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DETERMINATION OF THE KINETIC CONSTANTS OF FRUCTOSE TRANSPORT AND PHOSPHORYLATION IN THE PERFUSED RAT LIVER

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SUMMARY

The kinetics of fructose uptake was determined in perfused rat liver during steady-state fructose elimination. On the basis of the corresponding values of fructose concentration in the affluent and in the effluent medium, and the fructose and ATP concentration in biopsies, the kinetics of membrane transport and intracellular phosphorylation in the intact organ was calculated according to a model system. Carrier-mediated fructose transport has a high K_m (67 mM) and V (30 μmoles $\cdot$ min^{-1} $\cdot$ g^{-1}). The calculated kinetic constants of the intracellular phosphorylation were compared with values obtained with an acid-treated rat liver high speed supernatant (values given in parentheses). K_m with fructose 1.0 mM (0.7 mM), K_m with ATP 0.54 mM (0.37 mM), V 10.3 μmoles $\cdot$ min^{-1} $\cdot$ g^{-1} (10.1 μmoles $\cdot$ min^{-1} $\cdot$ g^{-1}, calculated on the basis of the highest measured rate of fructose uptake correcting the ATP concentration to saturating values). The kinetics of fructose uptake reveals that at physiological fructose concentrations the membrane transport limits the rate of fructose uptake, thus protecting the liver from severe depletion of adenine nucleotides.

INTRODUCTION

Experiments with perfused pig and rat liver [1, 2] revealed that the elimination rate of fructose increases with increasing fructose concentration in the affluent medium over a concentration range which by far exceeded what had previously been assumed [3]. The reason for assuming a low K_m for the elimination was that carbohydrates were thought to be distributed by diffusion between extra- and intracellular water phases in the liver [4] and hence the elimination rate was determined by the kinetics of the fructose phosphorylating enzyme which has a low K_m with fructose [5].

In the present investigation, experimental data on fructose elimination kinetics obtained from isolated perfused rat liver, demonstrates the tenability of a simple model system including reversible carrier-mediated membrane transport and intracellular phosphorylation by a two substrate ping-pong mechanism.

In contrast with previously proposed models for elimination of substrates by an intact organ, this model also includes intracellular metabolic events in pertinent biochemical terms.

MATERIALS AND METHODS

Analytical grade reagents were employed when available. D-Fructose and phlorhizin were supplied by E. Merck, Darmstadt, Germany. Enzymes and coenzymes were from Boehringer and Soehne, Mannheim, Germany. Heparin was from Leo, Ballerup, Denmark. Animals were female Wistar rats. Fructose and ATP were determined enzymatically [6,7].

List of symbols

Symbol	*Unit*	*Definition*
x	—	Distance from the portal end of sinusoid/its entire length
V_1	μmoles $\cdot$ min^{-1} $\cdot$ g^{-1}	Maximal velocity of the membrane transport of fructose
K_1	mM	Michaelis constant of the membrane transport of fructose
V_2	μmoles $\cdot$ min^{-1} $\cdot$ g^{-1}	Maximal velocity of the ketohexokinase reaction
K_2	mM	Michaelis constant of the ketohexokinase reaction with fructose as substrate
K_2'	mM	Michaelis constant of the ketohexokinase reaction with ATP as substrate
m	g	Liver weight
S	mM	Concentration of fructose in extracellular water phase
S_I	mM	Concentration of fructose in intracellular space
S_{ATP}	mM	Concentration of ATP in intracellular space
F	ml/min	Flow of total perfusate
Htc	—	Hematocrit
ρ	g/ml	Specific gravity of liver
γ	—	Interstitial volume of liver/entire liver volume
δ	—	Vascular volume of liver/entire liver volume
$\bar{S}$	mM	Mean value of S
$\bar{S}_I$	mM	Mean value of S_I
$\bar{S}_{ATP}$	mM	Mean value of S_{ATP}
S_B	μmoles/g	Concentration of fructose in biopsy
S_A	mM	Value of S at $x = 0$
S_E	mM	Value of S at $x = 1$

Ketohexokinase (EC 2.7.1.3) assay

The kinetics of fructose phosphorylation was determined on a high speed supernatant. A 10% rat liver homogenate was centrifuged at $30\,000 \times g \cdot \text{min}$ and the supernatant from this centrifugation was centrifuged at 60 min and $100\,000 \times g$. The supernatant from the last centrifugation was incubated for 20 min at 4 °C at pH 5.1 to abolish the sorbitol dehydrogenase activity and to diminish the activity of hexokinase. The measurements were based on the rate of formation of ADP [5]. The reactions were initiated by fructose. Blanks without ATP or fructose were run in parallel.

