Biochemical Pharmacology, Vol. 34, No. 6, pp. 873–882, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

EFFECTS OF *STEVIA REBAUDIANA* NATURAL PRODUCTS ON RAT LIVER MITOCHONDRIA

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(*Received* 20 *August* 1983; *accepted* 18 *July* 1984)

Abstract—The effects of several natural products extracted from the leaves of *Stevia rebaudiana* on rat liver mitochondria were investigated. The compounds used were stevioside (a non-caloric sweetener), steviolbioside, isosteviol and steviol. Total aqueous extracts of the leaves were also investigated. *S. rebaudiana* natural products inhibited oxidative phosphorylation, ATPase activity NADH-oxidase activity, succinate-oxidase activity, succinate dehydrogenase, and L-glutamate dehydrogenase. The ADP/O ratio was decreased. Substrate respiration (state II respiration) was increased at low concentrations (up to 0.5 mM) and inhibited at higher concentrations (1 mM or more). In uncoupled mitochondria, inhibition of substrate respiration was the only effect observed. Net proton ejection induced by succinate and swelling induced by several substrates were inhibited. Of the compounds investigated, the sweet principle stevioside was less active. It was concluded that, in addition to the inhibitory effects, *S. rebaudiana* natural products may also act as uncouplers of oxidative phosphorylation. The possible physiologic consequences of the ingestion of stevioside and *S. rebaudiana* aqueous extracts are discussed.

Stevia rebaudiana is a shrub from Brazil and Paraguay belonging to the Compositae [1]. The leaves of this plant contain large amounts (5% of dry weight) of stevioside, a sweetener 300 times as sweet as sucrose [1]. It is now becoming important in Japan and Brazil as a new non-caloric sweetener. Stevioside is a glycoside formed by three glucose molecules and steviol, a diterpenic carboxylic alcohol (Fig. 1; [2–4]). In addition to stevioside, the leaves of *S. rebaudiana* contain several related compounds, such as rebaudioside A (2% of dry weight), rebaudioside B, steviolbioside, and steviol (Fig. 1) [5], several of which are sweet. As a consequence of its high content in sweet glycosides, *S. rebaudiana* leaves are remarkably sweet.

Besides its organoleptic properties, the plant is also known for its physiologic and therapeutic effects. It was reported that aqueous extracts of *S. rebaudiana* are able to decrease blood sugar levels [6] and also that they have cardiotonic [7] and contraceptive properties [8]. More attention has been devoted by workers to the hypoglycemic effect. Regulation of blood sugar levels in human diabetic patients was reported by Miquel [6]. The same effect was observed in experiments performed with alloxanized rabbits [9]. It was also reported that administration of *S. rebaudiana* extracts to normal humans significantly increases glucose tolerance [10]. These effects were observed only with total extracts of the leaves. Stevioside, the major sweet principle, has no hypoglycemic effect.

A hypoglycemic action may be an indication of alterations in cell metabolism. Stevioside itself, which has no effect on blood sugar levels, seems not to be completely inert. In fact, as shown by Vignais *et al.* [11], stevioside and steviol inhibit ADP/ATP exchange in isolated rat liver mitochondria. Since the use of stevioside and *S. rebaudiana* leaves (in the form of aqueous infusions) in Japan and Brazil continues to increase, precise and detailed investigations of their effects on cell metabolism are indis-

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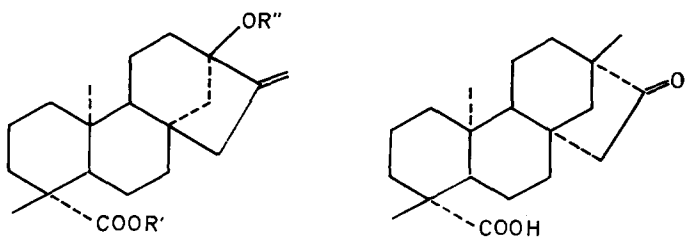

Steviol: R' = R" = H
Steviolbioside: R'=H, R"=sophorose
Stevioside: R'=glucose; R"=sophorose

Isosteviol

Fig. 1. Structural formulae of steviol, steviolbioside, stevioside and isosteviol [5].

pensable. With this purpose, *S. rebaudiana* was introduced recently in our laboratory. Preliminary experiments performed with isolated rat liver mitochondria and intact cells showed us that the effects of *S. rebaudiana* natural products are not restricted to an inhibition of ADP/ATP exchange. Since a previous knowledge of the effect on isolated mitochondria may be important for the interpretation of subsequent experiments with intact cells and organisms, we decided to perform first a systematic study with four purified compounds and the total aqueous extract of the leaves. The compounds investigated were stevioside, steviolbioside, steviol and isosteviol (Fig. 1). Stevioside and steviolbioside are representative of the two main types of glycosides occurring in the leaves of *S. rebaudiana* whereas steviol and isosteviol are the two main forms of the free aglycone [5]. The results may provide information about the mechanism of action of *S. rebaudiana* natural products and help in the interpretation of subsequent experiments on cell metabolism.

MATERIALS AND METHODS

Materials. Enzymes and coenzymes (ADP, ATP, NAD^+, NADH, $NADP^+$ and NADPH), antimycin A, rotenone, oligomycin, sodium atractylate, 2,4-dinitrophenol and carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) were purchased from Sigma. The reagent grade chemicals were from Merck (Darmstadt), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

Crystalline stevioside was purified from dried *S. rebaudiana* leaves as described previously [12]. Steviolbioside was obtained by strong base hydrolysis and isosteviol by strong acid hydrolysis of stevioside, essentially as described by Kohda *et al.* [5]. Steviol was obtained by enzymatic hydrolysis of stevioside with pectinase (poly(1,4-α-D-galacturonide)-glycano-hydrolase; EC 3.2.1.15) as described elsewhere [13].

The total aqueous extract of *S. rebaudiana* leaves was prepared by extraction with hot water. Dried *S. rebaudiana* leaves (100 g) were suspended in 1000 ml of hot water (100°). After 30 min, the suspension was filtered through several layers of cheesecloth, and the filtrate was used in the experiments.

Steviol, isosteviol and steviolbioside were added to the incubations either as aqueous alkaline solutions (pH 8–9) or as alcoholic solutions. Adequate controls were run in order to exclude unspecific solvent effects. Stevioside was directly dissolved into the incubation medium.

Isolation of rat liver mitochondria. For oxygen uptake and ATPase activity measurements, mitochondria were isolated according to Voss *et al.* [14], using a mannitol–sucrose medium. Swelling and proton efflux measurements, on the other hand, were carried out with mitochondria isolated as described by Packer *et al.* [15], using a sucrose medium.

