weight gained as a percent of feed consumed is generally similar for test article treatment groups as compared to the control group (Table 4).

Additionally, there were no clinical pathology or microscopic correlates to suggest a direct malnutritive effect of the high rebaudioside A concentrations. There were no treatment-related adverse effects on clinical observations and survival, body weights, food consumption, functional observational battery (i.e., home cage, handling, open field, sensory, neuromuscular, and physiological observations), locomotor activity, hematology, serum chemistry, urinalysis, ophthalmic examinations, macroscopic examination, organ weights, macroscopic pathology, and microscopic histopathology. Therefore, based on the results of this study, the no-observed-adverse-effect level

TABLE 7
Serum chemistry values (mean ± *SD*) at week 13

	Target Dietary Levels (mg/kg/day) of Rebaudioside A									
	Alb (g/dl)	TP (g/dl)	Glob. (g/dl)	A/G ratio	Bili. (mg/dl)	Urea (mg/dl)	Creat. (mg/dl)	ALP (U/L)	ALAT (U/L)	ASAT (U/L)
Males	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0	4.3 ± 0.20	7.1 ± 0.33	2.8 ± 0.22	1.52 ± 0.139	0.2 ± 0.03	14.9 ± 1.39	0.3 ± 0.06	98 ± 9.9	44 ± 9.4	107 ± 31.2
500	4.3 ± 0.21	7.0 ± 0.36	2.7 ± 0.22	1.59 ± 0.104	0.2 ± 0.05	14.7 ± 1.75	0.4 ± 0.05	82 ± 14.0	38 ± 8.2	84 ± 10.6
1000	4.3 ± 0.28	7.1 ± 0.37	2.8 ± 0.17	1.55 ± 0.110	0.2 ± 0.06	14.5 ± 2.15	0.4 ± 0.05	82 ± 14.4	45 ± 5.5	93 ± 22.0
2000	4.2 ± 0.14	6.9 ± 0.20	2.7 ± 0.24	1.56 ± 0.161	0.2 ± 0.03	14.1 ± 2.14	0.3 ± 0.06	94 ± 20.2	40 ± 8.4	100 ± 24.3
Females										
0	4.8 ± 0.52	7.3 ± 0.42	2.5 ± 0.26	1.98 ± 0.382	0.2 ± 0.05	17.4 ± 2.1	0.4 ± 0.06	57 ± 20.0	41 ± 20.2	103 ± 44.7
500	4.9 ± 0.58	7.4 ± 0.59	2.5 ± 0.28	2.00 ± 0.342	0.2 ± 0.03	18.7 ± 1.94	0.4 ± 0.06	53 ± 19.9	47 ± 28.2	112 ± 65.4
1000	4.8 ± 0.26	7.3 ± 0.26	2.5 ± 0.17	1.93 ± 0.194	0.2 ± 0.03	17.6 ± 2.74	0.4 ± 0.06	58 ± 14.3	36 ± 9.2	100 ± 21.1
2000	4.8 ± 0.41	7.3 ± 0.46	2.5 ± 0.19	1.94 ± 0.236	0.2 ± 0.07	16.6 ± 4.09	0.4 ± 0.10	52 ± 6.5	35 ± 6.3	89 ± 11.6

Note. Alb = albumin; TP = total protein; Glob. = globulin; A/G ratio = albumin/globulin; Bili. = total bilirubin; Creat. = creatinine; ALP = alkaline phosphatase; ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase.
N = number of rats/sex/group.

