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INHIBITION OF MONOSACCHARIDE TRANSPORT IN THE INTACT RAT LIVER BY STEVIOSIDE

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Abstract—The transport and metabolism of D-glucose and D-fructose in the isolated perfused rat liver and the influence of stevioside and its derivatives were investigated. The transport parameters were measured by the multiple indicator dilution technique. The maximal exchange rate of D-glucose was 700 μmol·min^{-1}·ml^{-1} and the K_m was 38 mM. Stevioside and its derivatives (isosteviol and steviolbioside) inhibited D-glucose and D-fructose transport across the cell membrane. The half-maximal effect at 1 mM D-glucose occurred at 0.8 mM stevioside. The inhibitory action of stevioside was of mixed type. Isosteviol was more potent than stevioside (half-maximal effect at 0.4 mM), whereas steviolbioside was less active (50% inhibition at 2.5 mM). Stevioside was without effect on D-glucose metabolism, except for transient changes in D-glucose release, reflecting changes in the intracellular concentration. D-Fructose consumption, however, was specifically affected (half-maximal effect at 2.8 mM), as well as all parameters depending on D-fructose transformation (D-glucose production, L-lactate and pyruvate production, and extra oxygen uptake). In livers releasing D-glucose from endogenous glycogen, strong inhibition of transport increased the intracellular to extracellular D-glucose concentration ratio (C_i/C_e). The control values of C_i/C_e, representing an average over the total intracellular water space, were always smaller than unity. The latter observation may indicate that D-glucose does not have access to the whole intracellular water space.

Stevioside is a glycoside which exists in large amounts in the leaves of *Stevia rebaudiana*, a Compositae shrub from Paraguay and Brazil. It is composed of steviol, a diterpenic carboxylic alcohol, and three glucose molecules (Fig. 1) [1, 2]. Stevioside is 300 times sweeter than sucrose and is presently employed as a non-caloric sweetener. Aqueous infusions of *S. rebaudiana* leaves are very popular, and a new branch of industry, dedicated to the extraction and purification of stevioside and other *S. rebaudiana* natural products for commercial purposes, is growing up rapidly in Japan, Brazil, and other countries.

The acute and subacute toxicity of stevioside has been investigated extensively [3–5]. From the results of these investigations it may be concluded that stevioside presents low toxicity in mammals. On the other hand, the effects of stevioside and other natural products of *S. rebaudiana* on metabolic fluxes, either *in vivo* or in intact cell systems, have been the subject of relatively few investigations. Well demonstrated are the effects of stevioside and other *S. rebaudiana* components on oxidative phosphorylation in isolated mitochondria. The first experiments were done by Vignais *et al.* [6]. More recently, Kelmer Bracht *et al.* [7] confirmed in our laboratory the earlier observations of Vignais *et al.* [6] and added new data about the action of *S. rebaudiana* natural products on isolated rat liver mitochondria. The latter compounds inhibit oxidative phosphorylation by a complex mechanism which includes uncoupling of phosphorylation, inhibition of the adenine nucleotide carrier, and several other effects [6, 7]. Yamamoto *et al.* [8] measured the effects of *S. rebaudiana* components on oxygen consumption and gluconeogenesis in isolated rat renal tubules. They found inhibition of both variables by steviol, isosteviol and glucosylsteviol, but stevioside was inactive.

The inability of stevioside to inhibit energy metabolism in intact cells, suggested by the experiments of Yamamoto *et al.* [8], was confirmed by us in the

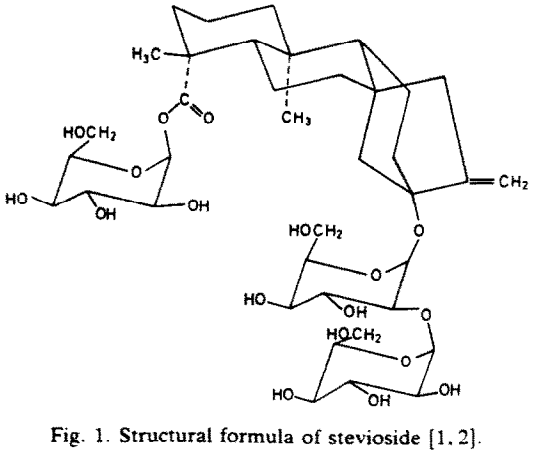

Fig. 1. Structural formula of stevioside [1, 2].

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isolated perfused rat liver, but at the same time we found that this compound inhibited the monosaccharide carrier of the plasma membrane. This carrier is responsible for the transport of D-glucose, D-fructose and D-galactose across the plasma membrane [9, 10]. The present article describes and discusses the experiments which were done in order to characterize this effect. D-Glucose, which is only slowly transformed by the isolated perfused rat liver, was utilized as the model substance [10], and the transport was measured by the multiple-indicator-dilution technique [10, 11]. Parameters of the transport of D-glucose across the liver cell membrane were also investigated, as well as the consequences of the transport inhibition for the metabolism of D-fructose. Our results are compatible with earlier reports, based on *in vivo* experiments, on the effects of stevioside and other *S. rebaudiana* components on carbohydrate metabolism [12–15].

MATERIALS AND METHODS

Hemoglobin-free liver perfusion. Male albino rats (Wistar strain; 220–300 g) received a standard laboratory diet (Purina) and water *ad lib.* prior to the surgical removal of the liver under pentobarbital anesthesia. For the measurement of D-fructose metabolism the rats were starved for 24 hr. The perfusion technique described by Scholz *et al.* [16] was used. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer, pH 7.4, saturated with an oxygen/carbon dioxide mixture (95/5). It contained bovine serum albumin (25 mg/100 ml) and, when required, 0.5 to 5.0 mM stevioside. D-Glucose, D-fructose, isosteviol, steviolbioside and sodium atractylate were infused into the perfusion fluid, and the final concentrations were calculated from perfusate flow and infusion rate. The fluid was pumped through a temperature-regulated (37°) membrane oxygenator prior to entering the liver via a cannula inserted in the portal vein. The effluent perfusate flowed past an oxygen electrode before being discarded or, in pulse experiments, collected by a fraction collector. The flow rate was adjusted to the metabolic activity of the liver as judged from the venous oxygen concentration. It varied between 4 and 5 $ml \cdot min^{-1} \cdot g^{-1}$, but it was constant in each individual experiment.

Pulse-labeling experiments. Pulse-labeling was performed by injection of labeled substances into the perfusate immediately before entering the liver. Up to three substances were dissolved in 200 μl perfusion fluid and were injected within less than 0.5 sec employing a microsyringe. The following combinations of substances were studied: (a) [^{14}C]sucrose (0.5 μCi), [^{32}P]phosphate (0.1 μCi), [^{3}H]water (10 μCi); (b) [^{14}C]glucose (0.5 μCi), [^{32}P]phosphate, [^{3}H]water; (c) [^{32}P]phosphate, [^{3}H]water and D-fructose (4.5 μmol). The effluent perfusate was collected in 0.5-sec fractions over a period of 90 sec following injection by means of a specially designed fraction collector [11, 17]. Up to four pulse experiments were performed consecutively with one liver over a period between 20 and 80 min of perfusion. When the experimental conditions were changed (e.g. infusion of substrates or inhibitors), at least 5 min passed before the next pulse experiment was performed.

Analytical. Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for D-glucose, L-lactate and pyruvate by standard enzymatic procedures [18]. D-Fructose was assayed colorimetrically by a modification of the procedure originally described by Roe [19]. The samples were pipetted into the reaction medium (10 N HCl and 9 mM resorcinol) and heated to 60° for 90 min. Stevioside hydrolysis, under these conditions, was minimized. Color development due to D-glucose (from the metabolic activity of the liver or from hydrolysis of stevioside) was less than 1% in all tests, as shown by control measurements. Stevioside was determined by the colorimetric procedure described by Dubois *et al.* [20] for total sugars (phenol-sulfuric reagent). Corrections were made for the exclusion of the interference due to free D-glucose.

The oxygen concentration in the perfusate was monitored continuously, employing a teflon-shielded platinum electrode. Metabolic rates were calculated from arterio-venous differences and the flow rate and were referred to the wet weight of the liver.

The radioactivity of the injected substances in each of the fractions of the effluent perfusate was measured by liquid scintillation spectroscopy (three channel spectrometer, Beckman LS 6800). The following scintillation solution was used: toluene/ethanol (2/1) containing 5 g/l PPO (2,5-diphenyloxazole) and 0.15 g/l POPOP [2,2'-*p*-phenylene-bis(5-phenyloxazole)].

Materials. The liver perfusion apparatus and the fraction collector for pulse-labeling experiments were built in the workshops of the University of Maringá. The platinum electrode was built in the workshops of the Institute of Physiological Chemistry, the University of Munich, and was a gift from Dr. Roland Scholz. Sodium atractylate, bovine serum albumin, and all enzymes and coenzymes used in the metabolite assays were products of Sigma (St. Louis, MO, U.S.A.). The reagent grade chemicals were from Merck (Darmstadt, F.R.G.), Carlo Erba (São Paulo, Brazil), and Reagen (Rio de Janeiro, Brazil). Stevioside was extracted and purified from dried *S. rebaudiana* leaves as described elsewhere [21]. Isosteviol was obtained by strong acid hydrolysis and steviolbioside by strong base hydrolysis of stevioside, essentially as described by Kohda *et al.* [22].

The following radiochemicals were purchased from New England Nuclear (Boston, MA, U.S.A.): [U-^{14}C]sucrose (NEC-100, 4 Ci/mol), D-[1-^{14}C] glucose (NEC-043, 8 Ci/mol) and [^{3}H]water (NET-001C, 25 mCi/g). Carrier-free [^{32}P]sodium phosphate was purchased from the Comissão Nacional de Energia Nuclear (São Paulo, Brazil).