Freeze–thawing disruption of mitochondria and extraction of soluble enzymes. Intact mitochondria, isolated according to Voss *et al.* [14] and suspended in extraction medium (30 μg protein/ml), were frozen at −10° for 24 hr, thawed at room temperature, and maintained at 0–4° for use. This preparation was used for the assay of membrane-bound enzymatic activities. For the assay of soluble enzymes, the disrupted membranes were eliminated by centrifugation at 30,000 *g* for 60 min. The supernatant (soluble fraction) was used as enzyme source for the assays.

Oxygen uptake and determination of ADP/O ratios. Oxygen uptake was measured polarographically as described by Voss *et al.* [16], using the following incubation medium: disodium phosphate, 5 mM; tris-(hydroxymethyl)aminomethane (Tris–HCl), 20 mM (pH 7.4); disodium ethylenediaminetetraacetate (disodium EDTA), 0.2 mM; potassium chloride, 10 mM; and mannitol 0.25 M. The P/O ratios were calculated as ADP/O ratios according to Chance and Williams [17].

Mitochondrial swelling. Respiration driven and ATP induced swelling of rat liver mitochondria were followed spectrophotometrically at 575 nm, as described by Packer *et al.* [15]. The reaction medium contained: sucrose, 100 mM; Tris–HCl, 10 mM (pH 7.3); and disodium-EDTA, 0.3 mM. Sodium acetate (20 mM) was added as co-permeant anion.

Proton efflux measurements. Respiration driven proton efflux was measured with a glass electrode, using succinate (8 mM) as electron donor [18]. The incubation medium contained: potassium chloride, 0.15 M; Tris–HCl, 1 mM (pH 7.4); and rotenone, 7 μM. Calibration was performed by addition of known amounts of HCl.

ATPase activity. The ATPase activity was assayed by measuring phosphate release [11] or by following the stoichiometric proton liberation with a glass electrode [19]. When phosphate release was measured, mitochondria (2 mg protein/ml) were incubated in reaction medium containing potassium chloride (0.15 M), Tris–HCl (50 mM, pH 7.4), 2,4-dinitrophenol (0.1 mM) and ATP (5 mM). Temperature of incubation was 25°. After 5 min of incubation, the reaction was stopped by addition of an ice-cold perchloric acid solution (3%), and phosphate was assayed as described by Lowry and Lopez [20]. The stoichiometric proton liberation was measured in reaction medium containing potassium chloride (0.15 M), Tris–HCl (1 mM, pH 7.4), 2,4-dinitrophenol (0.1 mM) and ATP (0.28 mM).

Polarographic assay of enzymatic activities. NADH-oxidase, succinate-oxidase and cytochrome *c*-oxidase activities were assayed polarographically [21]. The assay medium contained: Tris–HCl, 20 mM (pH 7.4); potassium chloride, 10 mM; and 0.5 mg mitochondrial protein per ml (freeze–thawing disrupted mitochondria). In the case of NADH- and succinate-oxidase, reaction was started by addition of NADH (1 mM) and succinate (8 mM) respectively. For the cytochrome *c*-oxidase assay, a mixture of *N, N, N', N'* - tetramethyl - *p* - phenylenediamine (TMPD) and ascorbic acid (0.3 and 2 mM, respectively) was used to reduce endogenous cytochrome *c* in the presence of antimycin A (5 μg/ml) [22].

Spectrophotometric assay of membrane bound enzymatic activities. Kinetic data of NADH-oxidase inhibition were obtained spectrophotometrically at 340 nm using Tris–HCl buffer (20 mM, pH 7.4), at 25°. Protein concentration was between 20 and 100 μg/ml and the NADH concentration was equal

to 0.15 mM, except when systematically changed. NADH-dehydrogenase (EC 1.6.99.3) and succinate dehydrogenase (EC 1.3.99.1) were assayed at 420 nm using ferricyanide as electron acceptor, according to Singer [21]. NADH cytochrome *c*-reductase activity was followed at 550 nm (reduction of ferricytochrome *c*) according to Hatefi and Rieske [23].

Assay of soluble enzymes. The mitochondrial L-malate dehydrogenase (L-malate:NAD oxidoreductase; EC 1.1.1.37), the $NADP^+$-specific isocitrate dehydrogenase (*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase, decarboxylating; EC 1.1.1.42), L-glutamate dehydrogenase (from rat liver and beef liver; L-glutamate $NAD(P)^+$ oxidoreductase, deaminating; EC 1.4.1.3) and fumarase (L-malate hydrolyase; EC 4.2.1.2) were assayed as described by Bergmeyer *et al.* [24]. The NAD^+-dependent isocitrate dehydrogenase (*threo*-D_s-isocitrate:NAD oxidoreductase, decarboxylating; EC 1.1.1.42) from rat liver mitochondria was assayed according to Plaut [25]. Hexokinase from yeast (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1) was assayed by measuring the pH decrease with a glass electrode. The incubation medium contained: Tris–HCl, 1 mM (pH 7.4); ATP, 0.3 mM; $MgCl_2$, 5 mM; and glucose, 7 mM.

Treatment of data. Each experimental point is the mean of at least three independent measurements. Concentrations for half-maximal inhibition (I_{50} values) were evaluated by interpolation. Inhibition constants (K_i) were obtained by fitting the Michaelis–Menten equation to the experimental data, according to Wilkinson [26].

RESULTS

Effect on phosphorylation coupled to L-glutamate oxidation. The effects of stevioside, steviolbioside, isosteviol and steviol on the respiration rates coupled to ADP phosphorylation and on the ADP/O ratios are shown in Table 1. The coupled respiration rates (oxygen uptake after ADP addition minus oxygen uptake before ADP addition—oligomycin and atractyloside sensitive) were inhibited by the four compounds and the ADP/O ratios were decreased. Of the four compounds, stevioside was the least effective as an inhibitor. High concentrations of stevioside were required (5 mM) to abolish activation of respiration by ADP, whereas a comparable effect occurred with 0.6 mM steviolbioside and 0.45 mM steviol or isosteviol. The effect on the ADP/O ratios was more evident with steviol and isosteviol. The control value of 2.6 was reduced to values around 1.0 by isosteviol or steviol at a concentration of 0.23 mM.

