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Metabolism of Stevioside by Chickens

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In intubation experiments (643−1168 mg per animal), most of the stevioside administered to chickens was recovered unchanged in the excreta, and only about 2% was converted into steviol. Neither stevioside nor steviol could be found in the blood. In chronic studies (667 mg of stevioside/kg of feed) with laying hens and meat-type chickens, no significant differences were found in feed uptake, weight gain, and feed conversion as the result of stevioside administration. The egg production and egg composition of laying hens were not influenced. Most of the stevioside taken up was found untransformed in the excreta, and about 21.5% or 7.3% was converted to steviol by meat-type chickens or laying hens, respectively. No stevioside or steviol could be detected in the blood or in the eggs of the different groups of animals. In anaerobic incubation experiments with chicken excreta, only a 20% conversion of stevioside into steviol was found. No harmful effects were observed in the chronic stevioside supplementation experiments nor in the intubation experiments in which very high stevioside doses were given.

KEYWORDS: ***Stevia rebaudiana*** **(Bertoni) Bertoni; stevioside; rebaudioside A; steviol; metabolism; chicken;** ***Gallus gallus domesticus*****; toxicology**

INTRODUCTION

Stevia rebaudiana (Bertoni) Bertoni is a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America (Paraguay and Brazil). The native Guaraní people know it by several names, all of which refer to the sweet taste of the leaf and especially to its use in "maté" tea (*Ilex paraguariensis*). It is often referred to as "the sweet herb of Paraguay". *Stevia* is grown commercially today mainly in Paraguay, Brazil, and the People's Republic of China (*1*).

Stevioside is a high-intensity sweetener that tastes about 300 times sweeter than sucrose (0.4% solution). In many countries, it is used as a low-calorie sweetener in a wide range of food products and beverages. The plant, its extracts, and stevioside have been used for several years as a sweetener in South America, Asia, Japan, China, and in different countries of the European Union, and in the United States it has been used as a dietary supplement since 1995. *S. rebaudiana* products are approved for sweetening purposes in Brazil, Korea, and Japan (*1*). The advantages of stevioside as a dietary supplement for human subjects are manifold: it is stable, it is noncalorific, it maintains good dental health by reducing the intake of sugar, and it opens the possibility for use by diabetic and phenylketonuria patients and obese persons.

Structures of the sweet components of *S. rebaudiana* (Bertoni) Bertoni, occurring mainly in the leaves, are given in **Figure 1**. Their content varies between 4% and 20% of the dry weight of the leaves, depending on the cultivar and growing conditions. Stevioside (**3**) is the main sweet component. Other compounds present but in lower concentration are dulcoside A (**9**), steviolbioside (**2**), and rebaudiosides A (**4**), B (**5**), C (**6**), D (**7**), and E (**8**). The presence of steviolbioside and rebaudioside B in extracts might be due to artifacts of the extraction procedure (references cited in ref *2*).

Many papers have been published describing the safety of stevioside used as a sweetener (see ref *3* for a review). In a chronic toxicity study with F344 rats, it was concluded that there were no significant increases in the incidence of neoplastic lesions in any organ or tissue in the stevioside-treated groups (daily doses of 385 and 775 mg per rat, i.e., 1 and 2 g/kg of body weight!). In male animals, the number of testicular tumors had the tendency to decrease. Moreover, the incidence of adenomas of the mammary gland in the stevioside-treated females was significantly lower than that in the controls. The severity of chronic nephropathy in males was also clearly reduced by both stevioside concentrations (*4*). No significant changes in the growth, general appearance, hematological and

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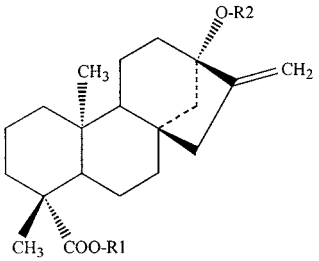

	Compound name	R1	R2
1	steviol	H	H
2	steviolbioside	H	β-Glc-β-Glc(2→1)
3	stevioside	β-Glc	β-Glc-β-Glc(2→1)
4	rebaudioside A	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
5	rebaudioside B	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
6	rebaudioside C (dulcoside B)	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
7	rebaudioside D	β-Glc-β-Glc(2→1)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