Rat liver perfusion technique

The technique has been described in detail recently [2]. The liver was perfused in a non-recirculating system by a medium consisting of Krebs–Ringer bicarbonate, 2% bovine albumin (Armour Pharmaceutical Company Ltd, England) and 28% bovine erythrocytes at 38 °C. The flow was kept constant at 3.75, 5.0 and 7.5 ml/min by means of a UNITA II B pump (Braun, Melsungen, Germany). To ensure a fairly constant liver weight, we employed fed rats weighing between 165 and 175 g, in which the liver was $4.14 \pm 0.10\%$ ($n = 8$) of the body weight. The perfusion pressure was between 5 and 10 cm water. The gas phase was a mixture of atmospheric air and carbon dioxide (95 : 5, by vol.).

Two types of perfusions were employed: (1) perfusion for 40 min with constant fructose concentration and flow, terminated by a biopsy performed by immediate freeze clamping, and (2) perfusion with stepwise changing fructose concentrations and/or flow. Biopsies were taken either from the left lower or from the right upper liver lobe and weighed approx. 0.5–0.7 g.

Metabolic rates were calculated as $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ liver wet weight. Biopsy concentrations were measured as $\mu\text{moles} \cdot \text{g}^{-1}$. The extracellular space was determined by perfusion for 10 min with [^{14}C]carboxyinulin (NEN Chemicals, Dreieichenhain, Germany) and calculated from the radioactivity in freeze-clamped liver and in erythrocytes-free medium. The erythrocyte space was determined by means of ^{51}Cr (Philips Duphar, Holland) marked erythrocytes.

At a hematocrit value of 28%, only 6.6% of 10 mM fructose in the medium was distributed in the water phase of the erythrocytes.

The values are given $\pm$ standard error.

Model for fructose elimination kinetics by perfused rat liver

The liver is considered to consist of a number of sinusoids of equal length, an interstitial space and hepatocytes. The intrahepatic events are described in terms of a dimensionless spatial coordinate x which is 0 at the portal end of the sinusoids and 1 at the central vein. It is assumed that the total areas of sinusoids, interstitial space, and hepatocytes perpendicular to the sinusoids are independent of x.

The concentration of fructose in the interstitial space is assumed to be identical with the concentration in the sinusoids, as the diffusion gradient may be neglected in cases with flow-limited extracellular distribution of substrates of small molecular weight [8]. It is further assumed that fructose is equally distributed within each hepatocyte, and that the erythrocytes are impermeable to fructose (cf. above).

ATP is likewise assumed to be equally distributed in each hepatocyte. The concentration in the erythrocyte can be neglected.

For both metabolites, the concentrations in the different compartments are considered to depend only on x.

Based on these assumptions, the following expressions for fructose phosphorylation and transport may be derived:

The rate of intracellular fructose phosphorylation is governed by the ketohexokinase reaction

$$\text{Fru} + \text{ATP} \rightarrow \text{Fru-1-phosphate} + \text{ADP}$$

V_2 (μmoles $\cdot$ min^{-1} $\cdot$ g^{-1}) is V for this reaction, K_2 (mM) is the K_m value with fructose and K_2' (mM) the K_m value with ATP as substrate. The process is supposed to be a two substrate process following ping-pong kinetics. The amount of fructose metabolized by this process between x and $x+\Delta x$ is

$$\frac{1}{1+\frac{K_2}{S_I}+\frac{K_2'}{S_{ATP}}} V_2 \cdot m \cdot \Delta x \tag{1}$$

where S_I (mM) is the intracellular fructose concentration, S_{ATP} (mM) is the intracellular ATP concentration and m(g) is the total liver weight.