Effect on substrate respiration. In addition to the coupled respiration, substrate respiration was also affected. The nature (activation or inhibition) and the extent of the effect depended not only on the concentration of the inhibitor but also on the incubation conditions and metabolic state of the mitochondria. The most pronounced effect was observed with isosteviol. Figure 2 illustrates typical results obtained with this compound. Mitochondria were preincubated (2–3 min) with variable isosteviol concentrations, in the presence or absence of uncoupler (0.1 mM 2,4-dinitrophenol). Respiration was initiated by addition of substrate (L-glutamate or succinate), and the computed initial rates of oxygen uptake were plotted against the isosteviol concentrations. In the presence of uncoupler, oxygen uptake was inhibited by isosteviol. When succinate was the substrate, 50% inhibition was observed with 0.5 mM isosteviol. When L-glutamate was the substrate, 50% inhibition occurred at 0.4 mM isosteviol.

Table 1. Effects of stevioside, steviolbioside, isosteviol and steviol on phosphorylation coupled to L-glutamate oxidation in rat liver mitochondria*

Expt.	Inhibitors	Concn (mM)	Respiration rate (nmoles/min/mg protein)	ADP/O ratio
1	None		50 ± 4	2.5 ± 0.3
	Stevioside	0.6	47 ± 3	2.5 ± 0.2
		1.2	39 ± 3	2.3 ± 0.3
		5.0	0	
2	None		38 ± 2	2.7 ± 0.3
	Steviolbioside	0.15	25 ± 4	2.2 ± 0.3
		0.3	13 ± 1	2.1 ± 0.2
		0.6	0	
3	None		59 ± 3	2.6 ± 0.2
	Isosteviol	0.1	25 ± 2	1.6 ± 0.2
		0.23	16 ± 2	1.2 ± 0.2
		0.45	0	
4	None		66 ± 6	2.6 ± 0.3
	Steviol	0.1	24 ± 3	1.7 ± 0.3
		0.23	11 ± 1	1.0 ± 0.2
		0.45	0	

* Mitochondria (0.5 to 1.0 mg protein/ml) were incubated in reaction medium as described in Materials and Methods. The L-glutamate concentration was 8 mM. Oxygen uptake was recorded polarographically. ADP (200 nmoles/ml) was added at appropriate times. The rate of respiration refers to the difference between oxygen consumption after ADP addition and the resting respiration (i.e. before ADP addition). Values are means ± S.E.

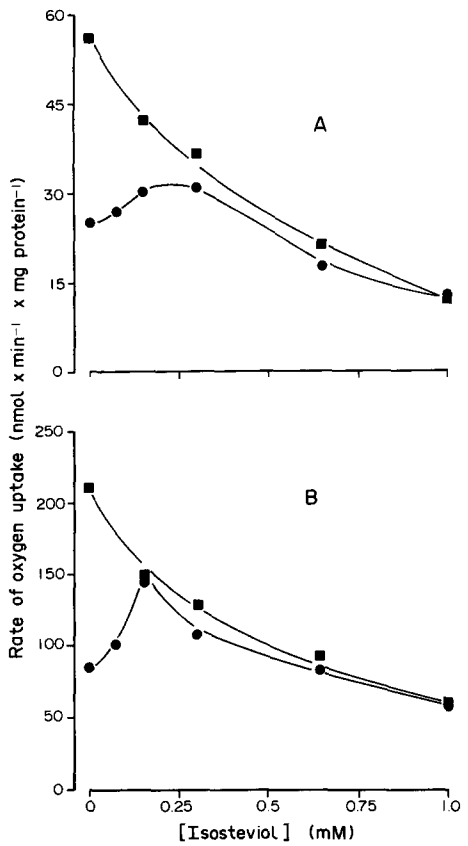

Fig. 2. Effect of isosteviol on substrate respiration. Mitochondria (0.5 mg protein/ml) were incubated at 25° in the reaction medium [14] in the presence (■) and absence (●) of 0.1 mM 2,4-dinitrophenol. Oxygen uptake was followed polarographically. The computed initial rates of oxygen uptake were expressed in nmoles O_2 per min per mg protein and plotted against the isosteviol concentration. (A) with 8 mM L-glutamate as substrate; (B) with 8 mM succinate.

With no uncoupler in the incubation medium, however, an activation of oxygen uptake took place at the lower concentrations (0.25 mM). An inhibition relative to the control rates was observed only at the higher concentrations. At high isosteviol concentrations, the respiration rates with and without uncoupler were practically the same. An experiment similar to that shown in Fig. 2 was carried out with ascorbate plus TMPD as electron donor in the presence of antimycin A. No inhibition was found with isosteviol up to 1 mM. In the absence of uncoupler, however, a small activation of respiration was found (maximally 20%).

Experiments similar to those shown in Fig. 2 were also performed with steviolbioside (up to 1 mM), stevioside (up to 5 mM) and steviol (up to 1 mM). Qualitatively the results were comparable to those obtained with isosteviol. An activation of respiration in the absence of uncoupler, similar to that observed with isosteviol, was found with steviol. Maximal activation (45% relative to the control values) occurred at 0.5 mM steviol. The effect was practically the same with succinate and L-glutamate as substrates. Steviolbioside, on the other hand, activated succinate respiration (maximally 40% activation at 0.15 mM steviolbioside), but no such effect was found with L-glutamate as substrate. Stevioside, finally, in concentrations up to 5 mM was unable to increase the rate of substrate oxidation. At the higher concentrations used, all compounds inhibited substrate respiration. With L-glutamate as electron donor, as may be judged from data in Table 2, the effectiveness of the compounds as inhibitors decreased along the series isosteviol > steviolbioside > steviol > stevioside.

Effect on ATPase activity. Figure 3 shows the results from a systematic investigation of the effects of isosteviol, steviol, steviolbioside and stevioside on the 2,4-dinitrophenol-stimulated ATPase of intact rat liver mitochondria. All the compounds inhibited ATP hydrolysis. The concentrations of steviol, isosteviol, steviolbioside and stevioside which produced 50% inhibition were 0.02, 0.1, 0.6 and 1.2 mM respectively. The results in Fig. 3 were obtained by measuring the stoichiometric proton liberation that follows ATP hydrolysis [19]. Measurement of phosphate release, however, yielded virtually identical results. Identical results were also obtained with both methods when atractyloside was the inhibitor. With 1 μM atractyloside, 50% inhibition of the 2,4-dinitrophenol-stimulated ATPase was found, a result which was also reported by Mitchell and Moyle [19].