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

blood biochemical findings, organ weights, and macroscopic or microscopic observations (41 organs were analyzed) were found related to the doses of stevioside or rebaudioside A administered to male and female F344 rats. It was also concluded that any neoplasms that occurred were not attributable to the administration of stevioside. Even at the highest dose of 1%, no significant effects were found (*5*). It was shown that so-far unknown steviol metabolites in *Salmonella typhimurium* TM677 after metabolic activation caused mutations, i.e., transitions, transversions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene. However, steviol was completely negative in the reverse mutation assays using *Escherichia coli* WP2uvrA/pKM101 or using different *S. typhimurium* TA strains, even when activation S9 mix was present (*6*). When steviol was given to hamsters on days 6−10 of pregnancy at daily doses of 500−1000 mg/kg of body weight (BW), it induced toxicity (*7*). The numbers of live fetuses per litter and mean fetal weight decreased. The maternal kidneys showed a dose-dependent increase in severity of convoluted tubules in the kidneys. The no-observable effect level (NOEL) for maternal and fetal toxicity was at a daily dose of 250 mg/kg of BW. However, this study with steviol is not relevant for the use of stevioside as a sweetener. When stevioside is fed to hamsters, no toxic effects were found, even in three successive generations (*8*). When steviol is given in the feed, it can be resorbed directly by the intestines, whereas stevioside is not. Stevioside is transformed only by the bacteria of the cecum or the colon from which steviol eventually may be resorbed or taken up by coprophagy. Moreover, hamsters are known to be very sensitive to steviol and stevioside (*9*). The NOEL of steviol was 250 mg/kg of BW (*7*), which corresponds to 625 mg of stevioside/kg of BW. Even under these very unfavorable conditions, an acceptable daily intake (ADI) of 2.5 mg of steviol/kg of BW, which corresponds to 6.25 mg of stevioside/kg of BW, can be calculated, which is close to 7.9 mg/kg of BW obtained by Xili et al. (*10*). However, this ADI should be considered as a minimum value, as the authors did not test concentrations of stevioside higher than 793 mg/kg of BW (safety factor 100). Considering many reports from the literature, an ADI of more than 20 mg of stevioside/kg of BW is likely (*3*).

However, because mutagenic effects of steviol (**1**, **Figure 1**) and/or its metabolites have been published (*6*, *11*), one of the most urgent problems to solve is the possible breakdown of stevioside into steviol and other metabolites in vivo. A second important issue is the question whether steviol, should it be produced, is being taken up by the intestine and to what extent.

It has been shown that none of the digestive enzymes from the gastrointestinal tract of different animals and humans were able to degrade stevioside into steviol (*12*). Stevioside was incubated with salivary α-amylase, pancreatic α-amylase, saliva, pepsin, gastric secretion, pancreatin, and intestinal brush border membrane enzymes of mice, rats, and hamsters. None of these enzymes digested stevioside. Nevertheless, in feeding experiments with rats and hamsters, stevioside was metabolized to steviol by the bacterial flora of the cecum. After several hours, steviol was found in the blood of the animals, the maximum concentration occurring after 8 h (*13*−*15*). However, in rodents coprophagy might occur. In the cited studies, it was not indicated that coprophagy was prevented, so it is not clear whether the steviol occurring in the blood was taken up directly from the colon or indirectly from the ingested excreta (after passing through the intestines again). Although bacteria isolated from the human colon were able to transform stevioside into steviol in vitro (*12*), it has never been proven that this was also the case in humans in vivo nor that the steviol possibly formed was taken up directly from the colon. Moreover, studies with roosters indicated that stevioside is eliminated from the body within 48 h, largely untransformed (*16*). Only the bacteria from the cecum or colon were able to degrade stevioside into steviol in vitro (cecum of mice, rats, and hamsters; colon of humans) (*12*). The bacteria from the human colon also showed in vitro formation of steviol-16α,17-epoxide, which was again metabolized to steviol. However, this epoxide formation probably will not occur in vivo due to the anaerobic conditions of the human colon (*12*). Moreover, steviol-16α,17-epoxide has been tested for its mutagenicity and was shown to be inactive (*11*).

This research aims to investigate whether stevioside is metabolized in vivo in the alimentary tract of the chicken. The advantage of using chickens is that cecal activity is rather low and coprophagy is easily avoided by keeping them on a wire floor. Moreover, an additional factor is that the sweetness of the diet does not seem to affect the feed intake of broiler chicks (*17*).

MATERIALS AND METHODS

Chemicals. The experiments were performed using stevioside that was purified by repeated crystallization from MeOH to a purity level of more than 96%. Steviolbioside (around 3%) and rebaudioside A (around 0.5%) were the main impurities. Steviol was made according to ref (*17*) and repeatedly crystallized from MeOH. Solvents of HPLC grade were from Acros (H_2O, acetonitrile, $CHCl_3$), BDH (MeOH, EtOH, *N*,*N*-dimethylformamide), and Biosolve (acetone). Triethylamine was from Acros and 4-(bromomethyl)-7-methoxycoumarine from Fluka.