In a steady state, this amount equals the net transport of fructose across the membrane between x and $x+\Delta x$:

$$\left(\frac{1}{1+\frac{K_1}{S}} - \frac{1}{1+\frac{K_1}{S_I}}\right) V_1 \cdot m \cdot \Delta x \tag{2}$$

where S (mM) is the concentration of fructose in the extracellular water phase and K_1 (mM) and V_1 (μmoles $\cdot$ min^{-1} $\cdot$ g^{-1}) are the kinetic constants of the membrane transport, which is assumed to be an equilibrative membrane carrier-transport governed by the same kinetic constants for the inward and outward flux direction.

This amount in turn equals the amount of substrate that has disappeared from the sinusoids between x and $x+\Delta x$:

$$-F(1-\text{Htc})\frac{dS}{dx} \cdot \Delta x \tag{3}$$

where F (ml/min) is the flow of total perfusate and Htc is the hematocrit.

The biopsy concentration of fructose is

$$S_B = \frac{1}{\rho}(\bar{S}_I(1-\gamma-\delta)+\bar{S}(\gamma+(1-\text{Htc})\delta)) \tag{4}$$

where ρ (g/ml) is the specific gravity, γ the relative interstitial volume and δ the relative vascular volume of the liver. $\bar{S}$ and $\bar{S}_I$ are the mean extra- and intracellular fructose concentrations, respectively, defined by

$$\bar{S} = \int_0^1 S\,dx; \quad \bar{S}_I = \int_0^1 S_I\,dx$$

If the Eqns 1, 2, and 3 are equated and S_I eliminated from the resulting two equations, the following equation is obtained

$$-\frac{y(v+1)+c+\sqrt{ay^2+by+c^2}}{y}\,dy = I \cdot dx, \tag{5}$$

where

$$y = S/K_1$$

$$a = (v-1)^2+4vk;\ b = 2(v-1)(v+k)+4k(v+1);\ c = v+k \tag{6}$$

$$k = \frac{K_2}{K_1}\cdot f;\quad v = \frac{V_2}{V_1}\cdot f;\quad f = \frac{1}{1+\frac{K_2'}{S_{ATP}}}$$

$$I = \frac{vV_1}{K_1}\,\frac{2m}{F(1-Htc)}$$

If the concentrations of fructose in the affluent and effluent medium are denoted S_A and S_E, respectively, integration of Eqn 5 between $x = 0$ and $x = 1$ gives

$$\int_{S_E/K_1}^{S_A/K_1}\frac{y(v+1)+c+\sqrt{ay^2+by+c^2}}{y}\,dy = I \tag{7}$$

It is assumed that S_{ATP} can be replaced by a suitable mean value without any significant error, implying that I, k, v, a, b, and c are independent of x and therefore independent of the integration variable y. Then S_E can be calculated from Eqn 7, when S_A, S_{ATP} and the kinetic constants are known. In the appendix is shown that S_E is uniquely determined under these circumstances, and an iterative procedure for determination of the value is given.

The intracellular mean ATP-concentration (mM) is given by

$$\bar{S}_{ATP} = (\text{biopsy concentration})\frac{\rho}{1-\gamma-\delta}$$

an equation analogous to Eqn 4 where $\bar{S}$ vanishes according to the assumptions.

The mean values of fructose concentration, $\bar{S}$ and $\bar{S}_I$, may be expressed in terms of S_A, $\bar{S}_{ATP}$, S_E and the kinetic constants by means of Eqn 5

$$\bar{S}_I = \frac{K_1}{I}\int_{S_E/K_1}^{S_A/K_1}\frac{(1-v+2vk)y+2k(v+1)-c+\sqrt{ay^2+by+c^2}}{vy+v+1}\,dy \tag{8}$$

$$\bar{S} = \frac{K_1}{I}\int_{S_E/K_1}^{S_A/K_1}(y(v+1)+c+\sqrt{ay^2+by+c^2})dy \tag{9}$$

Hence S_B is readily obtained from Eqns 4, 8, and 9, when S_E has been determined from Eqn 7.