A different picture resulted from an investigation of ATP hydrolysis in the absence of uncoupler. The results of such an investigation are shown in Fig. 4. As can be seen, the oligomycin (and atractyloside) sensitive ATPase was activated by isosteviol and steviol, when those compounds were present at low concentrations. With isosteviol, the effect was more pronounced and maximal activation occurred at a

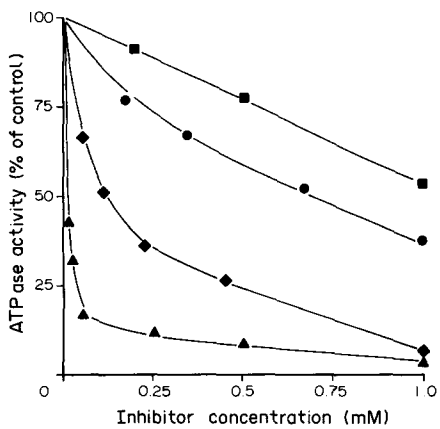

Fig. 3. Effects of isosteviol, steviol, steviolbioside and stevioside on the 2,4-dinitrophenol-stimulated ATPase of intact rat liver mitochondria. Mitochondria (0.2 mg protein/ml) were incubated in reaction medium containing 0.15 M KCl, 1 mM Tris–HCl and 0.1 mM 2,4-dinitrophenol. Temperature of incubation was 25°, and the initial pH was 7.4. Reaction was started by addition of a neutralized solution of ATP (final concentration 0.3 mM). The stoichiometric proton liberation was followed with a glass electrode. The rates obtained in the presence of inhibitors were expressed as percentage of the corresponding control values. Key: steviol (▲), isosteviol (◆), steviolbioside (●), and stevioside (■).

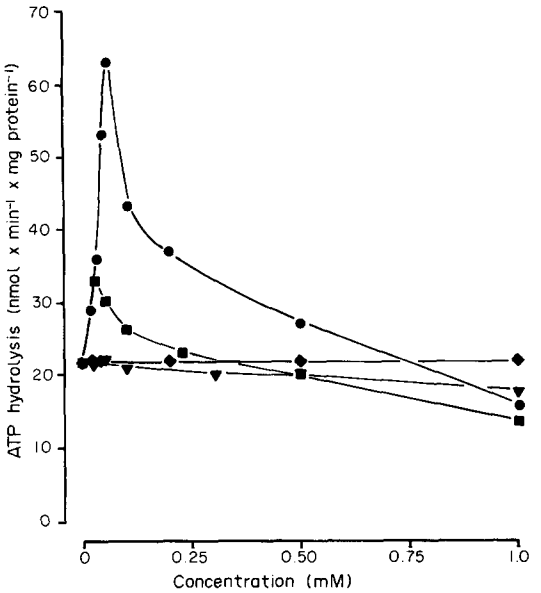

Fig. 4. Effects of isosteviol, steviol, steviolbioside and stevioside on ATP hydrolysis in intact rat liver mitochondria. The mitochondria were incubated at 25° in reaction medium containing potassium chloride (0.15 M), Tris–HCl (50 mM, pH 7.4) and ATP (5 mM). Protein concentration was equal to 2 mg/ml. After 10 min of incubation, the reaction was stopped by addition of an ice-cold perchloric acid solution, and phosphate was assayed as described by Lowry and Lopez [20]. The rates of phosphate liberation (expressed in nmoles per min per mg protein) were plotted against the concentration of each compound. Key: isosteviol (●), steviol (■), steviolbioside (◆), and stevioside (▼).

concentration of 0.06 mM. The effect of steviol was smaller by far, yet reproducible. Maximal activation occurred at 0.03 mM steviol. No activation was found with steviolbioside up to 1 mM or with stevioside up to 5 mM. The type of curve shown in Fig. 4 (activation followed by inhibition) suggest, at least for isosteviol and steviol, that inhibition of phosphorylation was not due solely to inhibition of adenine nucleotide exchange [11].

Effect on respiration-dependent net proton ejection. To obtain information about the mechanism of action of *Stevia rebaudiana* natural products, the effect on net proton efflux induced by succinate in rat liver mitochondria was investigated. Mitochondria were isolated in a sucrose medium (0.33 M sucrose) and incubated in 0.15 M KCl solution, buffered with 1 mM Tris and containing 7 μM rotenone. Proton concentration was monitored potentiometrically. A typical experiment is shown in Fig. 5. Addition of 8 mM succinate induced a significant acidification of the incubation medium; 50 ng-ions per mg protein were ejected until steady state was reached. Preincubation with antimycin A prevented proton efflux, an indication that it depends on electron transport. Preincubation with isosteviol, on the other hand, also prevented net proton efflux. The effect was already pronounced with 0.13 mM isosteviol (70% inhibition). At this concentration, isosteviol increased oxygen uptake in the absence of uncoupler (see Fig. 2B). At 0.4 mM isosteviol, net proton efflux was 90% inhibited. Even at this concentration, respiration was not inhibited by isosteviol (Fig. 2B). Control experiments showed that the effect of isosteviol was not due to alterations in the buffering capacity of the incubation medium.

Similar experiments were also performed with steviolbioside and stevioside. Concentrations were investigated which either increased or were without effect on substrate respiration. The effects of both compounds were less pronounced. A 25% decrease in net proton efflux, for example, was found with 0.15 mM steviolbioside; 0.15 mM steviolbioside maximally activates oxygen uptake with succinate as substrate. Half-maximal effect was observed at 0.4 mM steviolbioside. In the case of stevioside, on the other hand, only concentrations above 1 mM had an effect on net proton efflux.

Effect on mitochondrial swelling. Study of the effects of inhibitors on mitochondrial swelling may provide useful information about its mechanism of action. Osmotic work in mitochondria depends on energy generated by electron transport or by ATP hydrolysis [27]. From the results just described, one may expect a pronounced effect of isosteviol and its derivatives. Since isosteviol had the most pronounced effect, a more detailed investigation was