In Vivo Experiments. (1) Intubation Experiments. In a first series of experiments, broiler chickens (Cobb) were kept individually in digestibility cages and fed ad libitum a commercial grower diet. Under each cage, 70 cm below the bottom grid, a polystyrene container with solid carbon dioxide was placed. The excreta were collected quantitatively upon a sheet of aluminum foil in direct contact with the solid CO_2. This method was used in order to avoid ex vivo bacterial

decomposition of stevioside or steviol at room temperature. Three broiler chickens weighing about 0.9 kg were intubated into the crop with 643 mg of stevioside (96% purity) dissolved in 6 mL of 35% glycerol solution (714 mg/kg of BW). To ensure that all stevioside was in solution, the stevioside/glycerol mixture was heated at 70 °C and cooled to 40 °C before intubation. At this temperature, the stevioside remained in solution for at least 12 h (at the body temperature of the chickens). First, a small amount of water was sucked into the syringe to ensure a quantitative transfer of all the stevioside solution when intubating the animals.

Immediately after intubation, the excreta were collected at regular time intervals as indicated in the tables, weighed, and stored frozen at −20 °C, after which they were freeze-dried before analysis for stevioside and steviol. A blood sample was taken from a wing vein using a heparinized syringe at 2, 4, 6, 8, 24, and 48 h after intubation, and the whole blood was stored frozen before being freeze-dried and analyzed for stevioside and steviol.

The same experimental protocol was repeated with four other broiler chickens from the same batch but weighing about 1.9 kg. These chickens received 1168 mg of stevioside dissolved in 6 mL of 35% glycerol (≅614 mg of stevioside/kg of BW).

(2) Chronic Supplementation of Stevioside to Growing Broiler Chickens and Laying Hens. (a) Sixteen broiler chickens (Cobb) of about 0.5 kg body weight were placed on digestibility cages (two animals per cage). Half of the chickens were provided with a commercial broiler grower diet supplemented with 667 mg of stevioside/kg, whereas the other half received the unsupplemented diet. For this experiment that lasted for 2 weeks, the following parameters were used:

(i) Food intake was checked daily except for days 5 and 6, and 12 and 13, and the body weight gain was checked every 3 days during the trial.

(ii) Blood sampling was carried out at regular time intervals. These blood samples were examined for the absence or presence of stevioside or steviol.

(iii) Quantitative collection of excreta was carried out for determination of stevioside or steviol. From the amount of food consumed, digestibility coefficients were calculated.

(b) Four highly productive laying hens (Hisex brown), kept individually in battery cages, were fed ad libitum for 10 days a commercial laying hen diet supplemented with stevioside at a concentration of 667 mg/kg of diet (in this way, the hens will take up an amount of stevioside that is about 6 × ADI). Another four hens received only the unsupplemented diet and served as controls.

The food intake of each chicken was determined every day, and hens were weighed just before and at the end of the 10-day trial. On particular days (see **Table 3**), the excreta of the stevioside-supplemented hens were collected quantitatively for 24 h on solid CO_2 (see above) and weighed, and an aliquot of 5 g was stored for analysis. On these days, a 5-mL blood sample was collected from a wing vein, and the blood was stored frozen.

All eggs produced were collected, weighed, boiled for 5 min, and then separated into yolk and egg white. The blood samples, excreta, and egg components were freeze-dried and analyzed for their stevioside and steviol content.

In Vitro Experiments: Anaerobic Incubation of Excreta. Fresh chicken excreta were collected. Suspensions of 2 g of excreta were made in triplicate in 90 mL of pH 6.8 phosphate buffer. The suspensions were made anaerobic by admitting a flow of a gas mixture composed of nitrogen/hydrogen/carbon dioxide (80/10/10) and were incubated in an anaerobic flow cabinet provided with a flow through of the same gas mixture. When the color indicator (methylene blue) was fully reduced (colorless), 10 mL of anaerobic stevioside solution was added to attain a final concentration of 50 mg/L. At regular time intervals (see **Figure 2**), samples of 1 mL were taken, frozen in solid carbon dioxide, and freeze-dried. The dried samples were exhaustively extracted with acetone for steviol or with methanol for stevioside dosage.

In the tables, the mean is given together with the standard error. All data were analyzed using the Student's *t*-test.

Detection of Stevioside and Steviol. *Stevioside.* Weighed aliquots of about 100 mg of powdered excreta, blood, or egg samples, pulverized in a Retsch mixer mill (MM200), were extracted with methanol (4 × 1 mL). Next, 6 mL of H_2O was added to the pooled extract fractions, and the solution was acidified with HCl to pH 6.5 to enable the binding of steviol and steviolbioside onto C-18 cartridges. This solution was quantitatively applied to a conditioned 500-mg C-18 cartridge (Alltech) that was rinsed with 5 mL of 40% MeOH. Stevioside and steviolbioside were eluted with 3 mL of a 70% methanol solution. The extracts were evaporated at 50 °C under a flow of nitrogen, and the residues were redissolved in 0.2 mL of ethanol containing 3% diethyl ether. Extracts of excreta samples could be directly injected onto the HPLC column for stevioside and steviolbioside quantification (ODS-silica column, 25 cm × 4.6 mm i.d.; 35% AcCN as solvent; detection of stevioside and steviolbioside by UV at 210 nm). Extracts of blood samples were purified by TLC with ethyl acetate/EtOH/water (130/27/20) as solvent. The stevioside bands (R_f = 0.20) were eluted by MeOH.