Computations. The outflow profiles of the labeled D-glucose in the effluent perfusate were analyzed, employing a mathematical model of the liver which was originally proposed by Goresky and Nadeau [10]. Its application to the erythrocyte-free perfused rat liver was discussed previously [11]. According to this model, the outflow profiles of labeled D-glucose ($Q(t)$) may be described by a function which contains the outflow profile of a reference substance ($Q_{ref}(t)$)

representing the heterogeneity of the sinusoidal transit times (T), the transit time in the large vessels (t_0), the ratio of intracellular to extracellular volumes of the liver (θ'), and the parameters for the transport of labeled D-glucose across the plasma membrane of the liver cells (k_1 and k_2):

$$Q(t) = Q_{ref}(t)\exp(k_1\theta'(t - t_0)) + \exp(-k_2(t - t_0)) \times \int_0^{t-t_0} \exp((-k_1\theta' + k_2)) \times Q_{ref}(T + t_0) \times \sum_{n=1}^{\infty} \frac{(k_1k_2\theta'T)^n(t - t_0 - T)^{n-1}}{n!(n-1)!}\,dt \quad (1)$$

Adequate reference substances ($Q_{ref}(t)$) are materials expected to freely enter the Disse space, to penetrate the cell surface, but not to enter the cells. The transit time in the large vessels (t_0) and the ratio of intracellular to extracellular water spaces of the liver (θ') can be derived from the superposition of the outflow profiles of extracellular reference substances (e.g. [^{14}C]sucrose or [^{32}P]phosphate) and [^{3}H]water following a linear transformation proposed by Goresky and Nadeau [10]:

$$Q_{transformed}(t) = \left[\frac{1}{1+\theta'}\right] \cdot Q\left(\frac{t - t_0}{1+\theta'} + t_0\right)_{measured} \quad (2)$$

The rate constants for uptake ($k_1\theta'$) and release (k_2) of D-glucose are derived by fitting equation 1 to the experimental data, employing the numerical procedures for calculating the theoretical outflow profiles described elsewhere [23]. $k_1\theta'$ is related to the vascular space and k_2 to the cellular space, whereas k_1, which is calculated from $k_1\theta'$, has the same dimensions as those of k_2. The computation also provides a resolution of the theoretical profile into its components, i.e. the portion of labeled D-glucose injected that did not enter the cellular space (throughput, first term in equation 1) and the portion which exchanged at least once with the liver cells (second term in equation 1).

The radioactivity in the perfusate ($Q(t)$, $Q_{ref}(t)$, etc.) was expressed as the function of total radioactivity of each labeled substance injected that was recovered per second. The time integrals and the mean transit times [24] of the outflow profiles were determined by the trapezoid rule with exponential extrapolation. Parameter fitting was performed by the procedure of least squares [25] (for further details see Refs. 11 and 23). Experimental errors are given as ± standard errors. The Cyber 175 Computer (Control Data Corp.) at the Leibniz Rechenzentrum (Bayerische Akademie der Wissenschaften, Munich) was employed for all computations. The computer programs were written in FORTRAN using subroutines from the library of the Leibniz Rechenzentrum and from International Mathematical and Statistical Libraries (Houston, TX).

RESULTS

Outflow profiles of indicator substances. The outflow profiles of labeled substances injected into the portal vein will be distorted according to the events inside the liver (Fig. 2). The least distortion will be observed with substances, such as [^{14}C]sucrose, which do not exchange significantly with the cellular space during a single passage. Since water exchanges very rapidly with the intracellular spaces, its outflow profile was distorted to a greater extent (Fig. 2). As shown previously by Bracht *et al.* [26], labeled phosphate, at the relatively high flow rate of 4–5

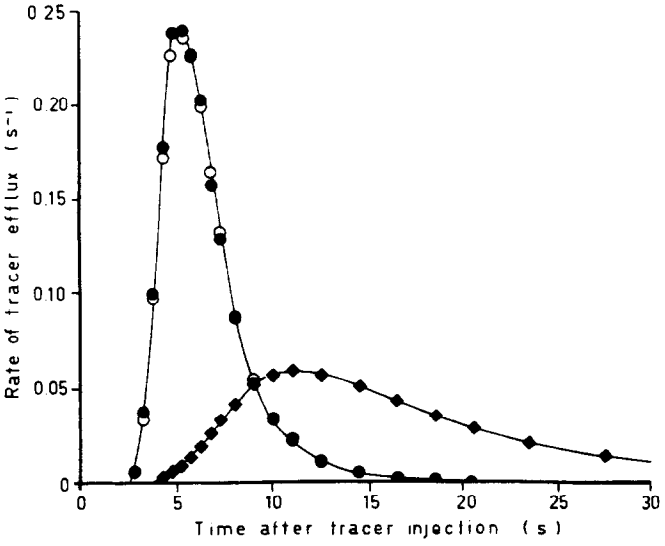

Fig. 2. Outflow profiles of [^{32}P]phosphate, [^{14}C]sucrose and [^{3}H]water. The liver from a fed rat was perfused in a nonrecirculating system with Krebs/Henseleit-bicarbonate buffer (pH 7.4). Trace amounts of [^{32}P]phosphate (●——●), [^{14}C]sucrose (○——○) and [^{3}H]water (◆——◆) were injected simultaneously into the perfusate prior to entering the liver. The fractions of the injected amount which appeared in the effluent perfusate per second were plotted versus the time following injection.

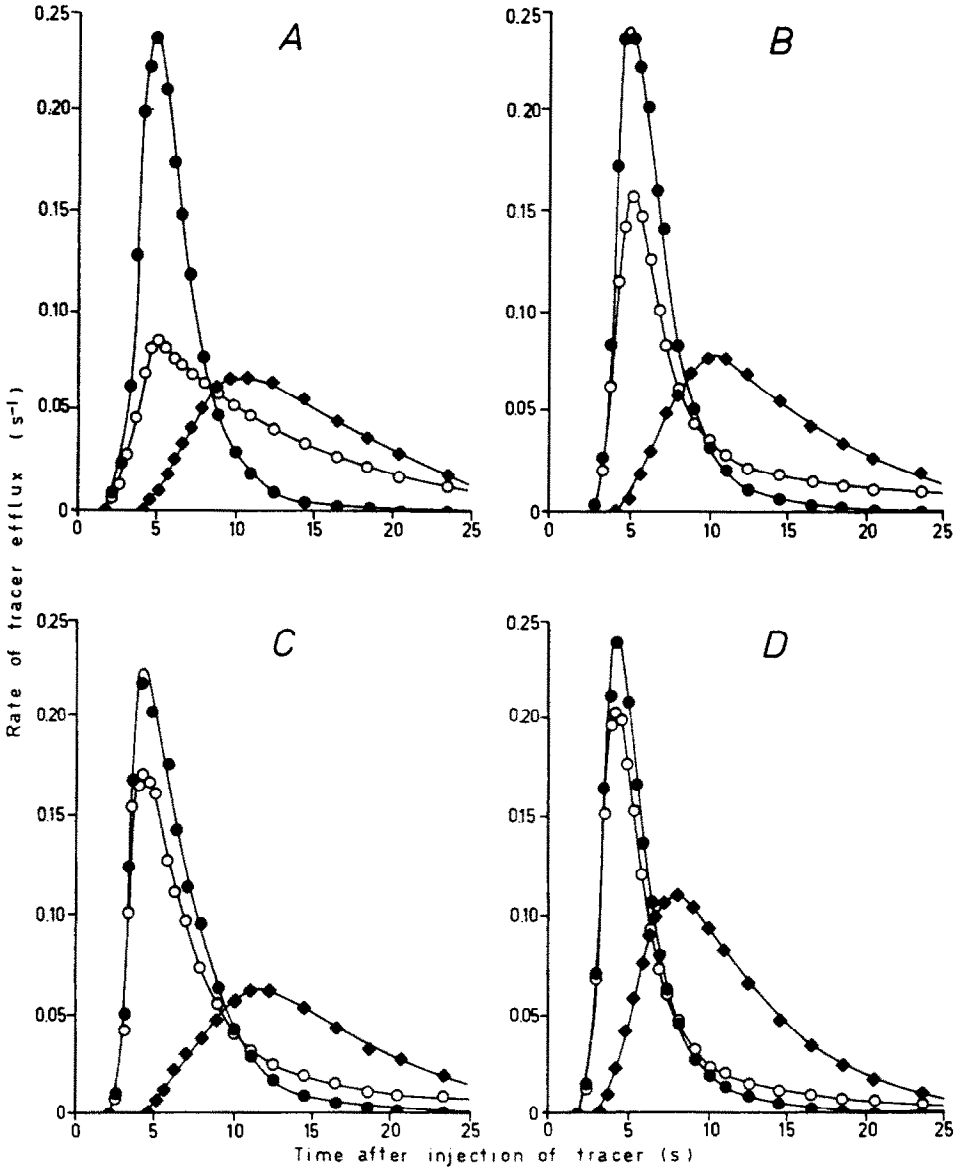

Fig. 3. Outflow profiles of [^{14}C]glucose and indicator substances: Effects of stevioside and isosteviol. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing D-glucose (0.9 to 1.2 mM). In each pulse-labeling experiment, trace amounts of D-[^{14}C]glucose (○—○), [^{32}P]phosphate (●—●) and [^{3}H]water (◆—◆) were simultaneously injected. The fractions of the injected radioactivity which appeared in the effluent perfusate per second were plotted versus the time following injection. Key: (A) control; (B) 1.5 mM stevioside; (C) 2.5 mM stevioside; and (D) 1.0 mM isosteviol.

ml·min^{-1} ·(g liver)$^{-1}$, shows outflow profiles that are indistinguishable from that of labeled sucrose, an observation with was confirmed by the experiment shown in Fig. 2. Since a negligible amount of [^{32}P]phosphate is taken up by the liver during a single passage, this compound may be used in place of labeled sucrose as a reference for the extracellular space. The injection of three different isotopes, which may be easily discriminated in a three-channel spectrometer, avoids laborious separation procedures. For this reason, [^{32}P]phosphate was used as the extracellular reference in the subsequent pulse experiments.

Effects of stevioside and its derivatives on the outflow profiles of D-[^{14}C]*glucose.* Figure 3 shows the results of four typical D-[^{14}C]glucose pulse experiments performed in the absence (A, control) and in the presence of 1.5 mM stevioside (B), 2.5 mM stevioside (C) and 1.0 mM isosteviol (D). The control experiment (Fig. 3A) shows that the position of the [^{14}C]glucose outflow profile was intermediate between that of [^{32}P]phosphate and [^{3}H]water. This reflects the fact that D-glucose exchanges rapidly with the intracellular space, but a portion seems to pass through the liver without exchanging. The areas under the D-glucose profiles are near 1.0, indicating

that the amount of radioactivity retained by the liver was negligible. Under the perfusion conditions of the experiments shown in Fig. 3 (livers from fed rats, 0.9 to 1.2 mM D-glucose in the perfusate), the livers were releasing D-glucose derived from endogenous glycogen (1.2 to 1.8 μmol·min^{-1}·g^{-1}) and were glycolyzing and respiring at the expense of endogenous substrates. Under such conditions, the [^{14}C]lactate, [^{14}C]pyruvate or $^{14}CO_2$ production amounted to less than 1% of the injected radioactivity.