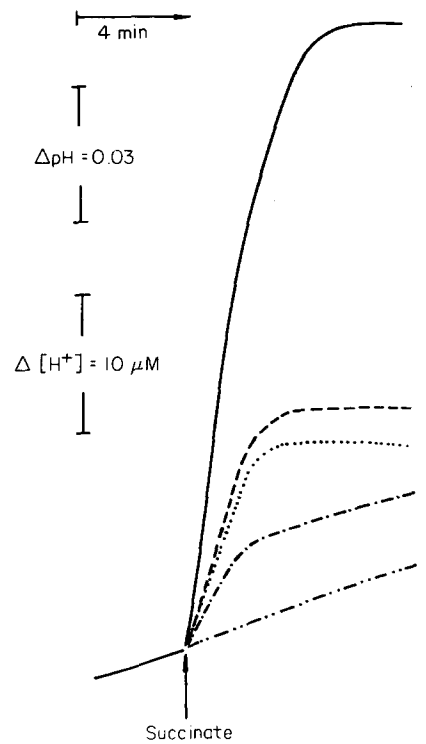

Fig. 5. Effect is isosteviol on net proton ejection induced by succinate. Rat liver mitochondria (0.9 mg protein/ml) were incubated at 25° in reaction medium containing 0.15 M KCl, 1 mM Tris–HCl and 7 μM rotenone, in the absence and presence of inhibitors. The initial pH was 7.4. Proton ejection was initiated by addition of 8 mM succinate and was recorded potentiometrically. Key: control (——), 0.13 mM isosteviol (– – –), 0.2 mM isosteviol (· · · · · ·), 0.4 mM isosteviol (– · – · –), and antimycin A, 3 μg/ml (– · · – · · –).

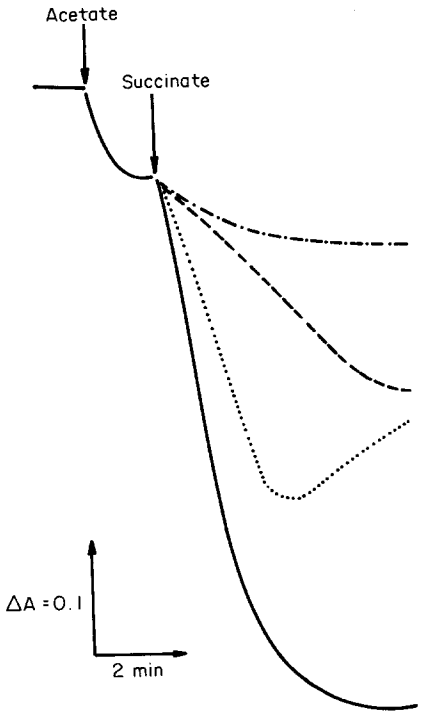

Fig. 6. Effect of isosteviol on mitochondrial swelling induced by succinate. Rat liver mitochondria (0.6 mg protein/ml) were incubated at 25° in medium containing 0.1 M sucrose, 0.3 mM EDTA, 10 mM Tris–HCl (pH 7.3), in the absence (control) and presence of various concentrations of isosteviol. Sodium acetate (20 mM) and succinate (8 mM) were added as indicated by arrows. The decrease in absorbance at 575 nm was recorded. Key: control (——), 0.2 mM isosteviol (·····), 0.4 mM isosteviol (– – – –), and 0.8 mM isosteviol (–·–·–).

performed with this compound. Figure 6 shows the results of an experiment in this series. The mitochondrial volume changes were measured as described by Mustafa *et al.* [22], using acetate as co-permeant anion, without addition of ionophores. As shown by Fig. 6, a decrease in absorbance at 575 nm occurred upon addition of succinate. This change was antimycin A and 2,4-dinitrophenol sensitive. Isosteviol strongly affected swelling induced by succinate. With concentrations between 0.13 and 0.2 mM, 50% inhibition took place. With 0.4 mM isosteviol, swelling was reduced to about 10% of the control. It should be noted that the results in Fig. 6 are consistent with the proton efflux measurements shown in Fig. 5.

L-glutamate-dependent swelling (rotenone sensitive), TMPD-ascorbate-dependent swelling (in the presence of antimycin A—cyanide sensitive) and ATP-dependent swelling (oligomycin sensitive) were also investigated. The effects of isosteviol, steviol, steviolbioside and stevioside are summarized in Table 2. From the concentrations for half-maximal effects, isosteviol was the most potent inhibitor, followed by steviol and steviolbioside. Stevioside was poorly active and, rigorously speaking, only the concentration for half-maximal effect on the ATP-dependent swelling could be measured. This was caused by the low solubility of stevioside in aqueous systems. Above 2 mM, stevioside precipitation rendered swelling measurements practically impossible.

Effect on enzymatic activities. The effects of steviol, isosteviol, steviolbioside and stevioside on several enzymatic activities are summarized in Table 3. Besides the mitochondrial ATPase activity (already discussed), there are a number of enzymatic activities which were inhibited by the compounds. NADH-oxidase was strongly affected by isosteviol. Steviol and steviolbioside were also active, but stevioside had no inhibitory action, at least with concentrations up to 5 mM. NADH-dehydrogenase (ferricyanide-reductase) was not inhibited. A kinetic study performed with isosteviol and steviolbioside (Fig. 7) showed that the inhibition was linear (complete) and non-competitive. The inhibition constants (K_i) for isosteviol and steviolbioside, evaluated by a least-squares fit [26], were 0.1 and 0.6 mM respectively.

Succinate-oxidase activity, on the other hand, was less strongly inhibited by isosteviol, steviol being the strongest inhibitor. Steviolbioside and stevioside were weak inhibitors of the succinate-oxidase

Table 2. Concentrations of isosteviol, steviol, steviolbioside and stevioside for half-maximal effects (I_{50} values) on several variables*

Variable	I_{50} (mM)			
	Isosteviol	Steviol	Steviolbioside	Stevioside
Coupled respiration (state III respiration)	0.07	0.05	0.25	2.5
Uncoupled respiration (with L-glutamate as substrate)	0.4	0.9	0.6	>2.0
DNP-stimulated ATPase	0.1	0.02	0.6	1.2
NADH-oxidase activity	0.1	0.6	0.6	>5.0
L-glutamate dehydrogenase	0.15	0.55	0.6	>5.0
ATP-dependent swelling	0.1	0.1	1.0	2.0
L-glutamate-dependent swelling	0.06	0.13	0.3	>2.0
Succinate-dependent swelling	0.1	0.2	0.4	>2.0
TMPD-ascorbate-dependent swelling (in the presence of antimycin A)	0.05	0.1	0.8	>2.0
Succinate-dependent proton efflux	0.1		0.4	>2.0

* Methods and incubation systems are described in Materials and Methods.