The most tenable set of kinetic constants (K_1, V_1, K_2, K_2', V_2) is then calculated by a computerized iterative process which gives the best fit of calculated to observed pairs of S_E and S_B including all values of S_A. The measure of the deviation is taken to be

$$M = \sum_{S_A} \left(\frac{S_E(\text{obsd}) - S_E(\text{calcd})}{S_E(\text{calcd})} \right)^2 + \beta \sum_{S_A} (S_B(\text{obsd}) - S_B(\text{calcd}))^2 \quad (10)$$

according to the method of least squares, where the error of S_E is assumed to be relative, while that of S_B is thought to be absolute. The actual errors observed confirm these assumptions. A constant (β) is introduced in order to obtain identical dimensions of the two terms of Eqn 10, and the magnitude of this value is chosen in order to weight the two terms equally.

For computation, a UNIVAC 1110 was employed.

RESULTS AND DISCUSSION

The extracellular space of the perfused rat liver was determined to be 179 ± 16 μl/g liver ($n = 8$), and the erythrocyte space was 26 μl/g. The interstitial space has been found to be about 7% [8], i.e. 70 μl/g and hence the vascular space amounts to 135 μl/g. Specific gravity (ρ) for liver tissue was 1.065 g/ml.

Table I shows the set of corresponding values of fructose concentrations in the affluent (S_A), in the effluent (S_E) and in the biopsies performed during steady-state fructose metabolism (40 min after addition of fructose). The deviations, calculated from the observed values of S_E and S_B are shown assuming either carrier-mediated membrane transport or diffusion (Table I). Diffusion clearly does not fit

TABLE I

The observed fructose concentrations on which the calculations are based and the result of the optimalization. Each set of data represents one single perfusion. The biopsy was performed after 40 min perfusion with a constant concentration of fructose in the affluent medium.

Observed concentrations of fructose			Deviation			
Affluent medium (mM) S_A	Effluent medium (mM) S_E	Biopsies (μmoles/g) S_B	Carrier transport		Diffusion	
			S_E (%)	S_B (μmoles/g)	S_E (%)	S_B (μmoles/g)
1.49	0.65	0.14	1.5	0.06	20.0	0.20
1.49	0.65	0.05	1.5	0.15	20.0	0.29
7.37	2.92	0.69	13.7	0.33	29.8	0.98
7.44	3.39	0.68	1.5	0.36	15.3	1.02
14.57	6.81	2.01	6.0	0.24	13.2	1.40
14.71	7.96	2.60	− 8.3	−0.29	− 2.4	0.85
15.10	7.93	2.31	− 6.7	0.05	0.9	1.21
15.40	7.62	2.38	− 4.7	−0.01	1.7	1.15
35.89	24.33	10.61	− 3.4	−0.43	−15.8	−1.37
36.81	22.79	10.70	8.7	0.42	−11.6	−1.07
114.20	104.44	61.84	1.1	−0.04	−18.1	−0.01
129.36	114.20	61.17	3.5	0.02	−20.5	0.20

TABLE II

THE KINETIC CONSTANTS OF MEMBRANE TRANSPORT AND INTRACELLULAR PHOSPHORYLATION OF FRUCTOSE IN RAT LIVER

The ketohexokinase assay was performed in Tris buffer at pH 7.5 with 11 mM $MgCl_2$ and either 27 or 108 mM KCl in the cuvette. Values are given ±S.E.

		Membrane transport		Intracellular phosphorylation		
		K_1 (mM)	V_1 (μmoles · min^{-1} · g^{-1})	K_2 (mM)	V_2 (μmoles · min^{-1} · g^{-1})	K'_2 (mM)
Kinetic constants of the integrated system		67	30	1.0	10.3	0.54
Kinetic constants of the high-speed supernatant	(K^+ = 27 mM)			0.46±0.06 (3)		0.30±0.03 (4)
	(K^+ = 108 mM)			0.53–0.80 (2)		