The form of the D-glucose outflow profile changed when stevioside or isosteviol was present in the perfusion fluid. The ratio of the peak values of [^{32}P]phosphate to [^{14}C]glucose, which was 2.6 in the control experiment, decreased to 1.5 at 1.5 mM stevioside, to 1.3 at 2.5 mM stevioside, and to 1.15 at 1.0 mM isosteviol. Thus, the D-glucose outflow profile tended to approach that of the extracellular reference, an indication that the rate of exchange of the former compound with the intracellular space is decreased by stevioside and isosteviol [10, 11].

Parameters of D-glucose transport in the isolated perfused rat liver. The quantitative evaluation of the experiments in Fig. 3 can be performed by means of the mathematical model proposed by Goresky and Nadeau [10]. As shown in Fig. 4, the theoretical curves obtained with constants (k_1 and k_2) with the highest probability are in good agreement with the measured outflow profiles, indicating that the model is a good approximation of the phenomena following a pulse injection of labeled D-glucose. Figure 4A shows a control experiment and Fig. 4B a pulse-labeling experiment performed in the presence of 2.5 mM stevioside. The portions of the labeled D-glucose injected which did not enter the cellular space (throughput; dotted lines) were also computed. It may be deduced from Fig. 4 that stevioside increased the throughput. It passed from 23% in the control experiment to 70% in the presence of 2.5 mM stevioside. This was a consequence of the inhibition of D-glucose exchange with the intracellular space caused by stevioside.

Table 1 summarizes the results of eight liver perfusion experiments and 18 pulse-labeling experiments performed with variable stevioside, isosteviol and steviolbioside concentrations and with a D-glucose concentration around 1 mM. In each perfusion experiment, a control injection was performed first. After the control, stevioside, isosteviol or steviolbioside was infused. In the case of Expt. 6, the concentration of stevioside was increased stepwise. At each step, a pulse-labeling experiment was performed. The D-glucose concentration in the perfusate shown in Table 1 is the arithmetic mean of the influent and effluent concentrations. The effluent D-glucose concentrations are higher than the influent concentrations because of the metabolic activity of the liver.

Table 1 shows several transport parameters derived from the calculations. For an average D-glucose concentration of 1.08 ± 0.06 mM, in eight control experiments, k_1 was equal to 0.240 ± 0.028 ml perfusate·sec^{-1}·(ml intracellular water space)$^{-1}$, and k_2 was equal to 0.402 ± 0.073 ml intracellular phase·sec^{-1}·(ml intracellular space)$^{-1}$. Since the extracellular concentration is known, the

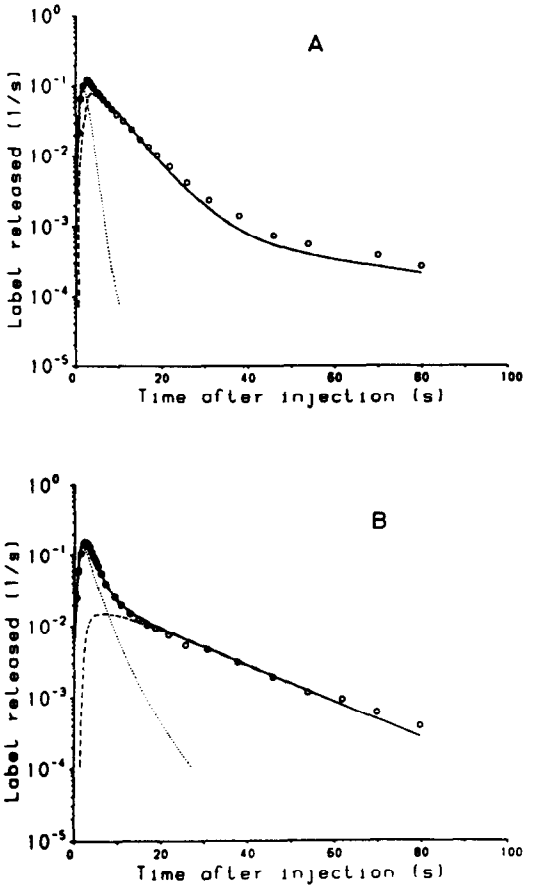

Fig. 4. Experimental and theoretical outflow profiles of D-[^{14}C]glucose. The pulse-labeling experiments were performed as shown in Fig. 3 in the absence (A) and presence (B) of 2.5 mM stevioside. The fractions of the injected [^{14}C]glucose which appeared in the effluent perfusate per second were plotted on a logarithmic scale versus the time following injection, corrected for t_0 (experimental data, ○). The solid lines represent the theoretical profiles computed with the following parameters [23]: (A) $k_1\theta' = 0.510\,sec^{-1}$, $k_2 = 0.497\,sec^{-1}$ and (B) $k_1\theta' = 0.093\,sec^{-1}$, $k_2 = 0.090\,sec^{-1}$. The resolution of the theoretical profiles into their components, throughput (.....) and exchanged tracer (----), is shown.

unidirectional influx rates may be calculated from the concentrations and the computed rate constants for influx. For 1.08 mM D-glucose in the perfusate, the unidirectional influx rate was 9.26 ± 1.0 μmol·min^{-1}·(g liver)$^{-1}$ [16.7 μmol·min^{-1}·(ml intracellular water space)$^{-1}$]. The mean net flux across the cell membrane, as revealed by the measurement of D-glucose release, was 1.5 μmol·min^{-1}·(g liver)$^{-1}$. Thus, the unidirectional flux was approximately six times greater than the net flux under these experimental conditions.

Isosteviol, the aglycone obtained from the total acid hydrolysis of stevioside [22], decreased the rate of D-glucose exchange. The unidirectional influx rate was decreased 92% by 2.0 mM isosteviol. A half-maximal effect, evaluated by numerical inter-

Table 1. Parameters of D-glucose transport across the plasma membrane in the isolated perfused rat liver and the effects of isosteviol, steviolbioside and stevioside

Expt.	Perfusate concentrations (mM)				Rate constants		Unidirectional influx rate (μmol·min^{-1}·g^{-1})	Inhibition of influx (%)	C_i/C_e calculated from rate constants and net flux
	D-Glucose C_e	Stevioside	Steviolbioside	Isosteviol	Influx k_1 (sec^{-1})	Efflux k_2			
1	1.15			0	0.213	0.367	8.60	0	0.65
	1.2			0.5	0.087	0.164	3.71	56	0.92
2	1.1			0	0.289	0.536	10.10	0	0.60
	1.2			1.0	0.056	0.142	2.20	78	0.72
3	1.1			0	0.230	0.390	8.80	0	0.66
	1.1			2.0	0.022	0.053	0.70	92	1.18
4	1.15			0	0.237	0.378	9.80	0	0.70
	1.2			1.0	0.069	0.127	2.90	70	0.89
5	1.0		0		0.232	0.380	11.05	0	0.73
	1.0		2.5		0.115	0.210	5.51	50	0.77
6	1.0	0			0.276	0.497	9.30	0	0.65
	1.05	1.0			0.111	0.193	4.20	54	0.79
	1.0	2.5			0.062	0.090	1.95	79	1.28
	1.05	5.0			0.029	0.053	0.90	91	1.70
7	1.1	0			0.199	0.290	7.50	0	0.84
	1.0	1.5			0.072	0.106	2.60	66	1.12
8	1.0	0			0.247	0.376	8.90	0	0.78
	1.0	1.5			0.083	0.130	3.00	66	1.00
N = 8	1.08 ± 0.06				0.240 ± 0.028	0.402 ± 0.073	9.26 ± 1.0		0.70 ± 0.08

Data are from eight liver perfusion experiments and eighteen pulse-labeling experiments with different stevioside, steviolbioside and isosteviol concentrations in the perfusate. The pulse-labeling experiments were similar to those shown in Fig. 3. The perfusate concentrations are the arithmetic means of influent and effluent concentrations. The rate constants for uptake (k_1) and release (k_2) were obtained by fitting the model proposed by Goresky and Nadeau [10] to the measured outflow profiles [23]. The unidirectional influx rates were calculated from k_1 and the extracellular concentration of D-glucose (C_e) and expressed as μmol·min^{-1}·(g liver wet weight)$^{-1}$. The extracellular volume and the total aqueous space of the liver were calculated from the mean transit times of [^{32}P]phosphate and [^{3}H]water respectively [24]. The ratio of the intracellular to extracellular D-glucose concentration (C_i/C_e) was calculated as explained in the text.