Table 3. Effects of isosteviol, steviol, steviolbioside and stevioside on enzymatic activities*

Activity	Inhibition (%)			
	Isosteviol (0.5 mM)	Steviol (0.5 mM)	Steviolbioside (0.5 mM)	Stevioside (1 mM)
DNP-stimulated ATPase	80	92	45	50
NADH-oxidase	85	45	40	0
Succinate-oxidase	24	42	11	8
Succinate dehydrogenase (ferricyanide-reductase)	24	46	10	10
NADH cytochrome *c* reductase†	82		50	0
L-Glutamate dehydrogenase (NADH)‡	80	46	60	0
L-Glutamate dehydrogenase (NADPH)‡	75		60	0
Cytochrome *c*-oxidase				
Hexokinase§				
Fumarase				
Isocitrate dehydrogenase (NAD^+-dependent)	0	0	0	0
Isocitrate dehydrogenase ($NADP^+$-dependent)				
L-Malate dehydrogenase (mitochondrial)				
NADH-dehydrogenase (ferricyanide-reductase)				

* Methods and incubation systems are described in Materials and Methods. The enzyme source was rat liver mitochondria, except when expressly indicated.
† Rotenone sensitive.
‡ Enzyme from rat liver or beef liver.
§ Yeast enzyme.

activity. Succinate dehydrogenase (ferricyanide-reductase) was also affected; the extent of inhibition was the same as for succinate-oxidase activity. This observation strongly suggests that the site of action on the succinate-oxidase activity was the flavoprotein moiety (succinate dehydrogenase). Moreover, in contrast to the kinetics of NADH-oxidase, the inhibition of succinate-oxidase activity was hyperbolic (see Fig. 7).

Of the soluble enzymes investigated, inhibition was found only with L-glutamate dehydrogenase. Isosteviol was the strongest inhibitor, followed by steviol and steviolbioside. Stevioside did not inhibit the enzyme. It should be noted that the effect was practically the same with both coenzymes, NADH and NADPH. Moreover, no difference was found with the enzyme from rat or beef liver mitochondria.

No effect was found on cytochrome *c*-oxidase activity, hexokinase, fumarase, isocitrate dehydrogenases (NAD^+- and $NADP^+$-dependent), and L-malate dehydrogenase. These negative results, however, provide useful controls for exclusion of unspecific effects or technical artifacts. This is particularly true for hexokinase, which was assayed potentiometrically. The negative results show that the compounds investigated in this work did not change the buffering capacity of an incubation medium buffered at pH 7.4 with 1 mM Tris–HCl.

Effect of the total aqueous extract of Stevia rebaudiana *leaves on selected variables.* The total extract of *S. rebaudiana* leaves has a complex composition. Stevioside and rebaudioside A account for 5% and 2% of the dry weight of the leaves respectively [5]. All other stevioside derivatives account individually for 0.04% or less of the dry weight of the leaves [5, 28–31]. Many other compounds are present in total extracts, and care must thus be taken when investigating their actions. Aqueous extracts are used

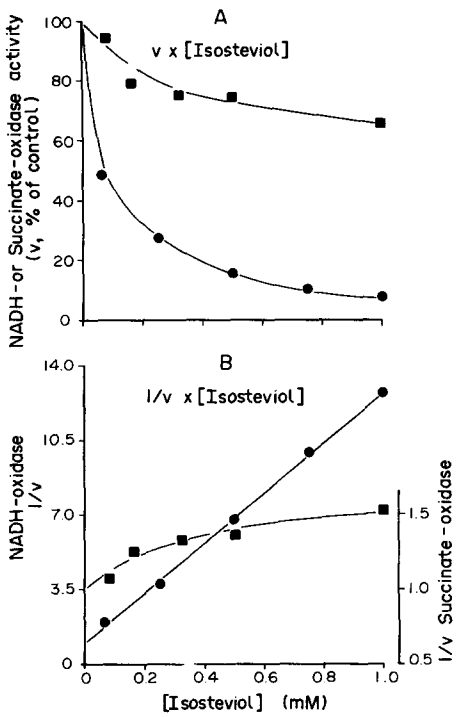

Fig. 7. Concentration dependence of the inhibition of NADH-oxidase and succinate-oxidase by isosteviol. Freeze–thawing disrupted mitochondria were incubated in Tris–HCl buffer (20 mM, pH 7.4) at 25°. NADH-oxidase (●) was assayed spectrophotometrically at 340 nm, with 100 μg protein/ml of the incubation medium. The initial NADH concentration was equal to 0.1 mM. Succinate-oxidase (■) was assayed polarographically with 0.5 mg protein/ml and with an initial succinate concentration equal to 8 mM. (A) reaction rates (expressed as percentage of the control) versus isosteviol concentration; (B) reciprocals of the reaction rates versus isosteviol concentration.

by a great number of people, however, and some knowledge about their action is highly desirable. Taking into account the observed effects of stevioside derivatives, three variables were selected in this work. They were: ATPase activity, NADH-oxidase activity and L-glutamate dehydrogenase. As expected, inhibition of the three enzymatic activities was found. ATPase, NADH-oxidase and L-glutamate dehydrogenase were inhibited 50% by final stevioside concentrations of 0.2, 0.007 and 0.04 mM respectively. Stevioside, the major sweet principle, contributed very little to the effect of the total extract. Most probably, all stevioside derivatives were contributing to some extent. The possibility, however, that other compounds, not belonging to the stevioside group, contributed to the observed effects of the total extract, cannot be excluded with the available data.

DISCUSSION

The results described in this article about the effects of *Stevia rebaudiana* natural products on rat liver mitochondria provide useful information for subsequent experiments on cell metabolism. The original observation of Vignais *et al.* [11] of the inhibitory action of steviol and stevioside on phosphorylation was confirmed in this work. As shown by those authors, this effect can be attributed to inhibition of the ADP/ATP exchange between intra- and extramitochondrial spaces. Our study also confirmed the effect of steviol on NADH-oxidase activity which was also originally demonstrated by Vignais *et al.* [11]. As shown by our experiments, the effects on phosphorylation and NADH-oxidase activity can be extended to steviolbioside and isosteviol. In addition to this, our work revealed some new aspects of the action of *S. rebaudiana* natural products on rat liver mitochondria. Evidence has been provided, for example, in favor of an uncoupling effect and of a pronounced effect on L-glutamate dehydrogenase.

