down during studies of glycolysis, and other NAD(PH) dependent reactions, in hemolysates[18]. Another reaction which was occasionally, but not reproducibly, seen with NAD in hemolysates (not in whole cell suspensions) was the reduction to NADH. This reaction results in the loss of the aromatic character of the nicotinamidyl moiety and a consequent diminution of the high frequency ^1H spin-echo NMR resonances. The emerging inequivalent geminal H^4 resonances of NADH appeared in the aliphatic region of the spectrum (~ 2.2 ppm). The reaction was inhibited with 3 mmol/l iodoacetate in hemolysates but this compound did *not* alter the NADase reaction rate in suspensions of whole cells.

In conclusion, ^1H spin-echo NMR spectroscopy was established as a valuable means for monitoring the NADase reaction in suspensions of cells (erythrocytes). Various reactions which complicate the interpretation of the results from lysates arise from ADP-ribose degradation, but since the cell is impermeable to this compound the kinetic analysis is simple for whole cells if the adeninyl H^8 resonance is monitored.

1 The work was supported by the Australian National Health and Medical Research Council.

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0014-4754/85/010053-03$1.50 + 0.20/0

Effect of steviol and its structural analogues on glucose production and oxygen uptake in rat renal tubules

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Summary. The effect of several natural products of *Stevia rebaudiana* on glucose production and oxygen uptake in rat renal cortical tubules was investigated. Steviol, isosteviol and glucosilsteviol decreased glucose production and inhibited oxygen uptake. The sweet principle stevioside, and steviolbioside, however, were without effect on gluconeogenesis and oxygen uptake.
Key words. Rat renal tubules; renal tubules, rat; *Stevia rebaudiana;* oxygen uptake, steviol; gluconeogenesis.

Steviol is the aglucone of stevioside (fig. 1), a sweet glucoside abundant in the leaves of *Stevia rebaudiana,* a shrub belonging to the Compositae[1]. Stevioside is 300 times as sweet as sucrose and is now becoming important in Japan and Brazil, mainly as substitute for other non-caloric sweeteners[1]. Aqueous extracts of the leaves of *Stevia rebaudiana,* on the other hand, are used not only for sweetening purposes, but also because they have physiologic and therapeutic effects. It has been claimed that they have cardiotonic[2], contraceptive[3] and hypoglycemic properties[4,5]. Furthermore, it has been demonstrated that stevioside, steviol and other natural products of *Stevia rebaudiana* significantly affect several mitochondrial functions[6,7]. In isolated rat liver mitochondria, oxidative phosphorylation is inhibited by a complex mechanism of action which includes inhibition of adenine nucleotide exchange [6], uncoupling of respiration[7], inhibition of NADH-oxidase and L-glutamate dehydrogenase[6,7], etc. Inhibition of ADP phosphorylation in mitochondria usually has important consequences for the whole organism and the question which now arises is whether stevioside, steviol and related compounds also affect mitochondrial functions in the intact cell. This communication represents our first contribution on this subject. It reports the effect of stevioside, steviol and other natural products of *Stevia rebaudiana* on gluconeogenesis, a biosynthetic route strictly dependent on the available energy in the intact cell. To our knowledge, this is the first report about the effect of *Stevia rebaudiana* natural products on intact cell systems.

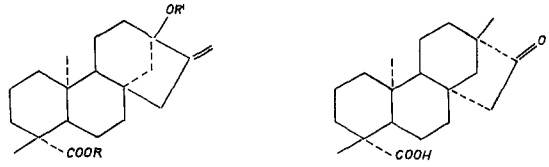
Materials and methods. Renal cortical tubules from rats were isolated with collagenase (clostridiopeptidase A; EC 3.4.24.3), essentially as described by Gordon and Hartog[8]. Male albino rats (Wistar strain, 180–250 g) were fasted for 24 h prior to the isolation of cortical tubules. Stevioside was purified from dried *Stevia rebaudiana* leaves as described previously[9]. Steviol was obtained from stevioside by enzymatic hydrolysis with pectinase (poly [1,4-α-D-galacturonide]-glycano-hydrolase; EC 3.2.1.15) as described by Mosettig and Nes[11]. Glucosilsteviol, on the other hand, was obtained by digestion of steviolbioside with the gastric juice of the marine snail *Megalobulimus paranaguensis* (M.L. Ferraresi et al., unpublished). Isosteviol and steviolbioside were prepared from stevioside by strong acid and strong base hydrolysis, respectively[10]. Glucose was assayed enzymatically with hexokinase (EC 2.7.11) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49)[12]. Oxygen uptake was measured polarographically[13]. Protein was determined according to Lowry et al.[14].