TABLE 8
Serum chemistry values (mean ± *SD*) at week 13

Target Dietary Levels (mg/kg/day) of Rebaudioside A								
	Glucose (mg/dl)	Chol. (mg/dl)	Ca (mg/dl)	Cl (mEq/L)	P (mg/L)	K (mEq/L)	Na (mEq/L)	TG (mg/dL)
Males	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0	113 ± 16.4	76 ± 13.6	10.7 ± 0.29	101 ± 0.8	7.2 ± 0.41	5.12 ± 0.82	143 ± 1.1	94 ± 27.5
500	112 ± 9.7	65 ± 10.2	10.6 ± 0.38	103 ± 1.7	6.9 ± 0.52	4.93 ± 0.562	144 ± 0.9	58 ± 18.9**
1000	114 ± 12.5	73 ± 19	10.7 ± 0.36	102 ± 1.8	7 ± 0.71	5.12 ± 0.486	145 ± 1.2**	77 ± 27.8
2000	105 ± 11.1	62 ± 15	10.4 ± 0.23	103 ± 1.7	7 ± 0.45	5.19 ± 0.572	145 ± 1.2**	61 ± 18**
Females								
0	109 ± 14.3	79 ± 14.5	10.6 ± 0.52	103 ± 2.5	5.8 ± 0.80	4.51 ± 0.567	144 ± 1.8	51 ± 13.7
500	107 ± 10.1	80 ± 24.4	10.9 ± 0.49	103 ± 1.3	6.3 ± 1.20	5.00 ± 0.923	145 ± 1.7	59 ± 17.1
1000	104 ± 8.6	65 ± 9.8	10.6 ± 0.18	103 ± 2.0	6.0 ± 0.65	4.76 ± 0.680	144 ± 2.1	53 ± 11.5
2000	109 ± 14.4	66 ± 7.9	10.7 ± 0.41	103 ± 1.2	6.3 ± 0.93	4.9 ± 0.646	144 ± 1.9	44 ± 4.7

Note. Chol. = total cholesterol; TG = triglycerides.
N = number of rats/sex/group.
**Significantly different from the control group at .01 using Dunnett's test.

TABLE 9
Urinalysis values (mean ± *SD*) at week 13

Target Dietary Levels (mg/kg/day) of Rebaudioside A				
	Bile acids (μmol/L)	Sorbitol dehydrogenase (U/L)	Insulin (ng/mL)	Glutamate dehydrogenase (U/L)
Males	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0	105 ± 116	14 ± 8.3	3.40 ± 1.665	15 ± 9.6
500	67 ± 56.3	15 ± 4.3	3.02 ± 2.137	15 ± 5.6
1000	33 ± 23.4	15 ± 4.7	2.58 ± 0.598	10 ± 3.2
2000	47 ± 54.3	12 ± 4.9	2.01 ± 0.333	11 ± 2.4
Females				
0	76 ± 73.5	16 ± 6.8	1.63 ± 0.331	32 ± 58
500	85 ± 81.2	20 ± 21.3	1.75 ± 0.333	77 ± 191.9
1000	69 ± 55.8	12 ± 4.3	1.74 ± 0.494	12 ± 10.5
2000	84 ± 81.7	15 ± 6.6	1.51 ± 0.234	12 ± 8.9

Note. N = number of rats/sex/group.

(NOAEL) was considered to be 2055 and 2050 mg/kg/day for males and females, respectively, the highest average dosage levels examined.

It has been reported that after oral administration, steviol glycosides are poorly absorbed in experimental animals and humans (JECFA 2005). The principal steviol glycosides, rebaudioside A and stevioside, are metabolized in experimental animals and humans by intestinal microflora by successive hydrolysis of glucose sugar moieties. However, this process does not appear to be efficient since these substances are essentially non-caloric. Based on its chemical structure, rebaudioside A (13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester) may be hydrolyzed by intestinal microflora to rebaudioside B (13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid) or stevioside (13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester), and given a long enough residence time in the lower intestine, theoretically could hydrolyze to the aglycone, steviol (ent-13-hydroxykaur-16-en-18-oic acid). There are some published studies that address the metabolism of rebaudioside A and its possible metabolite, stevioside, which are relevant to the safety assessment of rebaudioside A.