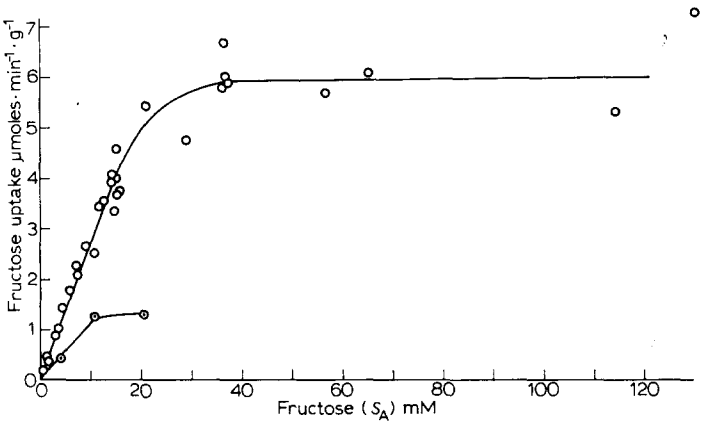

Fig. 1. Relationship between fructose concentration in the affluent medium and the rate of fructose uptake. The flow was 5 ml/min. ⊙, 10 mM phlorhizin added to the affluent medium, ○ without any additions.

the observed data set. In the case of diffusion, a positive deviation is seen at low fructose concentrations whereas a negative deviation is seen at high fructose concentrations. On the other hand carrier-mediated transport fits the observed data very well.

The carrier-mediated transport of fructose has a high K_m and V compared to the intracellular phosphorylation (Table II). The calculated K_m of the intracellular phosphorylation process with ATP, agrees with the value found in a high-speed supernatant of livers from rats of the same strain, but this value is about three times lower than the value found using a partially purified enzyme [5, 9]. In order to test the validity of a high K_m value of the ketohexokinase with ATP, K_1, V_1, K_2 and V_2 were calculated assuming K_2' to be 1.5 mM. This revealed a K_2 of 2.4 mM and a V_2 of 17 μmoles $\cdot$ min^{-1} $\cdot$ g^{-1} and a bad fit of observed to calculated values of S_E and S_B.

Fig. 2 shows the interrelation of fructose elimination and ATP concentra-

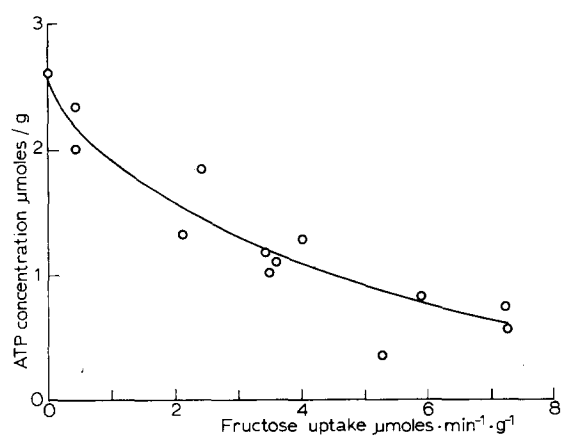

Fig. 2. Relationship of rate of fructose uptake to the concentration of ATP in biopsies. Biopsies were performed after 40 min perfusion with a constant fructose concentration in the affluent medium.

tion in perfused rat liver. The maximum rate of fructose uptake by the liver was 6.6 μmoles · min^{-1} · g^{-1} (Fig. 2). This value is in agreement with the calculated V_2 of 10.3 μmoles · min^{-1} · g^{-1} inasmuch as correction of the lowest ATP concentration measured (0.8 μmole/g) to saturating values, according to the K_2' and Michaelis kinetics, yields 10.1 μmoles · min^{-1} · g^{-1}. The rate of fructose uptake by the liver is inhibited 80% by 10 mM phlorhizin (Fig. 1).