Table 2. Dependence of D-glucose transport on D-glucose concentration: Effect of 2.5 mM stevioside

Expt.	Perfusate concn (mM)		Rate constants		Unidirectional influx rate (μmol·min^{-1}·g^{-1})	Inhibition of influx (%)	C_i/C_e calculated from:	
	D-Glucose C_e	Stevioside	Influx k_1 (sec^{-1})	Efflux k_2 (sec^{-1})			Rate constants	t
1	10	0	0.343	0.616	106.30	0	0.56	0.50
	10	2.5	0.078	0.132	25.12	76	0.63	0.60
2	20	0	0.201	0.313	135.30	0	0.64	0.59
	20	2.5	0.051	0.086	31.35	76	0.59	0.57
3	50	0	0.107	0.186	175.50	0	0.57	0.52
	50	2.5	0.036	0.072	56.97	68	0.50	0.50
4	100	0	0.096	0.146	303.00	0	0.66	0.65
	100	2.5	0.048	0.068	142.00	53	0.69	0.71
5	10	0	0.308	0.468	107.30	0	0.66	0.63
	10	2.5	0.050	0.100	17.66	84	0.50	0.50
6	20	0	0.165	0.265	110.20	0	0.62	0.62
	20	2.5	0.037	0.081	22.96	79	0.46	0.47
$N = 6$	10–100	0					0.62 ± 0.04	0.59 ± 0.06
	10–100	2.5					0.56 ± 0.08	0.56 ± 0.09

Data are from six liver perfusion experiments and twelve pulse-labeling experiments performed with different D-glucose concentrations. The pulse experiments were similar to those shown in Fig. 3. All calculations were performed as described in the legend of Table 1.

polation, can be expected at 0.4 mM isosteviol for an extracellular D-glucose concentration around 1 mM. Stevioside was less potent that isosteviol. A 91% decrease in the unidirectional influx rate occurred at 5.0 mM stevioside; half-maximal effect, for an extracellular D-glucose concentration of approximately 1 mM, would occur at 0.85 mM stevioside. Steviolbioside, which lacks the glucosyl residue involved in the ester bond (Fig. 1), was the least effective. When it was present at a concentration of

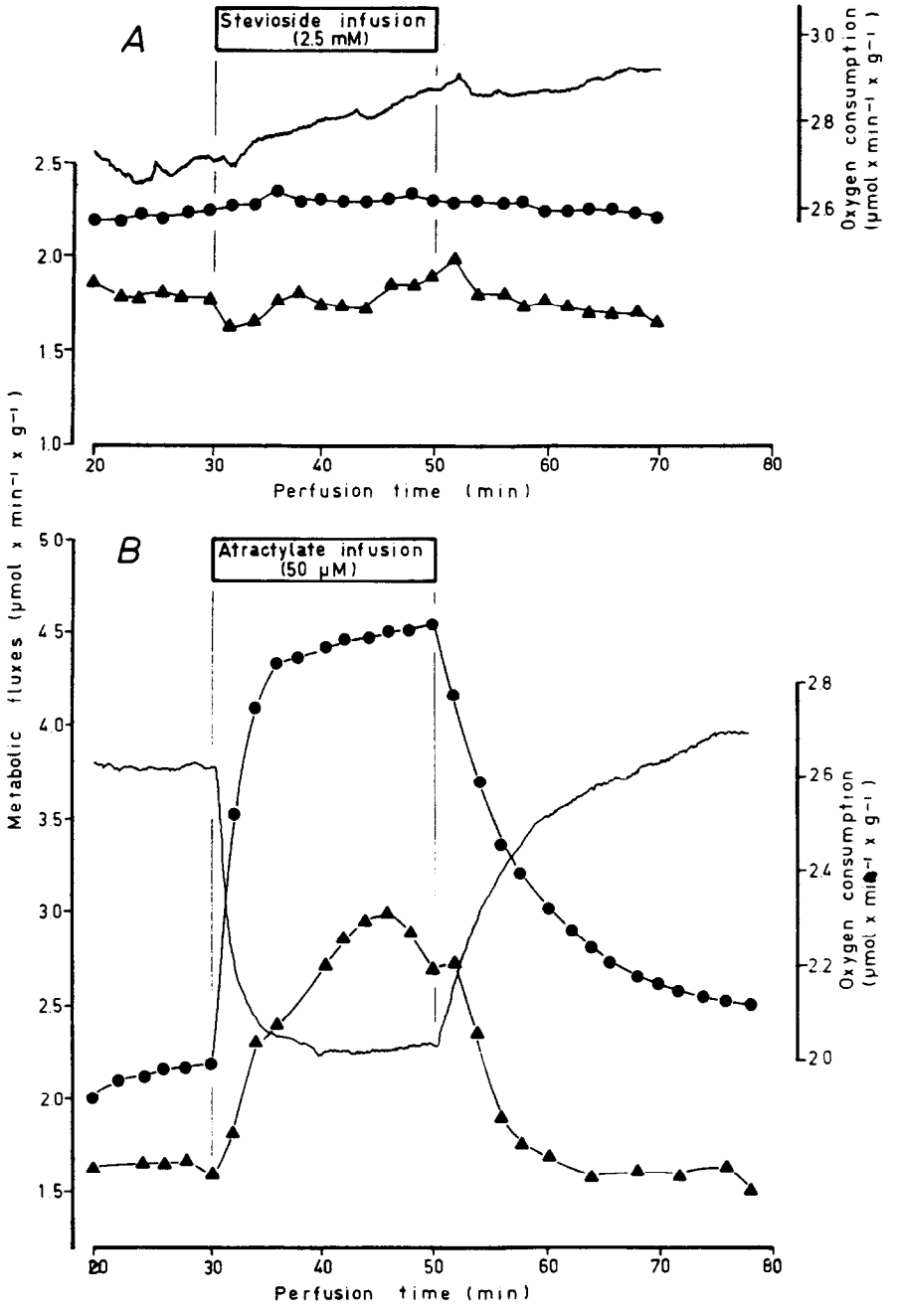

Fig. 5. Effects of stevioside and atractyloside on metabolic fluxes in the isolated perfused rat liver. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer in a non-recirculating system. In (A) stevioside (final concentration 2.5 mM) was infused into the perfusate prior to its entry into the liver at 30–50 min of perfusion; in (B) atractylate (final concentration 50 µM) was infused at 30–50 min of perfusion. The effluent perfusate was sampled in 2- or 4-min intervals. The concentrations of D-glucose, L-lactate and pyruvate were measured enzymatically. The oxygen concentration was monitored continuously employing a platinum electrode. Metabolic rates were calculated from the portal-venous differences and the constant flow rate and were expressed as $\mu mol \cdot min^{-1} \cdot (g\ liver\ wet\ weight)^{-1}$. Key: oxygen consumption (trace), D-glucose release (▲——▲), and L-lactate + pyruvate production (●——●).

2.5 mM, 50% inhibition of the unidirectional influx occurred.

Table 2 summarizes the results of another series of experiments in which the D-glucose concentration was varied in the range between 10 and 100 mM, and the stevioside concentration was constant and equal to 2.5 mM. With increasing D-glucose concentrations, the values of the rate constants for influx and efflux, k_1 and k_2, were decreased. This phenomenon reflects the saturation of the transport system, which has been shown to obey Michaelis–Menten kinetics [9]. Table 3 lists the kinetic constants K_m and V_{max} for D-glucose influx, obtained by fitting the Michaelis–Menten equation ($v_{in} = V_{max} \cdot C_e/(K_m + C_e)$) to the experimental data using the weighted least-squares procedure proposed by Wilkinson [27]. The K_m value for D-glucose exchange which gave the best fit was 38.9 ± 12 mM; the corresponding maximal rate was equal to 388 ± 61 $\mu mol \cdot min^{-1} \cdot g^{-1}$. Stevioside, when present at a concentration of 2.5 mM, changed both kinetic constants. The K_m was increased to 76.1 ± 40 mM and the maximal rate was decreased to 143 ± 61 $\mu mol \cdot min^{-1} \cdot g^{-1}$. This indicates that the inhibition caused by stevioside was of a mixed type.

Relationships between inhibition of D-glucose transport and D-glucose metabolism. Livers from fed rats release D-glucose from endogenous glycogen. An inhibition of the exchange of D-glucose with the intracellular space should not, in principle, affect glycogen breakdown. Figure 5A shows the results of

Table 3. D-Glucose concentrations for half-maximal rates of influx (K_m) and maximal rates of influx (V_{max}) in the absence (control) and in the presence of 2.5 mM stevioside

Condition	K_m (mM)	V_{max} ($\mu mol \cdot min^{-1} \cdot g^{-1}$)
Control	38.9 ± 12	388 ± 61
2.5 mM Stevioside	76.1 ± 40	143 ± 61

Data are derived from the experiments shown in Tables 1 and 2. The K_m and V_{max} values and the corresponding standard errors were obtained by fitting the Michaelis–Menten equation ($v_{in} = V_{max} \cdot C_e/(K_m + C_e)$) to the experimental data using the weighted non-linear least-squares procedure as proposed by Wilkinson [27].

a typical experiment in which the metabolic activity of a liver from a fed rat was measured in the absence of exogenous substrates. Glycolysis (L-lactate + pyruvate production), D-glucose release and oxygen consumption were measured and expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$. Upon 2.5 mM stevioside infusion, no significant alterations in the steady-state rates occurred. Since the perfusate samples were collected in 2-min intervals, however, rapid transient changes could have been obscured. The experiment in Fig. 6 provides a better time resolution of the effect of stevioside on D-glucose release. In this experiment, the perfusate was collected in 15-sec, 30-sec, 1-min

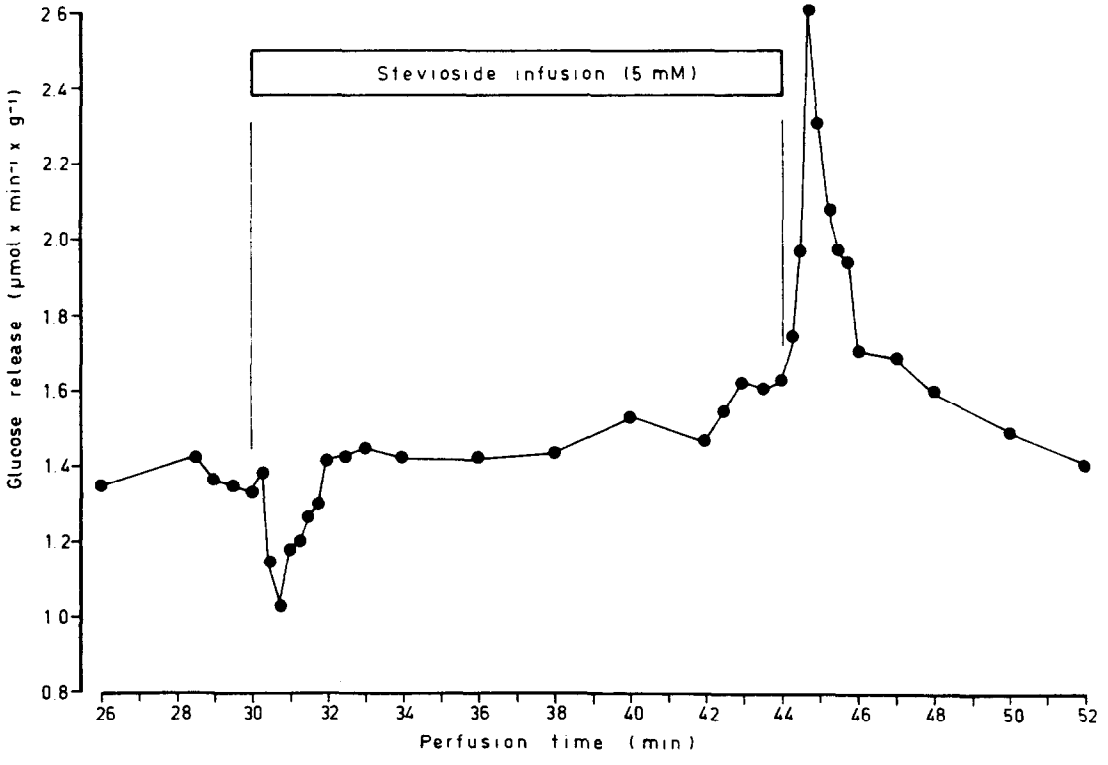

Fig. 6. Effect of stevioside on D-glucose release in the isolated perfused rat liver. A liver from a fed rat was perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) in a non-recirculating system. Stevioside (final concentration 5.0 mM) was infused at 30–44 min of perfusion. The effluent perfusate was sampled in 15-sec, 30-sec, 1-min and 2-min intervals. The concentration of D-glucose was measured and expressed as $\mu mol \cdot min^{-1} \cdot (g \text{ liver wet weight})^{-1}$.