Rebaudioside A and stevioside were studied in vitro by anaerobic incubation with microbial whole-cell suspensions from rat cecum and aerobic incubation with sonic cell-free extracts prepared from rat cecal contents to determine if they are metabolized to steviol. After 2 days of incubation of a 2.5 mg/ml dose

TABLE 10
Urinalysis values (mean ± *SD*) at week 13

Target Dietary Levels (mg/kg/day) of Rebaudioside A				
	Specific gravity	pH	Urobilinogen (mg/dl)	Total volume (ml)
Males	$N = 10$	$N = 10$	$N = 10$	$N = 10$
0	1.031 ± 0.0141	6.4 ± 0.39	0.2 ± 0.00	16.3 ± 18.76
500	1.047 ± 0.0179	6.6 ± 0.37	0.2 ± 0.00	6.8 ± 4.10
1000	1.048 ± 0.0193	6.5 ± 0.28	0.4 ± 0.34	5.9 ± 3.63
2000	1.032 ± 0.0148	6.4 ± 0.47	0.2 ± 0.00	14.1 ± 19.26
Females				
0	1.041 ± 0.0166	5.9 ± 0.39	0.3 ± 0.25	4.9 ± 2.56
500	1.042 ± 0.0204	5.9 ± 0.21	0.2 ± 0	5.5 ± 5.34
1000	1.052 ± 0.0321	6.1 ± 0.44	0.4 ± 0.57	3.9 ± 3.73
2000	1.051 ± 0.0297	6.1 ± 0.42	0.4 ± 0.35	4 ± 3.67

Note. N = number of rats/sex/group.

TABLE 11
Organ weights relative to final body weights (g/100 g) ± *SD*

	Target Dietary Levels (mg/kg/day) of Rebaudioside A						
	Adrenal glands	Brain	Heart	Kidneys	Liver	Ovaries/oviducts	Pituitary
Males	$N = 20$	$N = 20$	$N = 20$	$N = 20$	$N = 20$	$N = 20$	$N = 20$
0	0.011 ± 0.0016	0.402 ± 0.0412	0.312 ± 0.0249	0.716 ± 0.0679	2.99 ± 0.2508	NA	0.003 ± 0.0004
500	0.012 ± 0.0018	0.412 ± 0.0334	0.322 ± 0.0257	0.748 ± 0.0656	2.809 ± 0.2272	NA	0.003 ± 0.0004
1000	0.013 ± 0.0018**	0.415 ± 0.0385	0.340 ± 0.0231**	0.76 ± 0.0675	2.877 ± 0.2491	NA	0.003 ± 0.0003
2000	0.013 ± 0.0014**	0.434 ± 0.0458	0.327 ± 0.0146	0.755 ± 0.0625	2.893 ± 0.1993	NA	0.003 ± 0.0006
Females							
0	0.027 ± 0.0043	0.725 ± 0.0712	0.398 ± 0.0300	0.796 ± 0.0716	3.005 ± 0.3054	0.049 ± 0.0082	0.007 ± 0.0013
500	0.026 ± 0.0030	0.715 ± 0.0672	0.393 ± 0.0426	0.814 ± 0.0678	3.066 ± 0.2716	0.051 ± 0.0090	0.007 ± 0.0012
1000	0.027 ± 0.0042	0.732 ± 0.0836	0.401 ± 0.0325	0.805 ± 0.0589	3.077 ± 0.2392	0.051 ± 0.0095	0.007 ± 0.0013
2000	0.027 ± 0.0031	0.745 ± 0.0609	0.413 ± 0.0499	0.839 ± 0.058	3.146 ± 0.2879	0.053 ± 0.0059	0.007 ± 0.0011

Note. N = number of rats/sex/group.
NA = not applicable.
**Significantly different from the control group at .01 using Dunnett's test.