The K_m of the intracellular phosphorylation (K_2) with fructose is 1.0 mM. This is a little higher than the values obtained by a high speed supernatant (Table II) and with a partially purified ketohexokinase preparation [5, 9]. The cause of this discrepancy is not clear, but interference from hexokinase which has a high K_m with fructose [10] might be an explanation. On the other hand glucose at 5, 10 and 25 mM in the affluent medium did not significantly inhibit the fructose uptake at 10 mM fructose, although at 10 mM glucose the biopsy concentration of fructose was only 20% the value for glucose [2]. Under these conditions the hexokinase activity with fructose should be completely abolished. Interference from sorbitol dehydrogenase, which has a high K_m with fructose can also be ruled out since no net sorbitol formation takes place under steady-state conditions with a normal cytosolic NAD redox level [2]. The influence of K^+ on the kinetics of fructose phosphorylation [9] (see also Table II), suggests that a different intracellular ionic milieu may well account for the slight difference in the measured and calculated K_m for fructose phosphorylation.

Fig. 3 shows that the model system and the kinetic constants calculated at a flow of 5 ml/min do fit with the observed values of S_E (and S_B, not shown) at a flow of 3.75 and 7.5 ml/min. The apparent K_m value (K_p) of the net fructose uptake by the perfused rat liver, measured on the basis of the concentration of fructose in the affluent medium, was highly sensitive to changes in the flow, being 83 mM at

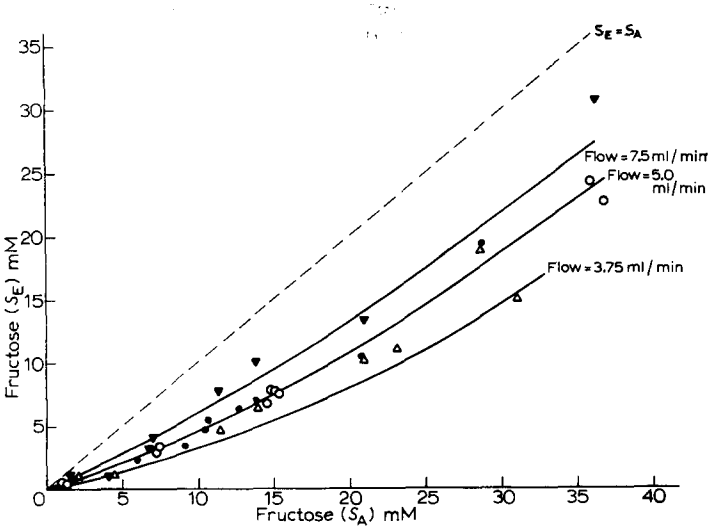

Fig. 3. The influence of flow on the concentration of fructose in the effluent medium. The kinetic constants were found on the basis of values obtained at a flow of 5 ml/min (○). The relationships of S_A to S_E calculated for different flows using these constants (Table II) are given as drawn lines. The corresponding observed values are given as points: △, 3.75; ●, 5.0 and ▼, 7.5 ml/min.

5 ml/min and 540 at 3.75 ml/min [11]. The agreement of calculated to observed values of S_E at different flows (Fig. 3) supports the tenability of the model. It also implies that apparent kinetics which are calculated without paying attention to the necessity of keeping the flow per g liver tissue constant are without any significance.

In contrast to the situation with glucose [2, 4, 12], a steep concentration gradient over the liver cell membrane is found with fructose at low fructose concentrations (Table I, Fig. 4). Under physiological conditions the fructose concentration in the portal vessel is low, being 2.59 ± 0.38 mM ($n = 4$) 10 min after 11 mmoles fructose in 2 ml water was given by stomach tube to Wistar rats weighing 170 g. This corresponds to 5.7 mM in the water phase when corrected for the in vivo hematocrit value of 55%.

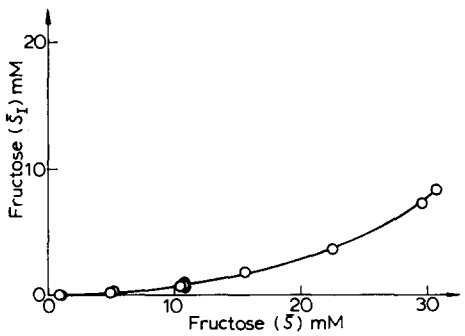

Fig. 4. Relationship of mean intracellular to mean extracellular fructose concentration. The concentrations were calculated according to Eqns 8 and 9.