and 2-min intervals as opposed to 2-min intervals in the experiment shown in Fig. 5. It may be seen that a transient decrease in D-glucose release occurred upon stevioside infusion. The same phenomenon, but in the opposite direction, took place when the stevioside infusion was stopped. The transient alterations did not exceed 2 min in the experiment shown in Fig. 6. In other experiments, depending on the flow rate, liver weight and metabolic activity, the phenomena were still evident 3 min after the onset/termination of stevioside infusion. Figure 6 shows the results obtained with 5 mM stevioside in the perfusate, but the transient phenomenon also occurred with 2.5 mM stevioside.

The above described phenomenon does not reflect stevioside hydrolysis by the liver because the latter compound is not metabolized. This was demonstrated by perfusion experiments in which stevioside was recirculated during 2 hr (not shown). Possible metabolic products were investigated by thin-layer (silica-gel) chromatography and by gas chromatography. The stevioside concentration, measured with the phenol-sulfuric reagent, remained constant over the entire recirculation period. All attempts to detect stevioside metabolites, especially hydrolysis products like steviolbioside, glucosylsteviol and steviol [21, 22], were unsuccessful.

Figure 5A also illustrates the observation that stevioside was without effect on energy metabolism. Since the compound inhibits the adenine nucleotide carrier in isolated rat liver mitochondria [6], an inhibition of oxygen consumption and an increase of the glycolytic activity can be expected. As shown in Fig. 5B, atractyloside, the classic inhibitor of the adenine nucleotide carrier, reversibly affected oxygen consumption and glycolysis. In addition to oxygen consumption and glycolysis, stevioside is also inactive on gluconeogenesis from both pyruvate and glycerol [28], two biosynthetic routes strictly dependent on energy in the form of ATP.

Intracellular D-glucose concentration. The transient decrease of the D-glucose release which occurred upon stevioside infusion may reflect an increase in the intracellular concentration. Similarly, the transient increase of D-glucose release, which followed the termination of stevioside infusion, may reflect the return of the system to the previous conditions. As revealed by the unidirectional influx rates in Table 1, at stevioside concentrations equal to or above 2.5 mM, the transport system was considerably inhibited and the unidirectional influx rate was now comparable to, or even smaller than, the net flux rate. Under such conditions, the unidirectional efflux rate may correspond to two or three times that of the influx, a situation which presupposes increased intracellular concentrations.

The ratio of the intra- to extracellular D-glucose concentrations (C_i/C_e) may be calculated from the computed rate constants, the net flux across the membrane, and the extracellular concentration. Under the conditions of our study, the rate of change of the intracellular D-glucose concentration (C_i) is given by

$$\frac{dC_i}{dt} = k_1 C_e - k_2 C_i + F, \tag{3}$$

where F is the metabolic D-glucose release. Under steady-state conditions, where $dC_i/dt = 0$, F also corresponds to the net flux across the membrane. The value of C_i/C_e may then be calculated by the following expression:

$$\frac{C_i}{C_e} = \frac{k_1 + F/C_e}{k_2} \tag{4}$$

It should be stressed that, at high D-glucose concentrations, $k_1 \gg F/C_e$, and the intracellular D-glucose concentration is then virtually independent of metabolism (i.e. $C_i/C_e = k_1/k_2$). It should also be noted that the rate constant k_1 is calculated from $k_1\theta'$, θ' being the ratio of the intracellular to extracellular water spaces [11]. Consequently, the intracellular concentration determined is an average over the intracellular water space of all subcellular compartments. The latter does not necessarily correspond to the intracellular space to which labeled D-glucose has access during a single and rapid passage through the liver.

Alternatively, the C_i/C_e value may also be calculated from the mean transit times of D-glucose and indicators, provided that equilibrium or near-equilibrium conditions prevail [26, 29], as it occurs at high D-glucose concentrations. The following expression

$$\frac{C_i}{C_e} = (\bar{t}_g - \bar{t}_p)/(\bar{t}_w - \bar{t}_p) \tag{5}$$

allows the calculation of C_i/C_e from the mean transit times of labeled D-glucose ($\bar{t}_g$), labeled phosphate ($\bar{t}_p$) and labeled water ($\bar{t}_w$) [26].

The results of the calculations are shown in Tables 1 and 2. For an extracellular D-glucose concentration of 1.08 mM, the mean value of C_i/C_e was 0.70 ± 0.08 (Table 1). Strong inhibition of transport leads to increased C_i/C_e values in comparison with the corresponding controls. This is particularly evident in Expt. 6 in which the stevioside concentration was increased stepwise. Each step resulted in an increase of the concentration ratio. At 5 mM stevioside, it was 2.6 times greater than the corresponding control.

In the concentration range between 10 and 100 mM D-glucose, the metabolic flux was negligible in comparison with the high unidirectional fluxes across the membrane (i.e. $C_i/C_e = k_1/k_2$). Consequently, the C_i/C_e values may also be calculated from the mean transit times. Table 2 allows the comparison of the results obtained by both methods. As can be seen, they agree fairly well. Moreover, no change in the intracellular to extracellular concentration ratio occurred in the range between 10 and 100 mM D-glucose, the mean value (around 0.6) being lower than that found at 1 mM. Stevioside, on the other hand, produced no significant changes at high D-glucose concentrations. This is to be expected if one takes into account the fact that, under the latter conditions, the metabolic activity of the liver has no influence on the D-glucose concentration ratio.

Effect of stevioside on the D-fructose outflow profiles. It has been shown that D-fructose is transported by the same transport system which transports D-glucose [9]. For this reason, stevioside should affect

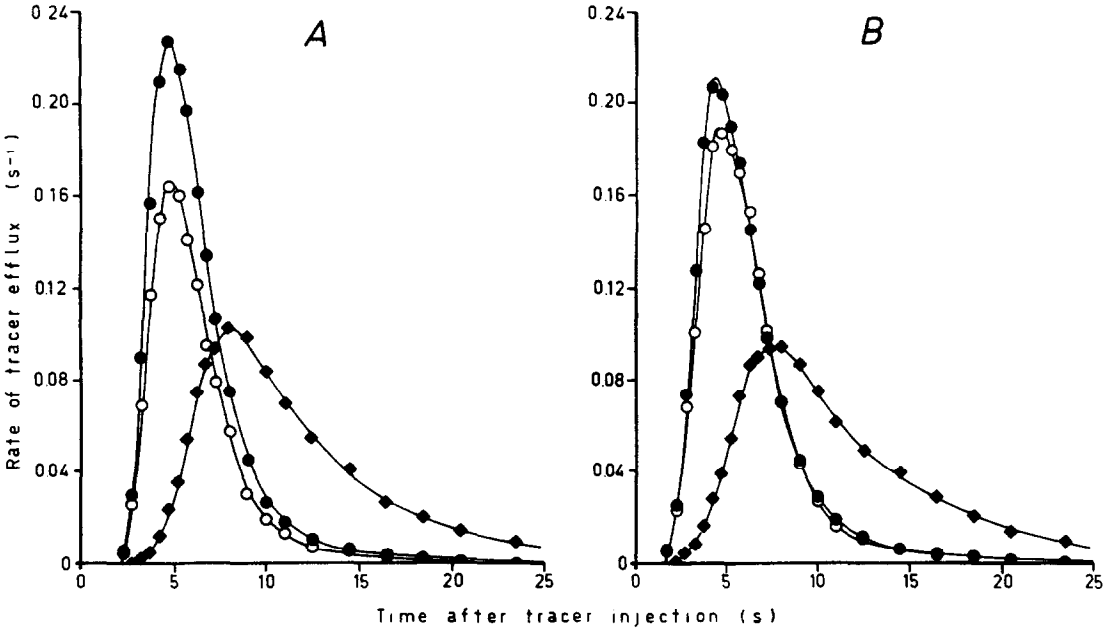

Fig. 7. Outflow profiles of D-fructose and indicator substances: Effect of stevioside. Two pulse-labeling experiments with simultaneous injection of trace amounts of [^{32}P]phosphate, [^{3}H]water and 4.5 μmol of D-fructose were performed consecutively in a liver perfusion experiment (liver from a starved rat), first, in the absence (A) and, second, in the presence of 2.5 mM stevioside (B). The fractions of the injected amounts which appeared in the effluent perfusate per second were plotted versus the time following injection. Key: D-fructose (○——○), [^{32}P]phosphate (●——●) and [^{3}H]water (◆——◆).

both transport and metabolism of D-fructose. Figure 7 shows representative results of the first approach to the latter question. Two pulse-labeling experiments were performed consecutively. Unlabeled D-fructose (4.5 μmol) was injected simultaneously with the usual references (labeled phosphate and water) in the absence (control, Fig. 7A) and presence (Fig. 7B) of 2.5 mM stevioside. The D-fructose outflow profile differed from that of labeled D-glucose (Fig. 3A) in that the experimental points of the former tend to remain below that of the extracellular reference. Another difference is that the area under the curve is equal to 0.7, meaning that 30% of the injected amount was transformed inside the liver. The ratio of the peak values of labeled phosphate to D-fructose was 1.4 in the control experiment. In the presence of 2.5 mM stevioside (Fig. 7B), the outflow profile of D-fructose presented two alterations. First, the area under the curve now is 0.9, i.e. the amount of D-fructose which was transformed decreased to 10% of the injected amount. Second, the D-fructose curve is now much closer to that of the extracellular reference. The ratio of the peak values of labeled phosphate to D-fructose is now equal to 1.1.