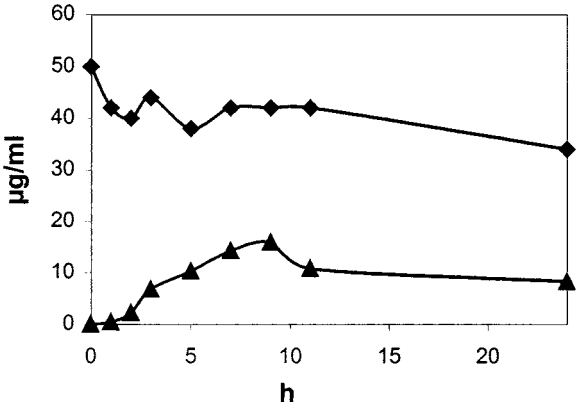

Figure 2. Anaerobic incubation of 5 mg of stevioside (ST) with 2 g of excreta/100 mL of phosphate buffer at 37 °C. The amounts of steviol formed are given as stevioside equivalents. ◆, ST; ▲, SV as ST equiv.

Steviol. Weighed samples of about 50 mg of dried and powdered excreta or egg samples or 100 mg of dry blood were extracted with acetone (4 × 1 mL). The extracts were concentrated and purified by TLC ($CHCl_3$/MeOH, 90/10). The bands corresponding to steviol (R_f = 0.35) were eluted with $CHCl_3$/MeOH (50/50), and following evaporation of the solvent, the residues were dried under a stream of nitrogen (traces of water interfere with the subsequent derivatization reaction). To enable sensitive fluorometric analysis of steviol in biological samples, steviol was derivatized by esterification of the free carboxyl group with the alkylating reagent 4-(bromomethyl)-7-methoxycoumarine (IUPAC name: 4-(bromomethyl)-7-methoxy-2*H*-chromen-2-one). This reaction was carried out in the aprotic solvent *N*,*N*-dimethylformamide (DMF). To ensure a quantitative derivatization, a 5-fold excess of reagent was used; e.g., 100 μg of steviol was dissolved in 200 μL of DMF containing 500 μg of reagent and 1 μL of *N*,*N*-diisopropylethylamine. For smaller sample sizes (under 5 μg of steviol), a larger excess of reagent (25 μg in 200 μL of DMF) was used. After being heated at 75 °C for 20 min, the reaction mixture was directly injected onto the HPLC column (ODS column, 25 cm × 4.6 mm i.d.; AcCN/water, 80/20; detection by fluorescence detector, λ_{ex} 321 nm, λ_{em} 391 nm). When, after derivatization, sample cleanup was necessary, the reaction was carried out in dry acetone, which was evaporated after the reaction was completed. The reaction mixture was applied onto TLC plates that were developed in $CHCl_3$/MeOH (98/2). After visualization under UV (blue fluorescence under UV 366 nm), the band containing the (7-methoxycoumarin-4-yl)methyl ester of steviol (R_f = 0.34) was scraped off and eluted with $CHCl_3$/MeOH (80/20). After being filtered over a plug of glass wool in a pipet tip, the solution was directly injected onto the HPLC column (see above). The (7-methoxycoumarin-4-yl)methyl ester of steviol was separated by HPLC on ODS-silica columns (25 cm length, 4.6 mm i.d.) using 80% AcCN as solvent. Detection of the steviol ester derivative proceeded by measuring the fluorescence intensity (λ_{ex} 321 nm, λ_{em} 391 nm).

RESULTS

Detection Limits and Recovery Experiments. The detection limit of stevioside was 50 ng per injection. With a more sensitive

Table 1. Stevioside (ST), Steviolbioside (SB), Steviol (SV), and Steviol as Stevioside Equivalents Found in the Excreta of Three Animals Receiving 643 mg of Stevioside (96% Purity)[a]

hour	total excreta (g dry wt)	ST (mg)	SB (mg)	SV (mg)	SV as ST equiv (mg)
0–2	0	0	0	0	0
2–4	1.73	0.022	0	0	0
4–6	4	1.39	0	0	0
6–8	3.2	182	7.6	0.00008	0.00018
8–10	0.73	19.0	2.63	0.0029	0.0073
10–12	2.27	41.5	1.84		
12–24	9.3	75.0	4.16	0.55	1.37
24–48	7.8	115	0.83	1.2	2.97
total	29	434.3	17.1	1.74	4.35 (0.95%)[b]

[a] The weight of the chickens was around 900 g each. The excreta were collected during the intervals indicated. [b] The percentage value of steviol (given in parentheses) represents the amount of steviol, expressed as stevioside equivalents, relative to the total amount of steviol glycosides recovered (456 mg).