Results. The formulae of the five compounds investigated in this work are shown in figure 1. As can be seen, the number of glucose molecules attached to the aglucone moiety decreases

Experientia 41 (1985), Birkhäuser Verlag, CH-4010 Basel/Switzerland

along the sequence stevioside (three glucose molecules), steviolbioside (two glucose molecules), glucosilsteviol (one glucose molecule) and steviol or isosteviol (no glucose). As previously shown, the ability to inhibit mitochondrial functions increases along the same sequence[6,7].

Figure 2 summarizes the results of 38 experiments in which isolated rat renal tubules were incubated in Krebs/Henseleit-bicarbonate buffer in the presence of L-lactate (10 mM) and pyruvate (1 mM) and variable concentrations of the compounds listed in figure 1. The results obtained were plotted against the concentration of each compound. The mean control value for glucose production was 80.8 ± 4.3 nmol/h/mg protein. As previously shown, the glycogen content of renal tubules from



Steviol: R = R' = H
Glucosilsteviol: R = H; R' = glucose
Steviolbioside: R = H; R' = sophorose
Stevioside: R = glucose; R' = sophorose

Isosteviol

Figure 1. Structural formulae of steviol, isosteviol, glucosilsteviol, steviolbioside and stevioside.

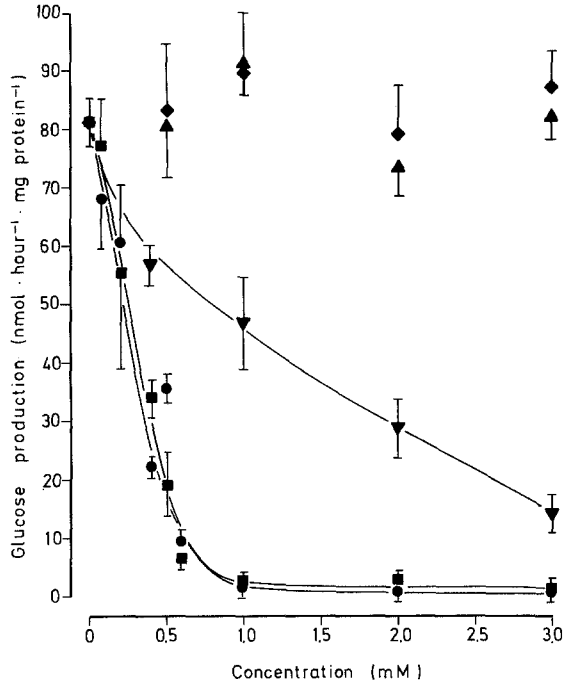


Figure 2. Effect of steviol, isosteviol, glucosilsteviol, steviolbioside and stevioside on glucose production of isolated renal cortical tubules. Renal cortical tubules were isolated from 24 h fasted rats and incubated (7 to 15 mg protein/ml) in Krebs/Henseleit-bicarbonate buffer (pH 7.4), at 37 °C, under an atmosphere of oxygen and carbon dioxide (95:5%). After a short pre-incubation period (usually 5 min), drugs were added, followed by 10 mM L-lactate and 1 mM pyruvate. Samples were collected at zero time and after 60 min of incubation, deproteinized with an equal volume of ice cold perchloric acid solution (0.5 N), centrifuged and the supernatant used for glucose assay. Legends: isosteviol, ■—■; steviol, ●—●; glucosilsteviol, ▼—▼; steviolbioside, ◆—◆; stevioside, ▲—▲. Vertical bars represent mean SE.