No attempts have been made until now to evaluate the D-fructose outflow profiles by means of computer calculations because this would involve complications due to the complex interrelations between transport and metabolism. An evaluation of such interrelations is possible by means of the numerical procedures described elsewhere [23], but the experiments should be performed under steady-state conditions, with the injection of trace amounts of labeled D-fructose. This would imply a need for separation of the complex mixtures of labeled metabolites which result from the metabolism of D-fructose. Nevertheless, since the peak of the outflow profile is composed mainly by throughput (i.e. non-exchanged material), the experiments in Fig. 7 strongly suggest that the D-fructose transport was also inhibited by stevioside [29].

Effect of stevioside on the steady-state fluxes of D-fructose metabolism. In addition to pulse experiments, the effect of stevioside on D-fructose metabolism was also studied by measurements of the steady-state fluxes. Figure 8 presents the results of a representative experiment in which D-fructose and stevioside were infused at constant rates. Livers from starved rats were used in order to eliminate interference due to glycogen metabolism. As shown in Fig. 8, D-glucose, L-lactate and pyruvate release was minimal before D-fructose infusion. The onset of D-fructose infusion caused a sudden rise in D-glucose production as well as in oxygen consumption. Both variables, however, progressively decreased to a lower steady-state value. This steady-state was maintained for at least 2 hr of perfusion (not shown). The L-lactate and pyruvate production increased slowly and reached a plateau 20 min after the onset of D-fructose infusion. Concomitantly with the high rates of oxygen consumption and D-glucose production, D-fructose consumption also showed high values at the beginning of the infusion, but they soon declined to a steady-state around 2.7 $\mu mol \cdot min^{-1} \cdot g^{-1}$. Stevioside infusion at 46 min of perfusion caused an inhibition of D-fructose consumption and of D-

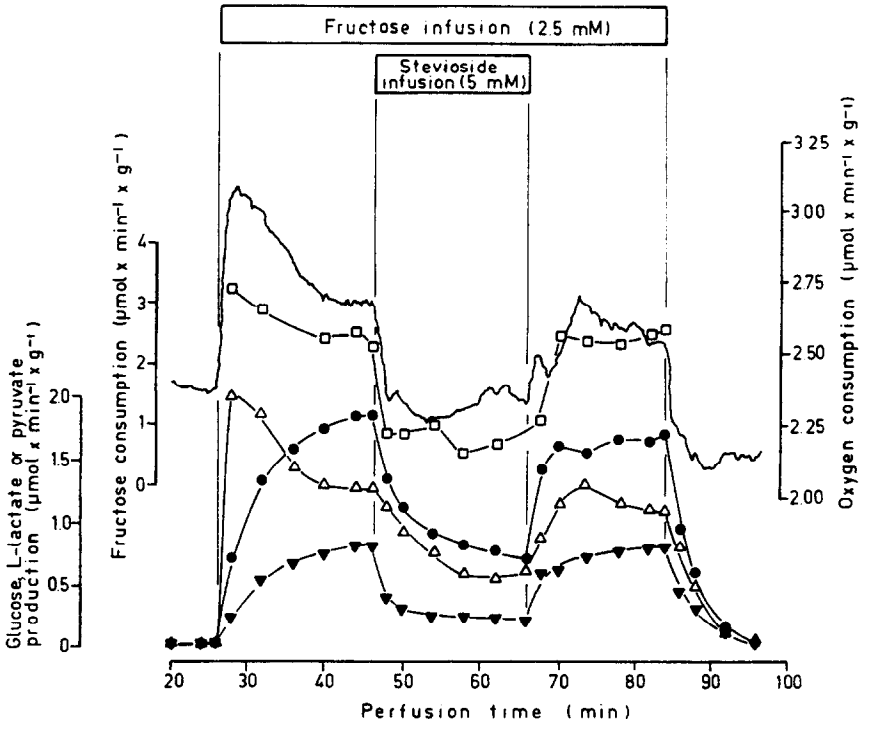

Fig. 8. Effect of stevioside on D-fructose metabolism in the isolated perfused rat liver. The liver from a starved rat was perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) in a non-recirculating system. D-Fructose (final concentration 2.5 mM) was infused into the perfusate prior to its entry into the liver at 26–84 min of perfusion and stevioside (final concentration 5.0 mM) at 46–66 min of perfusion, as indicated by horizontal bars. The effluent perfusate was sampled in 2- or 4-min intervals. The concentrations of D-glucose, L-lactate and pyruvate were measured enzymatically and the concentration of D-fructose colorimetrically. The oxygen concentration in the effluent perfusate was monitored continuously employing a platinum electrode. Metabolic rates were calculated from the arterio-venous differences and the constant flow rate and were expressed as $\mu mol \cdot min^{-1} \cdot (g \text{ liver wet weight})^{-1}$. Key: oxygen consumption (trace), D-glucose production (△——△), D-fructose consumption (□——□), L-lactate production (●——●), and pyruvate production (▼——▼).

glucose, L-lactate and pyruvate production. Oxygen consumption was also inhibited. Concerning the latter variable, one should remember that when no substrate was infused (Fig. 5A), stevioside was inactive. Moreover, several experiments performed with various stevioside and D-fructose concentrations revealed that the effect was always restricted to the extra oxygen uptake caused by D-fructose. All stevioside effects were reversible.

To quantify further the effect of stevioside, experiments were performed in which the D-fructose concentration was kept constant (1 mM), and the stevioside concentration was increased stepwise from 0.5 to 5.0 mM. Each stevioside concentration was maintained constant for 16 min. D-Fructose consumption, D-glucose production, and L-lactate + pyruvate production were evaluated and plotted versus the stevioside concentration. Figure 9 shows the mean values of three liver perfusion experiments. At 1 mM D-fructose in the influent (portal) perfusion fluid, approximately 1.8 μmol D-fructose per min per g liver was consumed under steady-state conditions. The sum of D-glucose, L-lactate and pyruvate production, expressed in terms of six carbon-units (C_6-units), amounted to 1.45 $\mu mol \cdot min^{-1} \cdot g^{-1}$. This means that 80% of the transformed D-fructose returned to the perfusion fluid in the form of D-glucose, L-lactate and pyruvate. This proportion remained approximately constant for all stevioside concentrations between 0.5 and 5.0 mM. At 5 mM stevioside, for example, for a D-fructose consumption rate of 0.65 $\mu mol \cdot min^{-1} \cdot g^{-1}$ (63% inhibition relative to the control), the D-glucose, L-lactate and pyruvate production amounted to 0.51 $\mu mol \cdot min^{-1} \cdot g^{-1}$, which corresponds to 83% of the total D-fructose metabolism. Nevertheless, fructolysis (L-lactate + pyruvate production) was inhibited more strongly than D-glucose production. This was revealed, for example, by the stevioside concentrations for half-maximal effects: in the case of D-glucose production, half-maximal inhibition occurred at 3 mM stevioside, but fructolysis was already inhibited 50% at a concentration of 1.8 mM.

The experiment shown in Fig. 8 was repeated twelve times, but with different D-fructose concentrations (in the range between 0.5 and 10 mM) and with two different stevioside concentrations (1.5 and 5.0 mM). Figure 10 shows the rates of D-glucose

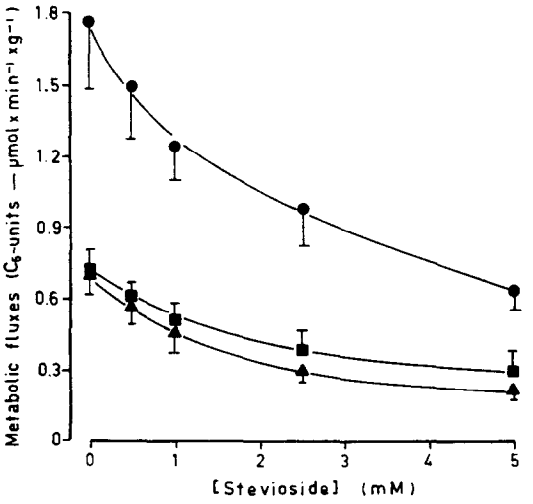

Figure 9. Dose–response curves of changes in D-fructose consumption (●——●), D-glucose production (■——■) and L-lactate + pyruvate production (fructolysis ▲——▲), due to stevioside in perfused livers from starved rats. The livers were perfused with Krebs/Henseleit-bicarbonate buffer in a non-recirculating system. D-Fructose (final concentration 1.0 mM) was infused into the perfusate at 26–122 min of perfusion. Stevioside was infused into the perfusate at 48–104 min and in stepwise increasing concentrations (0.5, 1.0, 2.5 and 5.0 mM). The effluent perfusate was sampled in 2- or 4-min intervals, and the concentrations of D-glucose, L-lactate and pyruvate were measured enzymatically. The arterial and venous D-fructose concentrations were measured colorimetrically. The metabolic fluxes were calculated from the new steady-state concentrations of the metabolites, which were reached between 10 and 14 min after each change in the stevioside concentration, and the constant flow rate. They were expressed as µmol C_6-units·min^{-1}·(g liver wet weight)$^{-1}$ and are the means of three experiments. The vertical bars represent standard errors of the means.