TABLE 12
Organ weights relative to final body weights (g/100 g) ± *SD*

	Target Dietary Levels (mg/kg/day) of Rebaudioside A					
	Prostate	Spleen	Testes	Thymus	Thyroids/ parathyroids	Uterus
Males	$N = 20$	$N = 20$	$N = 20$	$N = 20$	$N = 20$	$N = 20$
0	0.173 ± 0.0565	0.152 ± 0.022	0.676 ± 0.0686	0.058 ± 0.0172	0.005 ± 0.0007	NA
500	0.166 ± 0.0411	0.173 ± 0.0269*	0.699 ± 0.0881	0.063 ± 0.0167	0.005 ± 0.0008	NA
1000	0.164 ± 0.0446	0.163 ± 0.0198	0.709 ± 0.0826	0.063 ± 0.0132	0.005 ± 0.0009	NA
2000	0.19 ± 0.0394	0.163 ± 0.0223	0.750 ± 0.0920*	0.066 ± 0.0148	0.005 ± 0.0008	NA
Females						
0	NA	0.196 ± 0.0238	NA	0.105 ± 0.0285	0.008 ± 0.0017	0.234 ± 0.0416
500	NA	0.203 ± 0.0375	NA	0.105 ± 0.0205	0.007 ± 0.0012	0.269 ± 0.0821
1000	NA	0.199 ± 0.023	NA	0.112 ± 0.0239	0.007 ± 0.0013	0.263 ± 0.0971
2000	NA	0.209 ± 0.0324	NA	0.115 ± 0.0208	0.008 ± 0.0011	0.291 ± 0.0859

Note. N = number of rats/sex/group.
NA = not applicable.
*Significantly different from the control group at .05 using Dunnett's test.

of stevioside in whole-cell suspensions, 107% of theoretical was recovered as steviol. However, after 2 days of incubation of a 3.0 mg/ml dose of rebaudioside A in whole-cell suspensions, only 65% of theoretical was recovered as steviol. The experiment with rebaudioside A was continued and it was determined that after 4 and 6 days 83% and 108% of theoretical, respectively, were recovered as steviol. Incubation with sonic cell-free extracts resulted in much slower rates of hydrolysis with only 50 and 2% of theoretical yielded as steviol after seven days for stevioside and rebaudioside A, respectively (Wingard et al. 1980).

Degradation of rebaudioside A and stevioside at concentrations of 0.2 and 10 mg/ml was investigated in vitro by their incubation under anaerobic conditions with pooled human fecal homogenates from five healthy volunteers for 0, 8, and 24 h. Rebaudioside A and stevioside were degraded in a time- and concentration-dependent manner at both concentrations. At 0.2 mg/ml, rebaudioside A had degraded approximately 30% to 35% after 8 h and 100% after 24 h; whereas at 10 mg/ml, rebaudioside A had degraded only 5% to 10% at 8 h and 56% after 24 h of incubation. The conversion of rebaudioside A to steviol at substrate concentrations of 0.2 and 10 mg/ml after 24 h was determined to be 109% and 22%, respectively. In contrast to rebaudioside A, stevioside was determined to degrade more rapidly because 100% and 77% had degraded by 24 h at 0.2 and 10 mg/ml, respectively. On the other hand, it was reported that the conversion of stevioside to steviol at 0.2 and 10 mg/ml in 24 h was 84% and 63%, respectively. The 24-h conversion rate to steviol at the 0.2 mg/ml incubation concentration of 109% for rebaudioside A compared to 84% for stevioside is paradoxical since the authors propose the main route of rebaudioside A degradation goes through stevioside as its first metabolite. The authors concluded that there are apparently no species differences between humans and rats in anaerobic metabolism by intestinal microflora of rebaudioside A and stevioside (Koyama et al. 2003).

Stevioside and rebaudioside A were incubated for 72 h under anaerobic conditions with fecal suspensions provided by six male and five female volunteers aged 20 to 50 years. Stevioside completely degraded to steviol in a 10-h period. Steviolbioside (a metabolite of rebaudioside A and stevioside) concentration peaked after 2 to 4 h of incubation, and then decreased to zero with steviol detected after 3 to 4 h of incubation. These results suggest that stevioside was initially hydrolyzed to steviolbioside and then this intermediate was subsequently metabolized to steviol. After a period of 6 to 7 h, rebaudioside A was hydrolyzed to steviolbioside and completely metabolized to steviol after 24 h. The results of this study do not elucidate whether rebaudioside A preferentially hydrolyzes to rebaudioside B or stevioside before further hydrolysis to steviolbioside. Steviol remained unchanged during the 72-h incubation and no other metabolites were observed. No steviol epoxide derivatives were found after incubation of rebaudioside A or stevioside samples with intestinal microflora from 11 human volunteers (Gardana et al. 2003).