starved rats is insignificant, and measurement of glucose liberation is a good approximation for the gluconeogenetic activity[8]. Figure 2 shows that steviol and isosteviol inhibit glucose production. 50% inhibition takes place at 0.3 mM steviol or isosteviol. At 1 mM, inhibition is virtually complete. Differences in the chemical structure of the two compounds seem not to play a significant role. Additional experiments in which glucose production was followed during 90 min and measured at 15-min intervals revealed that inhibition is independent of the incubation time. Inhibition is also independent of the gluconeogenetic precursor. Gluconeogenesis from fructose, glycerol and succinate is also inhibited by steviol and isosteviol, the degree of inhibition being comparable to that found when L-lactate and pyruvate were the gluconeogenetic precursors.

Among the glucosides, glucosilsteviol also inhibits glucose production. As can be deduced from figure 2, 50% inhibition occurs at concentrations around 1.2 mM, a much higher value when compared to steviol or isosteviol. Stevioside and steviolbioside up to 3 mM, on the other hand, do not significantly affect gluconeogenesis.

Oxygen uptake was measured with 10 mM pyruvate as exogenous substrate. An inhibition of respiration was found with steviol and isosteviol. The concentration which produced 50% inhibition of oxygen uptake was 0.4 mM. Stevioside and steviolbioside up to 3 mM, on the other hand, were without effect on oxygen uptake.

Discussion. The simultaneous inhibition of glucose production and respiration by steviol, isosteviol and glucosilsteviol is consistent with the inhibitory action of these compounds on oxidative phosphorylation and electron transport in isolated intact mitochondria[6,7]. In this respect they behave like atractyloside, a potent inhibitor of adenine nucleotide exchange and of oxidative phosphorylation in mitochondria[6,17]. In the intact cell, atractyloside and its analogues inhibit gluconeogenesis and respiration, the same effect which was observed in this work with steviol and its analogues[15]. It should be noted that, chemically, steviol and the aglycone moiety of atractyloside (atractyligenin) differ only in few details[7]. This seems to justify their similar action on mitochondria and intact cells, but whereas atractyloside is more active than atractyligenin, the opposite occurs with stevioside and its aglycone[6,7]. In fact, steviol (and isosteviol) is more active than stevioside and the other glycosides in isolated mitochondria[6,7] as well as in intact cells. Stevioside and steviolbioside are indeed without effect on oxygen uptake and gluconeogenesis in isolated renal tubules. In isolated mitochondria, however, they are active. Oxidative phosphorylation is 50% inhibited by 1 mM stevioside and 0.5 mM steviolbioside[6,7]. In addition to this, steviolbioside also inhibits NADH-oxidase with a K_i of 0.6 mM[7]. These concentrations are well below the maximal ones used in this work (3 mM) and consequently, in the intact cell, one would expect some effect on respiration and gluconeogenesis. It may be that stevioside and steviolbioside do not freely permeate the cell membrane. This possibility seems more plausible if one remembers that disaccharides and glycosides – stevioside and steviolbioside have a disaccharide (sophorose) in their structure – frequently do not freely permeate the cell membrane. Sucrose is the most common exlample[18], but even atractyloside and carboxyatractyloside, as concluded by Stubbs et al.[15], permeate the cell membrane at a low rate. No data concerning the transport of stevioside and related glycosides across the cell membrane are available, however; further studies are required on this point.

The principal question which arises from the results described in this work is related to the hypoglycemic action of *Stevia rebaudiana* aqueous extracts[4,5]. It is generally accepted that inhibition of gluconeogenesis leads to hypoglycemia. Phenformin[8], 3-mercapto-picolinate and other indole and quinoline derivatives[16], are examples of substances which inibit gluconeogenesis in vitro and cause hypoglycemia in vivo. Moreover,

poisoning by atractyloside, whose mechanism of action resembles that of steviol in some respects, leads to hypoglycemic convulsions[17]. In isolated perfused rat liver, on the other hand, steviol (but not stevioside) also inhibits gluconeogenesis and respiration (Ishii et al., unpublished). This observation proves that the effect of *Stevia rebaudiana* natural products is not restricted to a single tissue. It is thus possible that inhibition of gluconeogenesis plays a significant role in the mechanism of action of *Stevia rebaudiana* natural products.