UV detector, a detection limit of 1 ng per injection may be obtained. The recovery of stevioside from spiked excreta samples (1, 6.5, or 20 mg/g dry wt) was 72.1 ± 1.33% (n = 20). Recovery could not be improved by using EtOH (absolute) or EtOH/diethyl ether (97/3) as extraction solvent. The recovery of stevioside from spiked blood samples (6.67 mg/g dry wt) after TLC cleanup was 68.4 ± 2.62% (n = 9). To obtain such a recovery, it was very important to rinse the sample vials containing the stevioside extracts three times to allow for a quantitative transfer of stevioside to the TLC plates.

The detection limit of the (7-methoxycoumarin-4-yl)methyl ester of steviol was about 50 pg per injection. Using this procedure, steviol was measured with a sensitivity that is about 1000 times higher than that obtained for stevioside. Recovery of steviol from spiked excreta (100, 170, and 1000 μg/g dry wt) was 34.5 ± 2.14% for the lower and 55.2 ± 11.8% for the higher concentrations. Use of other solvents for the extraction did not improve recovery (MeOH, diethyl ether). Recovery of steviol from spiked blood (340 μg/g dry wt) was 39.7 ± 3.0%. The addition of 1% or higher concentrations of acetic acid to acetone did not significantly increase the recovery. In fact, care must be taken, as acid conditions can cause conversion of steviol into isosteviol. Therefore, in our experiments steviol was always extracted with acetone.

The extraction yield of steviol from blood could not be improved by a procedure involving treatment of fresh blood samples (10 μg of steviol/500 μL of blood) with 2 N KOH, removal of lipids with diethyl ether, acidification of the aqueous phase with acetic acid to pH 6, and final extraction with diethyl ether (recovery yield 26 ± 3.90%), nor by direct extraction of fresh blood samples with ethyl acetate. As in vivo steviol might bind to different blood fractions, we preferred to extract the total blood fraction. Therefore, in all experiments blood samples were freeze-dried before extraction.

Despite the poor recovery of steviol at the lower concentration levels, very minute amounts of steviol could still be measured due to the very sensitive detection after derivatization; e.g., at the lowest concentration level of steviol (100 μg of steviol/g dry wt), the steviol content in 20 mg of excreta was 2 μg. At 35% recovery, 0.7 μg would be detected, i.e., 14 000 times the detection limit (50 pg).

Intubation Experiments with Chickens. Table 1 gives the results of a typical intubation experiment with three chickens weighing about 0.9 kg (643 mg of stevioside/animal). The amount of steviolbioside was 3.7% of the total amount of sweeteners recovered, which is about the same concentration as that found in the original intubated stevioside preparation. The relative amounts of steviol formed were calculated as stevioside equivalents, as the molecular mass of steviol is only 39.6% of that of stevioside. Based on the total stevioside excreted, the conversion of stevioside into steviol (1.74 mg of steviol, or 4.35 mg of stevioside equivalents) was only 0.95%. The total recovery of stevioside, steviolbioside, and stevioside equivalents corresponding to the steviol formed was 456 mg of the 643 mg stevioside applied, i.e., a recovery of 70.8%. Taking into account the degree of recovery for low concentrations of steviol, the percentage of stevioside to steviol conversion would amount to ca. 2%.

Blood was sampled at 2, 4, 6, 8, 10, 24, and 48 h after intubating the chickens with stevioside. No traces of stevioside or steviol could be detected in the blood of these animals (data not shown), although the technique allowed detecting very minute amounts of steviol (detection limit 50 pg).

Intubation experiments (1168 mg of stevioside/animal) with four chickens weighing about 1.9 kg (≅614 mg/kg of BW) gave similar results: about 0.84% of total recovered stevioside and stevioside equivalents was detected as free steviol. Taking into account the degree of recovery for low concentrations of steviol, the percentage of stevioside-to-steviol conversion would amount to ca. 1.7%. The steviolbioside concentration was about 3%, corresponding to that in the stevioside intubated (results not shown). These results confirm those of Pomaret and Lavieille (*16*), who stated that stevioside was eliminated from the body of roosters within 48 h, largely untransformed. The amount of stevioside metabolized into steviol by cecal bacteria was about 2% of the stevioside applied but did not appear in the blood.

Chronic Stevioside Supplementation to the Diet of Adult Laying Hens. The following parameters were measured: ad libitum feed intake, body weight changes, and metabolism of stevioside.