Stevioside was administered via the diet to pigs at approximately 70 mg/kg bw/day for 14 days and daily blood and fecal samples were collected starting after 2 days. Analysis of fecal samples indicated that stevioside was completely converted to steviol; however, no stevioside or steviol was detected in blood samples. When intestinal transport was investigated using the Caco-2 system, only a small fraction of stevioside and rebaudioside A (apparent permeability coefficient, P_{app} of 0.16×10^{-6} and 0.11×10^{-6} cm/s, respectively) was transported through the cell layer, whereas steviol was transported readily (38.6×10^{-6} cm/s). The authors attribute the discrepancy between relatively high absorptive transport in the Caco-2 system and the absence of steviol in blood samples following repeated oral intake by pigs

to the fact that in the in vitro system steviol is in solution in direct contact with the Caco-2 cell layer, whereas in vivo steviol probably is absorbed to the contents of the colon (Geuns et al. 2003).

In studies examining pharmacological effects, although rebaudioside A increased insulin secretion from mouse islets in a concentration-dependent fashion, with the effects of rebaudioside A on insulin secretion glucose-dependent at glucose concentrations >6.6 mmol/L (Abudula et al. 2004), oral administration of rebaudioside A in the Goto-Kakizaki rat, an established animal model for type 2 diabetes, did not affect plasma glucose, insulin, and glucagon levels or systolic blood pressure during or after eight weeks of treatment at 0.025 g/kg bw/day (Dyrskog et al. 2005). The authors of this study concluded, "In the light of our previous *in vitro* study (Abudula et al. 2004) this appears puzzling and we cannot rule out that uptake of Rebaudioside A has been hampered [*in vivo*]."

The studies described above on the metabolism and/or absorption of rebaudioside A, stevioside, and steviol in a number of in vitro and in vivo animal and human models provide strong support for the following conclusions:

1. Rebaudioside A and stevioside are hydrolyzed in vitro relatively slowly by sequential removal of glucose units by animal and human lower intestinal microflora and there is evidence that stevioside hydrolyzes similarly *in vivo*.
2. Hydrolysis by lower intestinal microflora in vitro is time- and concentration-dependent for rebaudioside A and stevioside, suggesting the degradation pathways are saturated at higher concentrations.
3. Complete hydrolysis to steviol by lower intestinal microflora *in vitro* takes place more slowly for rebaudioside A than stevioside, probably because of its longer degradation pathway.
4. Transit time through the gastrointestinal tract may limit the opportunity for animal and human microflora to convert rebaudioside A to steviol as compared to the more rapidly degrading stevioside.
5. Rebaudioside A and stevioside probably are not well absorbed by animals or humans based on low apparent permeability coefficient results with the Caco-2 system.
6. Steviol with a high apparent permeability coefficient with the Caco-2 system, appears as though it could be absorbed readily, although in vivo results in pigs suggest steviol is not efficiently absorbed.
7. Because intestinal absorption is inversely related to molecular weight (MW), those differences between rebaudioside A, stevioside, and steviol (MW = 967, 805, and 318, respectively) probably influence their in vivo bioavailability.
8. In a type 2 diabetic animal model, rebaudioside A does not demonstrate insulinotropic, glucagonostatic, antihyperglycemic, or blood pressure lowering effects, which have been reported in similar studies with stevioside.

The NOAEL from the current 90-day toxicity study in rats is greater than 2000 mg/kg/day. The results of the present study as well as data reviewed for metabolism, absorption, and pharmacological end points verify the safety of rebaudioside A for human dietary use in foods.

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