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0014-4754/85/010055-03$1.50 + 0.20/0

Drosophila melanogaster aldehyde dehydrogenase

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Summary. Subcellular fractionation by differential centrifugation confirms the presence of aldehyde dehydrogenase in *D. melanogaster*. It is found principally in the heavy mitochondrial fraction.
Key words. Drosophila melanogaster; aldehyde dehydrogenase; mitochondria.

Drosophila melanogaster is a species frequently found in environments characterized by a high alcohol concentration, and presumably by the presence of some acetaldehyde as well. Both ethanol and acetaldehyde can be dangerous if their concentration is to high, but at lower concentrations they attract the flies and the larvae[3-8]; indeed, both ethanol and acetaldehyde can be used as energy source[8-12]. As it is highly dangerous, the acetaldehyde resulting from oxidation of ethanol by alcohol dehydrogenase (ADH) or catalase must be rapidly metabolized into non toxic products. In most animal species, it is converted into acetate by aldehyde dehydrogenase (ALDH), an NAD^+ dependent enzyme[13-15]. It is commonly assumed that *Drosophilae* do not have any ALDH and that aldehyde oxidase (AO) plays the major role in acetaldehyde conversion[16,17]. Several authors have recently questioned this almost exclusive role attributed to AO[18-20]. Experimental data are in favor of an NAD^+ dependent conversion of acetaldehyde[21-23]. But other authors have a quite different opinion: they consider that 'ADH not only catalyzes the oxidation of ethanol into acetaldehyde, but additionally catalyzes the oxidation of this highly toxic product into acetate'[24]. In an attempt to detect a presumed aldehyde dehydrogenase, and in order to avoid all the difficulties and confusions which could result from the presence of aldehyde oxidase or alcohol dehydrogenase, we chose as our biological material the *bAdh*^{n4} strain (kindly made available by R. Sofer) which lacks ADH as well as AO[24]. It is known that larvae of this strain are attracted by acetaldehyde at low concentrations, as are the larvae of the *Adh*^{n2} strain[7,8].

Flies of the *bAdh*^{n4} strain were submitted to homogenization and to subcellular fractionation by differential centrifugation, according to a method originally used by De Duve et al. for rat liver[25]. Five fractions were isolated. First, a nuclear fraction (N) was separated from a total cytoplasmic extract (E). From the cytoplasmic extract, four fractions were isolated; a heavy mitochondrial fraction (M), a light mitochondrial fraction (L), a microsomal fraction (P), and a final supernatant (S). The activities of nine enzymes were assayed. Of course, our main interest was in aldehyde dehydrogenase, and also in aldehyde oxidase, alcohol dehydrogenase, and catalase. As reference enzymes we used cytochrome *c* oxidase and malate dehydrogenase for mitochondria, acid phosphatase and beta-galactosidase for lysosomes, and NADPH cytochrome *c* reductase for endoplasmic reticulum.

Our main observations can be summarized as follows:

1. The specific activities of reference enzymes, as compared with similar data obtained for rat liver by the same methods, show a much higher value for NADPH cytochrome *c* reductase, a similar value for cytochrome *c* oxidase, and distinctly lower values for lysosomal and peroxisomal enzymes (fig. 1).

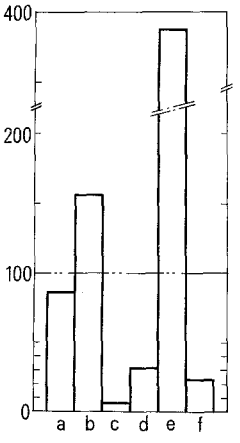

Figure 1. Reference enzymes specific activities: *a* cytochrome *c* oxidase, *b* malate dehydrogenase, *c* acid phosphatase, *d* β-galactosidase, *e* NAPDH cytochrome *c* reductase, *f* catalase. Ordinate: enzymatic activity in *Drosophila* fly, in percent of specific activity for the same enzyme in Rat liver.