No significant differences were found for the total feed consumption (1099 ± 60 and 1111 ± 83.2 g respectively for control and stevioside-treated animals), body weight gain, the total egg production (552 ± 18.6 and 604±9.8 g respectively for control and stevioside-treated animals), nor for the feed conversion calculated as the ratio between total feed uptake and total grams of egg mass produced during the 10 days of the experiment (respectively 1.98 ± 0.08 and 1.83 ± 0.13 for control and stevioside-treated animals).

The absolute weight of the eggs per chicken was very constant (**Table 2**) but may differ between different animals even in the same treatment (e.g., between ST1 and ST3). This is a normal between-animal variation and is probably due to differences in body weight, as the weight of the eggs as a percentage of the body weight is around 3.4 in both the control group and the stevioside-treated group. The percentage of yolk and egg white was not significantly different between the control group and the stevioside-treated group.

At different days after the start of the experiment, excreta was sampled for 24 h, freeze-dried, and analyzed for stevioside and steviol content (**Table 3**). The feed uptake during the 24 h preceding the excreta sampling is also indicated, as well as the stevioside amount taken up during these 24 h. The total recovery of stevioside in the excreta was about 67% and corresponded to values found earlier in intubation experiments. Only about 7.3% of the administered stevioside was found as steviol in the excreta of these chickens.

Because yolk formation occurs in the liver, which is also the pivotal place for metabolization and/or accumulation for com-

Table 2. Composition of the Eggs[a]

	wt of egg (g)	wt of egg yolk (g)	wt of egg white (g)	dry wt of yolk (g)	dry wt of white (g)
			Control Group		
Co1 ($n = 6$)	49.81 ± 0.52 (3.2%)	12.37 ± 0.17 (24.8%)	29.8 ± 0.28 (59.8%)	5.99 ± 0.11	4.23 ± 0.06
Co2 ($n = 6$)	58.34 ± 0.40 (3.3%)	15.37 ± 0.19 (26.3%)	34.7 ± 0.23 (42.3%)	7.81 ± 0.10	4.69 ± 0.02
Co3 ($n = 5$)	56.44 ± 0.69 (3.4%)	15.69 ± 0.24 (27.8%)	32.7 ± 0.39 (57.9%)	7.85 ± 0.13	4.43 ± 0.08
Co4 ($n = 6$)	56.26 ± 0.70 (3.6%)	14.78 ± 0.18 (26.3%)	30.9 ± 0.19 (54.9%)	7.51 ± 0.06	4.18 ± 0.09
mean (%)	55.21 ± 1.86 (3.4 ± 0.08%)	14.55 ± 0.75 26.3% ± 0.61	32.02 ± 1.07 53.7% ± 3.94	7.29 ± 0.44	4.38 ± 0.11
			Stevioside Treatment Group		
ST1 ($n = 10$)	61.12 ± 0.29 (3.7%)	15.22 ± 0.32 (24.9%)	36.3 ± 0.51 (59.4%)	7.78 ± 0.14	5.25 ± 0.10
ST2 ($n = 10$)	57.14 ± 0.89 (3.5%)	13.62 ± 0.22 (23.8%)	35.45 ± 0.69 (62.0%)	6.61 ± 0.13	4.90 ± 0.08
ST3 ($n = 10$)	53.13 ± 0.61 (3.3%)	14.55 ± 0.27 (27.4%)	30.36 ± 0.50 (57.1%)	6.91 ± 0.14	4.34 ± 0.06
ST4 ($n = 10$)	53.87 ± 0.37 (3.2%)	15.71 ± 0.17 (29.2%)	30.58 ± 0.20 (56.8%)	7.79 ± 0.10	4.47 ± 0.05
mean (%)	56.31 ± 1.82[b] (3.4 ± 0.11%)	14.77 ± 0.45[b] 26.3% ± 1.22	33.17 ± 1.57[b] 58.8% ± 1.21	7.27 ± 0.30[b]	4.74 ± 0.21[b]

[a] Weights of the eggs are given in grams and as a percentage of the body weight (in parentheses) of the animals. Co1–4, controls 1–4; ST1–4, stevioside treatment 1–4. The number of eggs analyzed (*n*) is given in parentheses (this does not equal the number of eggs produced). [b] Statistically not significantly different from controls ($p > 0.05$; Student's *t*-test).

Table 3. Excreta Sampled during 24 Hours Following the Days for Which Feed Uptake Was Measured from Four Laying Hens Receiving a Chronic Stevioside Supply of 667 mg/kg of Feed[a]

	mean ($n = 16$)
total excreta per day (g dry wt)	91 ± 7.0
feed uptake per day (g)	118 ± 7.9
ST taken up (mg)	79 ± 5.3
total ST recovered (mg)	53.2 ± 3.2
(as % of total recovery)	(67.3%)
SV formed as ST equiv	4.2 ± 0.8
(as % of total recovery)	(7.3%)
total recovered (mg)	57.3 ± 3.4
	(72.5%)

[a] Each animal was sampled four times during the experiment. The total daily feed uptake (in grams), the total amount of stevioside (ST) taken up (in milligrams), and the stevioside recovered as well as the steviol formed, expressed as stevioside equivalents (in milligrams), are given.

pounds taken up by the alimentary tract, and thus steviol or stevioside, if taken up, could be transported and accumulated in yolk, they were measured separately in yolk and albumen. The latter is only a secreted product from the oviduct. Each individual egg was separated into egg yolk and white. Of the 40 eggs from stevioside-treated animals being analyzed, no traces of stevioside or steviol could be found in the extracts of either egg white or egg yolk. No traces of stevioside or steviol could be detected in the blood samples of these animals.