An interesting feature of the effects of steviol and isosteviol is the observed increase in the rate of substrate oxidation in the absence of uncoupler when they are present in low concentrations. In this sense, they behave like long-chain fatty acids. As shown by Vázquez-Colón *et al.* [32], long-chain fatty acids also accelerate respiration in the absence of ADP when present in low concentrations. It is well known that uncouplers usually increase oxygen uptake [33]. In the present case, however, interpretation is complicated by the fact that the same compounds inhibit oxygen uptake when present in high concentrations and when an uncoupler such as 2,4-dinitrophenol (or FCCP) is present in the incubation medium. Nevertheless, a series of observations strongly suggest that steviol and isosteviol (and to a lesser extent steviolbioside and stevioside) act as uncouplers. The first argument is the decrease in the ADP/O ratios observed with steviol and isosteviol and to a lesser extent with steviolbioside and stevioside. For steviol indeed the same effect was observed by Vignais *et al.* [11]. The second argument is based on the comparison of the effect on oxygen uptake and on net proton efflux induced by succinate. Whereas oxygen uptake was increased, net proton ejection was inhibited, a combination of events commonly found among uncouplers [18, 34, 35]. Thus, it is possible that isosteviol and its analogues act as protonophores as in the case of the classic uncoupling agents [33]. A similar argument is furnished by the observed effect on mitochondrial swelling. Swelling generated by electron transport or by ATP hydrolysis in the presence of acetate as permeant anion depends on active proton ejection. Protons ejected are carried back by the undissociated acetic acid molecules [27]. The effect of uncouplers on swelling depends on the incubation conditions and on the type of swelling. At very low uncoupler concentrations, swelling may be increased [33, 36]. At higher concentrations, however, uncouplers such as 2,4-dinitrophenol and FCCP inhibit steady-state swelling as well as damped volume oscillations [36, 37]. In our experiments, inhibition was the only effect observed with isosteviol and its analogs. In the case of ascorbate plus TMPD-dependent swelling, for example, even 15 μM isosteviol produced shrinkage relative to the control. It should be recalled that, in the case of electron-transport-dependent swelling, inhibition already took place at concentrations which were either without effect or which increased respiration. ATP-dependent swelling, on the other hand, was 50% inhibited by 0.1 mM isosteviol, a concentration at which ATP hydrolysis was activated rather than inhibited. The most simple explanation for such a combination of events is the assumption that at low concentrations isosteviol and its structural analogues decrease swelling because they act as uncouplers. It should be pointed out that the effect is unspecific. It occurs not only with L-glutamate- and succinate-induced swelling, but also when swelling depends on ascorbate oxidation by cytochrome *c*-oxidase. Very significantly, even high concentrations of isosteviol do not inhibit ascorbate oxidation.

The concentration dependence of substrate oxidation in the absence of uncoupler (see Fig. 2) could be the resultant of two effects: uncoupling (which in principle tends to increase the respiration rate) and a direct inhibition of electron transport (which tends to decrease the oxidation rate). The same applies to the concentration dependence of ATP hydrolysis in the absence of uncoupler (Fig. 4): uncoupling tends to increase the rate of ATP hydrolysis, but inhibition of ATP transport [11] has a negative influence. When the respiratory chain is already uncoupled by 2,4-dinitrophenol, no activation of oxidation or of ATP hydrolysis occurs, since in the latter case the enzymatic system is already operating at its maximal or nearly maximal capacity. Consistent with the inhibition of L-glutamate and succinate oxidation in uncoupled mitochondria, an inhibition of NADH-oxidase and succinate-oxidase activities was also found in disrupted mitochondria (see Tables 2 and 3). The effects on both activities are possibly independent of each other. The available data are at least consistent with two independent sites of action, the first one between NADH-dehydrogenase and coenzyme Q and the second one between succinate-dehydrogenase and coenzyme Q. There are several arguments in favor of this supposition. A common site of action on both enzymatic activities (between

coenzyme Q and cytochrome *c*-oxidase) would in principle result in identical or nearly identical degrees of inhibition. This was the case with steviol (see Table 3), but with isosteviol and steviolbioside there was a great difference in the inhibition of the two enzymatic activities. The type of inhibition (linear complete in the case of NADH-oxidase and hyperbolic with succinate-oxidase) is a further argument for the existence of two independent mechanisms. The alternative possibility of two independent sites of action, the first one between NADH-dehydrogenase and coenzyme Q and the second one between coenzyme Q and cytochrome *c*-oxidase, on the other hand, would lead to a more complex type of inhibition on NADH-oxidase (parabolic or some more complex type [38]). This latter possibility is also improbable by virtue of the observation that succinate-dehydrogenase is affected to the same extent as succinate-oxidase activity. This observation strongly suggests that the site of action on succinate-oxidase activity is the flavoprotein moiety of the system. Vignais *et al.* [11] did not find inhibition of succinate-oxidase activity by steviol. They used digitonin particles of rat liver mitochondria as enzyme source. This discrepancy may be a consequence of the difference in structural integrity of digitonin particles and mitochondria disrupted by a freeze–thawing procedure. Moreover, the effect on succinate-oxidase was consistent with the effect on succinate oxidation by intact mitochondria (Fig. 2B). The NADH-oxidase activity of digitonin particles, on the other hand, is sensitive to steviol [11]. This latter observation reinforces the supposition that the effects on NADH-oxidase and succinate-oxidase activities have independent mechanisms.

The use of several structural analogues with biological activity frequently allows conclusions about the molecular groups involved. In the case of stevioside and its derivatives, the carboxylic group seems to play an important role. A free carboxylic group is a prerequisite for inhibition of NADH-oxidase activity and L-glutamate dehydrogenase. Possibly it also plays some role in the inhibition of phosphorylation and ATPase activity since stevioside is less effective as inhibitor. A comparison of the results of swelling inhibition (Table 2), proton ejection (Table 2), ATP hydrolysis activation (Fig. 4), substrate respiration activation and decrease in the ADP/O ratio reveals that the ability of the compounds to uncouple oxidative phosphorylation obeys the sequence isosteviol > steviol > steviolbioside ⩾ stevioside. The sweet principle stevioside is practically inactive as an uncoupler. This may be related to the fact that it lacks a free carboxylic group and also because of its reduced lipophilicity [12]. As may be deduced from literature data, the relative lipophilicities of the compounds obeys the sequence isosteviol ≈ steviol > steviolbioside > stevioside [3–5, 12, 39].

It was shown that total aqueous extracts (infusions) of *S. rebaudiana* leaves such as those commonly taken in by humans affect several mitochondrial functions, the same functions which are also affected by the natural products found in the leaves of the plant. The question which now arises is whether the action of *S. rebaudiana* on mitochondria explains some of the physiologic effects that have been reported by some authors [6–9]. A definitive answer requires more experimental evidence with intact cell systems and organisms. Inhibition of oxidative phosphorylation and respiratory enzymes, however, generally has important consequences for cell metabolism. It is well known that inhibition of ATP synthesis in mitochondria leads to an increase in the rate of glycolysis and a decrease in the rate of gluconeogenesis. It was shown recently that *S. rebaudiana* natural products are able to inhibit gluconeogenesis in isolated rat renal tubules [40]. In living organisms, this may lead to hypoglycemia [41], but whether this is really the mechanism responsible for the reported hypoglycemic action of *S. rebaudiana* needs to be confirmed. Nitrogen metabolism, on the other hand, may also be affected by virtue of the inhibitory effect on L-glutamate dehydrogenase, which plays a key role in urea production.