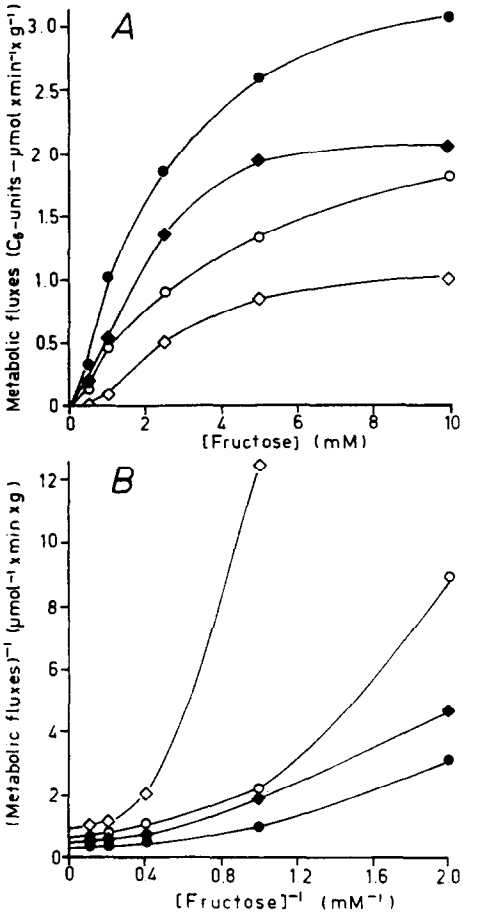

Fig. 10. Dependence of rates of D-fructose metabolism on D-fructose concentration: Effect of stevioside. Rates of fructolysis (L-lactate + pyruvate production) and of D-glucose production are from a series of experiments similar to that shown in Fig. 8, in which various D-fructose concentrations (0.5 to 10.0 mM) were infused. The concentration of stevioside was equal to 5.0 mM in all experiments. The metabolic fluxes were expressed as µmol C_6-units·min^{-1}·(g liver)$^{-1}$ and plotted versus the D-fructose concentration (A). A double-reciprocal plot is also shown (B). Key: D-glucose production (control, ●——●), fructolysis (control, ◆——◆), D-glucose production (5 mM stevioside, ○——○), and fructolysis (5 mM stevioside, ◇——◇).

production and the rates of fructolysis as a function of the arterial D-fructose concentration in the absence (control) and in the presence of 5.0 mM stevioside. Because of the low portal-venous concentration differences, the D-fructose consumption could not be measured accurately at concentrations above 2.5 mM. The curves in Fig. 10A are typical for saturation and show a tendency toward sigmoidicity. The latter phenomenon results in non-linear double-reciprocal plots (Fig. 10B). Regarding the effect of stevioside, one general aspect may be recognized: the degree of inhibition is a function of D-fructose concentration. For example, at 0.5 mM D-fructose, fructolysis was inhibited 90% by 5 mM stevioside; at 10 mM D-fructose, however, the inhibition decreased to 51%. D-Glucose production was inhibited 61% by 5 mM stevioside at 0.5 mM D-fructose, but only 40% at 10 mM D-fructose. A similar picture resulted from analogous experiments performed with 1.5 mM stevioside in the perfusate (not shown). Another parameter whose sensitivity to stevioside was also dependent on the D-fructose concentration was the extra oxygen consumption caused by the latter compound. As revealed by Fig. 8, the action of 5 mM stevioside at 2.5 mM D-fructose was practically complete. This did not hold for all D-fructose concentrations. At 10 mM D-fructose, for example, the extra oxygen consumption was inhibited only 60% by 5 mM stevioside (not shown).

DISCUSSION

Parameters of D-glucose transport in the rat liver. The transport of D-glucose in the rat liver has been measured by Baur and Heldt in isolated hepatocytes [9] and by Williams *et al.* in the isolated perfused liver [30]. In the latter study, tissue sampling was performed and a lumped model analysis was used to derive preliminary estimates of the transport parameters. Baur and Heldt [9], by measuring zero-trans influx rates in isolated hepatocytes, found a K_m of 30 mM for D-glucose. The K_m of 38.9 mM obtained

in the present work, in which near-equilibrium exchange rates were measured, agrees fairly well with the value reported for isolated hepatocytes. In this respect, the liver cells seem to differ from erythrocytes. In the latter cells, the K_m for D-glucose zero-trans influx has been reported to be 1.6 mM [31]. The corresponding parameter for equilibrium exchange, however, is 35 mM, i.e. approximately twenty times greater [31]. Williams *et al.* [30] reported a K_m of 17 mM for D-glucose in the perfused rat liver. Since the latter value is actually a minimum one, comparisons seem not to be appropriate in this case. The transport of D-glucose was also measured in the dog liver by Goresky and Nadeau [10]. The measurements were performed in anesthetized dogs *(in vivo)* by means of pulse-labeling experiments, the experimental and analytical procedures being similar to those of the present work. In the dog liver, the K_m of the monosaccharide carrier for D-glucose exchange is 121 mM.

In addition to the different K_m values for D-glucose, the monosaccharide carriers of rat and dog livers also differ in their maximal transport capacities. In the present work, a maximal exchange rate (V_{max}) of 388 μmol·min^{-1}·(g liver)$^{-1}$, corresponding to 700 μmol·min^{-1}·(ml intracellular water space)$^{-1}$, was found. In the dog liver, Goresky and Nadeau [10] found a maximal exchange rate of approximately 1700 μmol·min^{-1}·(ml cell water)$^{-1}$, which corresponds to 2.4 times that of the rat liver. The maximal exchange rate in the rat liver, on the other hand, is much higher than the maximal zero-trans influx rate. Baur and Heldt [9] reported a maximal zero-trans influx rate of 33.8 μmol·min^{-1}·(ml hepatocyte water)$^{-1}$ at 20°. By correcting this value to 37°, based on their own temperature dependence data, one arrives at 60 μmol·min^{-1}·(ml hepatocyte water)$^{-1}$ [9]. This is less than one-tenth of the maximal exchange rate found in this work. A similar difference between the maximal zero-trans influx rate and the maximal exchange rate was also found for the D-glucose transport system of erythrocytes [31].

With such high exchange rates, one cannot expect an influence of transport on metabolism. For example, the unidirectional influx rate of D-glucose at the physiological plasma concentration of 5 mM, calculated from the kinetic constants of Table 3, is equal to 44.2 μmol·min^{-1}·(g liver)$^{-1}$. The maximal net rate of metabolic D-glucose release under the influence of hormones has been found to be between 5 and 7 μmol·min^{-1}·(g liver)$^{-1}$ [32], whereas the rate of D-glucose consumption rarely exceeds 1.0 μmol·min^{-1}·(g liver)$^{-1}$ [33]. A limitation of metabolism by transport, under normal physiological conditions, is thus highly improbable. Under artificial experimental conditions, on the other hand, transient changes in the metabolic fluxes were demonstrated (Fig. 6). Such changes occurred when the transport system was strongly inhibited (80–90%) and were the consequence of alterations in the intracellular concentration, which follow the onset/termination of the infusion of an inhibitor. The transient alterations of the metabolic fluxes, however, were significant only at low and non-physiological D-glucose concentrations.

Intracellular D-glucose distribution. The intracellular to extracellular D-glucose concentration ratio (C_i/C_e) found in this work was between 0.6 and 0.7 in the absence of transport inhibitors. At low D-glucose concentrations the C_i/C_e ratio is somewhat higher, because of the metabolic activity of the liver. It may be increased further when the unidirectional fluxes across the membrane are decreased by inhibitors. The maximal value for C_i/C_e, measured in the presence of transport inhibitors, was 1.7. It should be noticed that the calculations performed using the mean transit times, which are independent of modelling according to Goresky, revealed values which are not different from those obtained employing the rate constants. Furthermore, it must be recalled that the intracellular concentration determined was an average of the intracellular water space of all subcellular compartments.

The fact that the calculated C_i/C_e values were always smaller than unity in the absence of transport inhibitors may have, basically, two reasons. First, if labeled D-glucose really had access to the whole intracellular water space, the C_i/C_e values calculated would indicate that a concentration gradient existed between cells and perfusate. This possibility, however, has little theoretical support. Since D-glucose is a neutral, undissociable molecule, its distribution across the cell membrane should be independent of membrane potential and pH difference. Moreover, the transport of D-glucose in the liver is passive [9], a fact which excludes an energy-dependent concentration gradient. Alternatively, the calculated C_i/C_e values may indicate that D-glucose does not have access to the whole intracellular water space. In fact, it is well known that sugars do not permeate the inner mitochondrial membrane. The water space of the mitochondria accounts for at least 10% of the total intracellular water space [9]. In addition to mitochondria and other organelles, one should also consider the hydration water of glycogen and of the cellular membranes. The latter space, for example, is believed to account for at least 14% of the total intramicrosomal water [34]. This seems to be the main factor that restricts the intramicrosomal sugar space (mono- and disaccharides permeate the microsomal membrane [35]) to approximately 77% of the total aqueous space [34]. Furthermore, there is a complex interrelation between transport across the endoplasmic reticulum membranes and glucose-6-phosphate hydrolysis by glucose-6-phosphatase [36], the exact mechanism of which is far from being understood. The available data, however, strongly suggest that the intracellular compartmentation of either free glucose or its phosphorylated derivatives may be very complex [36]. Finally, one must also consider that by means of the outflow profiles of labeled D-glucose one really senses the space to which that compound has access during a rapid and single passage through the liver. This space is not necessarily the total space accessible to D-glucose after total equilibration, in the way that the outflow profile of labeled phosphate, under our perfusion conditions, indicates solely the extracellular space, although the latter compound certainly permeates the cell membrane.

Concerning the intracellular distribution of D-glu-

cose, the data obtained by different authors show several discrepancies. The measurements of Cahill *et al.* [37] were carried out under *in vivo* conditions in both rat liver and dog liver. In normal rats, D-glucose was measured employing a colorimetric technique. The data presented by Cahill *et al.* [37] allow the calculation of the C_i/C_e ratio as defined in this work. For plasma D-glucose concentrations higher than 10 mM, they found C_i/C_e values between 0.6 and 0.7. Equivalence between plasma and liver was found at a plasma concentration of 8 mM. At concentrations below 7 mM the C_i/C_e ratio becomes higher than unity. Extrapolation of the data of Cahill *et al.* [37] to 1 mM plasma glucose gives a C_i/C_e value as high as 7.0. In diabetic rats and normal dogs, on the other hand, Cahill *et al.* used radioactively labeled D-glucose and found a C_i/C_e ratio of 0.97 for a plasma concentration around 30 mM. A similar equivalence between plasma and liver water was also found in dogs. Williams *et al.* [30] measured the radioactively labeled D-glucose distribution in isolated perfused rat livers. The latter authors found approximately 90% equilibration with the total water space of the liver (extra- plus intracellular) after 10 min of constant infusion, irrespective of the extracellular concentration. This corresponds to a C_i/C_e value of 0.85. Finally, Baur and Heldt [9], found a C_i/C_e ratio slightly smaller than unity after 1 min of incubation of labeled D-glucose in a hepatocyte suspension. After 3.5 min of incubation, the apparent C_i/C_e ratio had increased to 1.2.