Chronic Stevioside Supplementation to the Diet of Meat-Type Chickens. No significant differences in body weight gain or in feed uptake were recorded between the controls and the stevioside-treated group (**Table 4**). As these animals are selected for fast growth, the huge weight increase during the experimental period was as expected. No significant differences in feed uptake were found between the control and stevioside-treated animals (**Table 4**). Consequently, no significant difference in feed conversion could be calculated, either.

The stevioside recovered in the excreta fluctuated around 108 mg of the 175 mg taken up (61.7%) for the different groups of animals (**Table 5**). The amount of steviol in the excreta, given as stevioside equivalents in **Table 5**, is about 21.5% of the stevioside taken up, whereas in intubation experiments about 2% of the administered stevioside was converted to steviol, as mentioned previously.

No stevioside or steviol could be detected in the blood samples of these animals. In recovery experiments, it was shown that stevioside and steviol could be extracted from blood. The detection limits of stevioside and steviol permitted the estimation of the maximum amount of stevioside and steviol that might be present undetected in the blood. The hypothetical maximum blood concentration of stevioside would then be between 0.4 and 2.0 μM, and that of steviol between 0.5 and 2.5 μM.

Anaerobic Incubation of Excreta. The in vitro conversion under anaerobic conditions was also studied. Incubation of chicken excreta with stevioside resulted in a partial conversion of stevioside into steviol (given as stevioside equivalents). At the end of the experiment, about 20% of the added stevioside was converted into steviol (**Figure 2**). This conversion value approached the value observed in the chronic experiments.

DISCUSSION

Metabolism of Stevioside. In the intubation experiments and chronic stevioside supplementation studies, only a small amount (about 2%) of the stevioside was converted into steviol. The total recovery percentage of the applied stevioside (biological recovery) was similar to the values of the recovery experiments with spiked excreta (methodological recovery). This might explain the fact that not all of the administered stevioside was recovered in the excreta. It should be noted that a large part of the intubated stevioside was still excreted in the period between 24 and 48 h after application. These results are in agreement with the results of Pomaret and Lavieille (*16*), who found an excretion of stevioside within 48 h, largely untransformed. Steviol was the only compound that we could detect. This observation agrees with the results of Hutapea et al. and Koyama et al. (*12*, *14*), who found steviol as the only metabolite detected under strictly anaerobic conditions. In anaerobic incubations with chicken excreta, about 20% of the stevioside was converted into

Table 4. Body Weight (g) ± SE, Feed Uptake (g), and Excreta Production (g Fresh) by Broiler Chickens (Two Chickens per Cage) Receiving a Chronic Stevioside Supplementation of 667 mg/kg of Feed

	control group			stevioside-treated group		
day	wt (g)	feed uptake (g)	excreta (g fresh)	wt (g)[a]	feed uptake (g)[a]	excreta (g fresh)[a]
0	583 ± 42.9			598 ± 18.4		
1		138 ± 60.1	94 ± 54.7		224 ± 33.5	146 ± 22.0
2		186 ± 56.2	195 ± 70.9		239 ± 14.4	259 ± 10.9
3	871 ± 68.0	214 ± 30.7	281 ± 29.4	969 ± 33.6	237 ± 11.0	243 ± 3.4
4		253 ± 15.6	278 ± 11.4		264 ± 3.2	247 ± 21.8
5+6		552 ± 4.3	608 ± 61.7		578 ± 31.1	500 ± 32.0
7	1236 ± 68.9	236 ± 4.2	290 ± 14.5	1323 ± 33.7	248 ± 7.1	248 ± 15.0
8		241 ± 20.9	269 ± 18.7		240 ± 7.7	232 ± 4.1
9		247 ± 24.4	277 ± 30.5		294 ± 6.5	274 ± 9.5
10	1510 ± 75.13	255 ± 28.4	272 ± 38.9	1640 ± 42.6	294 ± 9.5	309 ± 7.3
11		261 ± 22.6	246 ± 9.1		274 ± 3.0	238 ± 13.8
12+13		694 ± 12.1	665 ± 6.2		710 ± 11.0	617 ± 42.4
14	1972 ± 66.78	281 ± 14	326 ± 19.1	2007 ± 49.2	278 ± 9.6	311 ± 27.1

[a] Not significantly different from control values at the same day of measurement ($p > 0.05$; Student's *t*-test).