Stevioside may be considered a weak inhibitor of oxidative phosphorylation. Since very low doses are normally taken in because of its high sweetening power, it is doubtful if the levels normally attained in the living organism are sufficient for a significant inhibition of phosphorylation. Attention should be directed to the possibility of hydrolysis of stevioside to steviol or isosteviol, either in the intestinal tract or in the circulation. This was demonstrated for the rat [42], and it deserves attention because steviol and isosteviol are much more potent inhibitors of mitochondrial functions than stevioside. An answer to this question and many other related ones is indispensable for the continued use of stevioside as a non-caloric sweetener. The same may be said about the use of aqueous infusions of *S. rebaudiana* leaves.

REFERENCES

1. M. Bridel and R. Lavielle, *C. r. hebd. Séanc. Acad. Sci., Paris* **192**, 1123 (1931).
2. M. Bridel and R. Lavielle, *Bull. Soc. Chim. biol.* **13**, 781 (1931).
3. H. B. Wood Jr., R. Allerton, H. W. Diehl and H. C. Fletcher, *J. org. Chem.* **20**, 875 (1955).
4. E. Mosettig and W. R. Nes, *J. org. Chem.* **20**, 884 (1955).
5. H. Kohda, R. Kasai, K. Yamasaki, K. Murakami and O. Tanaka, *Phytochemistry* **15**, 981 (1976).
6. O. Miquel, *Revta med. Parag.* **7**, 200 (1966).
7. G. A. von Schmeling, *Bolm Sanat S Lucas* **94**, 67 (1967).
8. G. M. Planas and J. Kué, *Science* **162**, 1007 (1968).
9. G. A. von Schmeling, F. N. Carvalho and A. D. de Espinoza, *Ciênc. e Cult.* **22**, 599 (1977).
10. M. Alvarez, R. B. Bazzote, G. L. Godoy, R. Cury and L. M. Botion, *Archos Biol. Tecnol., Curitiba* **24**, 178 (1981).
11. P. V. Vignais, E. D. Duee, P. M. Vignais and J. Huet, *Biochim. biophys. Acta* **118**, 465 (1966).
12. M. Alvarez, A. Bracht and E. L. Ishii, *Archos Biol. Tecnol., Curitiba* **24**, 179 (1981).
13. M. de L. Ferraresi, A. M. Kelmer Bracht and A. Bracht, *Archos Biol. Tecnol., Curitiba* **27**, 159 (1984).
14. D. O. Voss, A. P. Campello and M. Bacila, *Biochem. biophys. Res. Commun.* **4**, 48 (1961).
15. L. Packer, K. Utsumi and M. G. Mustafa, *Archs Biochem. Biophys.* **117**, 381 (1966).
16. D. O. Voss, J. C. Cowles and M. Bacila, *Analyt. Biochem.* **6**, 211 (1963).

17. B. Chance and G. R. Williams, *Nature, Lond.* **175**, 1120 (1955).
18. A. Villalobo and A. L. Lehninger, *J. biol. Chem.* **254**, 4352 (1979).
19. P. Mitchell and J. Moyle, *Eur. J. Biochem.* **4**, 530 (1968).
20. O. H. Lowry and J. A. Lopez, *J. biol. Chem.* **162**, 421 (1946).
21. T. P. Singer, in *Methods of Biochemical Analysis* (Ed. D. Glick), Vol. 22, p. 123. John Wiley, New York (1974).
22. M. G. Mustafa, M. L. Gowger, R. F. Labbe and T. E. King, *J. biol. Chem.* **243**, 1908 (1968).
23. M. G. Hatefi and J. S. Rieske, in *Methods of Enzymology* (Eds. S. P. Collowick and N. O. Kaplan), Vol. 10. Academic Press, New York (1974).
24. H. V. Bergmeyer, K. Gawehn and M. Grassl, in *Methods of Enzymatic Analysis* (Ed. H. V. Bergmeyer), Vol. 1, p. 425. Academic Press, New York (1974).
25. G. W. Plaut, in *Methods of Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 2, p. 650. Academic Press, New York (1974).
26. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
27. M. G. Mustafa, K. Utsumi and L. Packer, *Biochem. biophys. Res. Commun.* **24**, 381 (1966).
28. I. Sakamoto, H. Kohda, K. Murakami and O. Tanaka, *Yarugaku Zasschi* **95**, 1507 (1975).
29. M. Kobayashi, S. Horikava, I. H. Degrandi, J. Ueno and H. Mitsuhashi, *Phytochemistry* **16**, 1405 (1977).
30. I. Sakamoto, K. Yamasaki and O. Tanaka, *Chem. pharm. Bull., Tokyo* **25**, 844 (1977).
31. I. Sakamoto, K. Yamasaki and O. Tanaka, *Chem. pharm. Bull., Tokyo* **25**, 2437 (1977).
32. L. Vázquez-Colón, F. D. Ziegler and W. B. Elliott, *Biochemistry* **5**, 1134 (1966).
33. W. G. Hanstein, *Biochim. biophys. Acta* **456**, 129 (1976).
34. P. Mitchell and J. Moyle, *Biochem. J.* **105**, 1147 (1967).
35. S. Papa, *Biochim. biophys. Acta* **456**, 39 (1976).
36. V. D. Gooch and L. Packer, *Biochim. biophys. Acta* **346**, 245 (1974).
37. L. Packer, *J. biol. Chem.* **235**, 242 (1960).
38. K. M. Plowman, *Enzyme Kinetics*. McGraw-Hill, New York (1972).
39. O. Tanaka, *Proceedings of the International Symposium on Natural Products Research*, p. 111–9 (1980).
40. N. S. Yamamoto, A. M. Kelmer Bracht, E. L. Ishii, F. S. Kemmelmeier, M. Alvarez and A. Bracht, *Experientia,* in press.
41. E. E. Gordon and M. de Hartog, *Diabetes* **22**, 50 (1972).
42. R. E. Wingard Jr., J. P. Brown, F. E. Enderlin, J. A. Dale, R. L. Hale and C. T. Seita, *Experientia* **36**, 519 (1980).