In Fig. 5, the calculated rates of in- and outward fructose transport are outlined. At fructose concentrations below 10 mM the rate of outward transport is negligible indicating that the kinetics of the membrane transport is of overwhelming

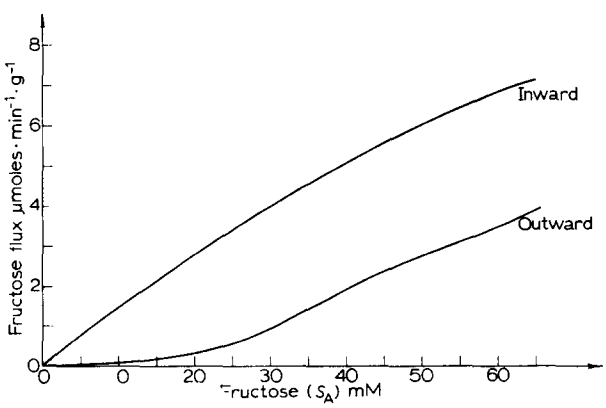

Fig. 5. Relationship of in- and outward fluxes of fructose to the fructose concentration in the affluent medium. The fluxes were calculated on the basis of the mean fructose concentration in the extracellular ($\bar{S}$) and in the intracellular water phases ($\bar{S}_I$). The rates are related to the fructose concentration in the affluent medium at a flow of 5 ml/min.

importance for the rate of fructose uptake by the liver at physiologic fructose concentrations. Glucose uptake on the other hand, is regulated by the kinetics of the hexokinase (IV), the K_m of which with glucose is of the same order of magnitude as the normal glucose concentration [10]. In the case of fructose metabolism, the membrane transport process is protecting the liver cell from severe depletion of adenine nucleotides under physiological conditions.

A carrier-mediated transport of glucose [13] and galactose [14] has previously been shown to occur in the liver. In liver the K_m of the fructose carrier is four times the value for glucose. A similar relationship between the K_m values of glucose and fructose transport has previously been found in erythrocytes [15], and in fat tissue the apparent K_m values suggest the same relationship [16].

In contrast to previous model systems, the present one gives the kinetic constants of both the carrier transport and the first intracellular metabolic step as the variables which are directly relevant for the molecular process per se.

APPENDIX

Determination of S_E from the equation

$$\int_{S_E/K_1}^{S_A/K_1} \frac{y(v+1)+c+\sqrt{ay^2+by+c^2}}{y}\,dy = I$$

where S_A, K_1, I, v, and k are positive constants, k only being implicitly used in the expressions

$$a = (v-1)^2+4vk;\ b = 2(v-1)(v+k)+4k(v+1);\ c = v+k.$$

Defining

$$y_A = S_A/K_1,\ y_E = S_E/K_1$$

and

$$g(y) = \frac{y(v+1)+c+\sqrt{ay^2+by+c^2}}{y},\ y > 0$$

y_E is a solution to the equation

$$f(y) = 0$$

where

$$f(y_E) = \int_{y_E}^{y_A} g(y)dy - I. \tag{A1}$$

Eqn A1 has only one solution in the range $0 < y < y_A$, since $f(y_A) = -I$ and $f'(y) = -g(y)$ are negative, and

$$\lim_{y\to 0} f(y) = +\infty.$$

An approximation to the solution of Eqn A1 is found by means of the Newton–Raphson iteration formula

$$y_{n+1} = y_n - \frac{f(y_n)}{f'(y_n)} \tag{A2}$$

starting with a suitable value of y_1.

It is known that A2 converges when $f(y)$ and $f''(y)$ have equal sign between y_1 and the solution. Since $f''(y)$ is positive for all $y > 0$, y_1 is chosen so that $f(y_1)$ is positive. Such a value is $y_1 = y_A \cdot e^{-(I/2c)}$:

$$f(y_1) = \int_{y_1}^{y_A} \left\{ g(y) - \frac{2c}{y} \right\} dy - I + 2c \ln \frac{y_A}{y_1} = \int_{y_1}^{y_A} \left\{ g(y) - \frac{2c}{y} \right\} dy$$

$g(y) - 2c/y$ is easily shown to be positive.

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