Table 5. Stevioside Analysis in the Excreta of Broilers (Two per Cage)[a]

chicken no.	sampling days	feed uptake (g)	excreta produced (g dry wt)	total ST uptake (mg)	ST recovered (mg)	SV as ST equiv recovered (mg)	total recovery (ST + SV) (mg)
ST1–2	9	239 ± 13.0	61 ± 5.8	151 ± 11.8	97 ± 12.8	21.2 ± 2.4 (17.8%)	118.6 ± 13.8
ST3–4	4	278 ± 13.8	70 ± 6.6	175 ± 8.3	132 ± 14.4	22.4 ± 6.2 (14.5%)	155 ± 10.8
ST5–6	5	277 ± 11.5	69 ± 4.4	185 ± 7.7	92 ± 9.7	42.5 ± 4.1 (31.7%)	134 ± 13.5
ST7–8	5	286 ± 20.4	60 ± 4.8	191 ± 13.5	114 ± 8.5	33.8 ± 2.5 (22.9%)	147.6 ± 10.6
mean		270 ± 10.5	65 ± 2.6	175.5 ± 8.8	108.7 ± 9.1	29.9 ± 5.0 (21.5%)	138.8 ± 8.0

[a] The excreta were sampled during 4–9 days as indicated in the table. Total feed uptake (in grams), total stevioside uptake (in milligrams), and recovery of stevioside (ST, in milligrams) and steviol as stevioside equivalents (SV, in milligrams) are given. The percentage conversion of stevioside into steviol is given in parentheses.

steviol. The result with chicken excreta is in agreement with the conversion (ca. 21.5%) in chronic in vivo experiments. The steviol formed in the excreta was not found in the blood or in the eggs produced by laying hens. These results suggest that steviol, which occurs as a carboxylic acid salt and is only sparingly soluble, was not taken up easily through the epithelia of the cecum or the colon. Taking into account the very low detection limits of steviol when analyzed as the (7-methoxy-coumarin-4-yl)methyl ester (50 pg), the amount of steviol possibly remaining undetected in our analyses (blood and eggs) can be estimated to be very low. The hypothetical maximum steviol concentration in the blood would probably not be toxic, as in hamsters daily fed steviol a NOEL of 250 mg/kg of BW was found (*7*). In this case, steviol concentration might be estimated to be about 0.786 mM on a body weight basis, or 11.23 mM on the basis of blood volume when assuming that all steviol fed was taken up by the intestines.

Effect on Nutrient Bioavailability. Modern broiler chickens are intensively selected for growth rate, and body weight increases by a factor of more than 50 in a time span of 6 weeks, making these animals especially suited to study the influence of feed additives on growth. However, they have become very susceptible to even slight deviations from optimal environmental and nutritional conditions. If such aberrations occur, this is readily reflected in feed intake and growth rate. As the supplementation of stevioside did not affect these parameters, it can be inferred that stevioside did not influence the uptake of other essential nutrients such as amino acids, vitamins, minerals, etc. Our results are in agreement with those of Wood et al. (*19*), who did not find any effects of stevia sweeteners at 0.042 and 0.085% in the feed on feed consumption or on the weight of 1000 broiler chickens. Notwithstanding the huge concentration of stevioside in the diet, no positive or negative effect on appetite could be discerned. Hence, the sweetness of the diet is not a discriminative factor for chickens. This observation is in line with literature reports. Adverse linear responses on broiler performance were observed when bitter lupins were added to the diet, but no effect was seen when sweet lupins were included up to 40% (*17*). It was also concluded that chickens reacted little to the sweetness of the diet in terms of appetite, but they strongly to the bitterness (*20*). Similar conclusions were found by Gentle (*21*). These results, showing the lack of effects on growth and hence on bioavailability of essential nutrients, are in good accordance with studies performed with other animal species (*5*, *6*, *10*, *22*−*25*).

A weak mutagenic effect of steviol (only 90% purity) to the sensitive *Salmonella typhimurium* TM 677 strain does not mean that stevioside used as a sweetener should be carcinogenic per se, even if the stevioside is transformed to steviol by bacteria in the colon (*26*). Moreover, it is not excluded that the mutagenic activity might be attributed to the 10% impurities present in the steviol sample used. The safety of stevioside in relation to carcinogenic activity was evidenced by the several published reports (*4*, *5*, *8*, *10*, *26*−*28*).

In view of the literature data, mainly with rats, and our results showing additionally that stevioside taken up by chickens was found largely untransformed in the excreta, while no traces of stevioside or its controversial metabolite steviol were found either in blood of broiler chickens and layers, or in eggs of the

laying hens, we can suggest that stevioside can be used in the diet as a sweetener.

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