The different results listed above may reflect, partly at least, the different methods, conditions and animal species employed by the several authors. Measurements in tissue samples after longer infusion or incubation periods, for example, are more subject to interference by metabolism. Incorporation of label into glycogen [30], phosphorylated intermediates [9] or monosaccharides producing the same phenylozasone [37] may lead to increased C_i/C_e values. Rapid measurements, on the other hand, may be influenced by cell heterogeneity. Whatever the different species concerns, it is clear that D-glucose exchange is more rapid in the dog (V_{max} = 1700 μmol·min^{-1}·(ml cell)$^{-1}$ [10]) than in the rat liver (V_{max} = 700 μmol·min^{-1}·(ml cell)$^{-1}$). The data of Goresky and Nadeau [10] indeed suggest a more even distribution of D-glucose between plasma and cell in the dog liver than in the rat liver. However, one should also take into account the fact that the flow rate per unit liver weight in our experiments with the isolated rat liver was three to four times higher than the flow rate in the living dog [10]. This means that the mean transit time of radioactively labeled glucose per unit liver weight in the experiments of Goresky and Nadeau [10] was three to four times higher than in our experiments. This difference may be important if the rates of diffusion of D-glucose in the different regions within the cell are not the same, because in this case some regions may be reached more rapidly and others more slowly. If this supposition is correct, Goresky and Nadeau [10] were sensing a larger portion of the liver cells than we in our experiments.

Irrespective of the correct interpretation of our data and of the data of other investigators, it seems to be clear that the intracellular distribution of D-glucose is still an open question.

Stevia rebaudiana *natural products as inhibitors of the monosaccharide carrier of the liver.* By virtue of the results of this work, stevioside and its derivatives can be added to the list of inhibitors of the monosaccharide carrier. The effect of stevioside does not primarily depend on the glucosyl residues of the molecule (Fig. 1). This may be concluded from the observation that isosteviol, the product of total hydrolysis of stevioside, also inhibited D-glucose transport. Isosteviol was indeed more potent than stevioside and steviolbioside, an indication that the aglycone portion plays a decisive role in the mechanism of action. Some type of interaction of the glucosyl residues of the glycosides with the transport system, however, cannot be excluded. There are two observations suggesting this. First, the inhibition caused by stevioside was most likely of a mixed type (K_m and V_{max} were both changed), indicating some degree of competition. Second, steviolbioside, which lacks the glucosyl residue involved in the ester bond in the case of stevioside (Fig. 1), was less potent than the latter compound. This observation also disproves, in principle at least, the necessity of a free carboxyl group for the enhanced inhibitory activity of isosteviol.

The observation that the aglycone isosteviol was more active than the glycoside stevioside resembles the results obtained by Baur and Heldt [9] with phloretin and phlorizin. The glycoside phlorizin is the classic inhibitor of sugar transport in mammalian cells [10]. In the liver, however, the aglycone phloretin is more potent than phlorizin. The potency of phloretin as an inhibitor is comparable to that of isosteviol, whereas phlorizin and stevioside have similar activities. Although less potent than isosteviol, stevioside, from the viewpoint of the experimentator, is the most interesting molecule. Whereas isosteviol affects several metabolic pathways in addition to the monosaccharide carrier, stevioside is relatively inert [8, 38] except for the case of the metabolism of exogenous sugars. It has been suggested that stevioside is without effect on energy metabolism in the intact cell, in contrast to its activity in isolated mitochondria, because it does not permeate the plasma membrane at an appreciable rate [8]. Corroborating this view, it has been shown that the volume of distribution of stevioside in a human red blood cell suspension, after 2 hr of incubation at 37°, is essentially the extracellular space [38]. This observation suggests that stevioside does not freely diffuse across biological membranes, its permeation requiring facilitated diffusion, as frequently happens with compounds bearing one or more polyhydroxylic units in their structure [11]. In the liver, specific and precise measurements of stevioside in tissue samples require radioactively labeled analogues which, unfortunately, are not currently available. Preliminary pulse-labeling experiments, in which stevioside was measured by the rather unspecific phenol-sulfuric technique [20], with the exclusion of the possible interference due to free glucose (not shown), revealed stevioside outflow profiles nearly identical to those of the extracellular references. It is thus probable that the plasma membrane of the

liver cells is practically impermeable to stevioside, an observation which makes this compound a highly specific inhibitor of the monosaccharide transport system.

Inhibition of D-fructose metabolism by stevioside. It is believed that D-glucose and D-fructose, as well as several other monosaccharides, are transported by the same carrier in the liver. The basis for this belief is provided by the reciprocal competition of D-glucose and D-fructose and by the sensitivity of the transport of both sugars to the same inhibitors [9]. Concerning the relationships between transport and metabolism, D-fructose differs from D-glucose. In the liver D-fructose is transported at rates well below the transport rates of D-glucose, but it is metabolized at higher rates [9, 39]. This situation leads to intracellular D-fructose concentrations below the extracellular ones [40]. Under such conditions, inhibition of metabolism by a transport inhibitor is an expected phenomenon. The effect of stevioside on D-fructose metabolism is most probably the consequence solely of transport inhibition. An additional effect on some enzymes of the metabolic pathways going from D-fructose to D-glucose or L-lactate and pyruvate is improbable. First, one must consider that stevioside does not affect glycolysis from endogenous sources and gluconeogenesis from pyruvate and glycerol. These observations exclude the majority of the enzymes also involved in D-fructose metabolism, except fructokinase and triosekinase [39]. Second, the cell membrane is practically impermeable to stevioside, and the inhibition of D-fructose consumption (see Fig. 8) took place very rapidly. In general, not more than 2 min after the onset/termination of stevioside infusion were required for the completion/reversion of the inhibitory effect on D-fructose consumption. In 2 min, the amount of stevioside which could have permeated the cell membrane is certainly very small.

The degree of inhibition of D-fructose metabolism decreased with increasing D-fructose concentrations. It is improbable that this phenomenon reflects the type of inhibition. The affinity of the monosaccharide carrier for D-fructose is lower than for D-glucose. In isolated hepatocytes, Baur and Heldt [9] did not find saturation in the range up to 100 mM. Sestoft and Fleron [41], however, reported a K_m of 67 mM in the perfused rat liver. The maximal concentration used in the present work was 10 mM, well below the reported K_m. Under such conditions (i.e. $K_m \gg$ [fructose]), the degree of inhibition is practically independent of the type of inhibition. Alternatively, the effect of D-fructose on the degree of inhibition could be related to the affinity of the intracellular enzymes responsible for the metabolic transformations. The introduction of a transport inhibitor, when metabolism is coupled to transport, shifts the intracellular concentration to a new steady-state. The intracellular D-fructose concentration, under normal conditions, is already lower than the extracellular one [40, 41]. Upon stevioside infusion, a further decrease should occur, the inhibition of metabolism being a consequence of the latter phenomenon. At high D-fructose concentrations, with the enzymatic system near to saturation, a certain decrease of the intracellular concentration will affect metabolism to a lesser extent than at low D-fructose concentrations. This is a consequence of the fact that, at concentrations around or below the Michaelis constant (half-saturation concentration), an enzymatic system responds better to concentration changes than at higher concentrations.

Another aspect calling for attention is the unequal sensitivity of the various parameters of D-fructose metabolism to stevioside. D-glucose production was less affected by stevioside than L-lactate + pyruvate production or activated oxygen uptake. With the available data no definitive explanation can be given. Possibly, the phenomenon reflects the complex interrelationships between the concentrations of the various intermediate metabolites (which are probably decreased) and the rate control coefficients of the rate-controlling steps.

Metabolic effects of S. rebaudiana *natural products and inhibition of sugar transport.* There are at least four reports about an effect of *S. rebaudiana* components on carbohydrate metabolism *in vivo*. Suzuki and co-workers [13] have studied the effects of feeding rats with a high carbohydrate diet containing dried *S. rebaudiana* leaves or pure stevioside. In both cases a significant decrease of liver glycogen was found. Rats fed a high fat diet containing stevioside had no significant changes in liver glycogen levels, compared to rats fed a high fat diet alone [13]. Alvarez *et al.* [14] evaluated the effect of the daily administration of an aqueous extract of *S. rebaudiana* leaves on D-glucose tolerance in humans. D-Glucose was given orally and two tolerance tests were performed in each person: the first one before treatment with *S. rebaudiana* extracts and the second one 4 days later. Appropriate controls were made. The tolerance tests revealed a significant increase of glucose tolerance in those patients who had received *S. rebaudiana* extracts [14]. Miquel [12] has reported that administration of *S. rebaudiana* extracts to diabetic patients has a regulatory action on blood sugar levels. A similar action on glycemy was reported by von Schmeling *et al.* [15]. The latter authors demonstrated that administration of *S. rebaudiana* extracts to alloxanized rabbits has a significant effect on blood sugar levels [15].

The question which now arises is whether the action of stevioside and other *S. rebaudiana* natural products on carbohydrate transport explains some of the physiological effects listed above [12–15]. In answering this question, one must consider the similar sensitivities to inhibitors of the carbohydrate transport systems of the various mammalian tissues. Phlorizin and phloretin, for example, do not only affect the passive transport of sugars in liver and erythrocytes, but also the active transport in the intestinal epithelium and in renal tubules [9]. If the same occurs with stevioside and its derivatives, inhibition of carbohydrate transport may be an important factor underlying the reported physiological effects on carbohydrate metabolism. This is particularly true for those cases in which *S. rebaudiana* components were given orally, and the measured variables were dependent on carbohydrate absorption from the intestine followed by metabolic transformation in the various tissues. This includes glucose tolerance [14] and liver glycogen levels [13]. The rate of gly-

cogen synthesis from D-fructose, for example, could be decreased both by inhibition of intestinal absorption and by inhibition of D-fructose transport into the liver cells. Experiments concerning the effects of stevioside and related compounds on the transport of carbohydrates in other tissues are, thus, highly desirable. Depending on the results, additional *in vivo* experiments may be adequately planned in order to further elucidate the physiological effects of *S. rebaudiana* natural products.

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