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A TEST FOR NON-SPECIFIC DIFFUSION STEPS IN TRANSPORT ACROSS CELL MEMBRANES, AND ITS APPLICATION TO RED CELL GLUCOSE TRANSPORT

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SUMMARY

1. The rate of transport of a substance across a cell membrane may be partly determined by its rate of diffusion to and from the specific transport sites. An experimental test is described which sets an upper limit on the effect of such diffusion steps on a transport system.

2. The test suggested that if glucose transport across the human red cell membrane has a carrier mechanism, perhaps 30% of the resistance to flux of a low concentration of glucose at 20 °C could be due to a diffusion step, activation-energy barrier or similar subsidiary rate-determining step.

3. In addition, equilibrium exchange of glucose and the inhibition by glucose of sorbose flux were measured at 20 °C using 0.8 and 8 mM glucose. The rate constant of efflux of 0.8 mM glucose was 0.35 s^{-1}. Comparison with other workers' data suggested that either K_m for glucose exchange is about 15 mM and K_i for inhibition of sorbose flux by glucose is about 9.5 mM or else these fluxes do not obey Michaelis–Menten kinetics. Zero-trans efflux of glucose almost certainly deviates from Michaelis–Menten kinetics.

4. It is argued that a carrier model with a diffusion step inside the cell explains many features of the kinetics of glucose transport.

INTRODUCTION

It has often been suggested that diffusion to and from the specific transport site, either through unstirred layers (for references see Winne [1]) or through a highly viscous or hydrophobic region in or close to the membrane [2, 3], may significantly influence the rate of transport of substances across cell membranes. This paper describes an experimental test which sets an upper limit for the influence of such diffusion steps on a transport system. The test can distinguish between bulk

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unstirred-layer effects and local diffusion barriers adjacent to individual transport sites. It does not assume any particular mechanism for the transport site, but to apply the test it is necessary to use a low concentration of substrate and it may be necessary to assume a model of transport in order to decide what is a sufficiently low concentration. The test is used to provide evidence that if glucose transport across the red cell membrane has a carrier mechanism then it may involve a diffusion step or some similar additional rate-determining step.

Studies of the irreversible inhibition of transport systems strongly support the carrier theories of transport (the mobile carrier [4] and the conformational-change theories [5–7]) by suggesting that transport systems undergo a conformational change like that postulated by the conformational-change theory [10, 11]. So the inability of current carrier models to explain the kinetics of glucose transport in red cells [8, 9] may just show that the models are too simple. A diffusion-like step in glucose transport was of interest because it might explain [3] some of the discrepancies between the models and the kinetics, for example the difference between the K_m of glucose exchange and the K_i for the inhibition by glucose of sorbose flux [12]. To test for diffusion the rate of efflux of 0.8 mM glucose was measured and the high result suggested that the ability of the carrier model to account for the kinetics of glucose transport should in any case be reassessed.

To see if the difference between K_i and the K_m of exchange could be explained by any apparent diffusion step, it was remeasured. Exchange efflux of 0.8 and 8 mM [^{14}C]glucose were measured and exchange efflux of [^{14}C]sorbose in the presence of 0.8 and 8 mM glucose. Previous determinations of the K_m of glucose exchange [8, 13] have used higher concentrations of glucose than the determinations of K_i [12, 14], so the apparent difference between the parameters could have just been deviations from Michaelis–Menten kinetics. A few measurements of zero-trans efflux of low concentrations of glucose were also made.

THEORETICAL

The test for diffusion

Consider a transport system consisting of the specific transport site in series with a diffusion pathway (Fig. 1, I or II). The resistance of the system to substrate flux will be due partly to the transport site, and partly to the diffusion pathway. The proportion due to diffusion is greatest for a rapidly-transported substrate at concentrations low compared to the concentration that half-saturates the transport site, and it is negligible both for a substrate that is transported very slowly and for any substrate when the transport site is saturated both sides or severely slowed down. Suppose that the diffusion pathway is through an unstirred layer overlying a group of specific transport sites, as in I of Fig. 1. The distance between the sites must not be much greater than the thickness of the unstirred layers or the situation becomes case II of Fig. 1. If for example 50% of the transport sites are blocked, flux of a slowly-transported substrate will be inhibited by 50% but flux of a low concentration of a rapidly transported substrate will be inhibited less. This will not necessarily be observed in Case II of Fig. 1, where the diffusion pathway is through a local region around each transport site and flux through each site is independent of the others. For example irreversible inactivation of 50% of the sites will inhibit all fluxes 50%.

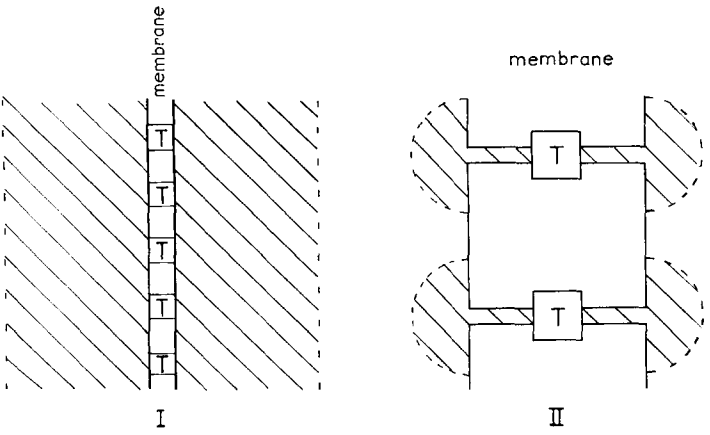

Fig. 1. Two types of diffusion pathway (not to scale). Type I overlying a large number of transport sites, for example unstirred layers. The thickness of the layers must not be small compared to the distance between the transport sites or the system becomes Type II. Type II associated with individual transport sites, perhaps representing diffusion through bound water or a hydrophobic region. Shaded areas represent regions where there is significant resistance to diffusion of substrate. The specific transport sites are labelled T.

The effect of inhibiting transport will be the same as in Case I provided either (a) that the inhibition is rapidly reversible so that binding and release of inhibitor is fast compared to the time taken for substrate to cross the whole system, or (b) that inhibitor is bound all the time but only slows the transport mechanism rather than completely stopping it. It is usually assumed that (a) is true for competitive inhibitors. Some non-competitive inhibitors will satisfy a or b. A change of pH would probably satisfy either a or b.

A difference between I and II of Fig. 1 is that an inhibitor which does not satisfy a or b, for example an irreversible inhibitor like fluorodinitrobenzene, will inhibit flux of a slowly transported substrate more than the flux of a rapidly transported substrate in Case I, provided that the thickness of the unstirred layer is not small compared to the distance between the transport sites, but in Case II it will inhibit both to the same extent.

So to test for the presence of a diffusion step, transport is inhibited and the degree of inhibition determined both for a low concentration of a rapidly transported substrate and for a low concentration of a slowly transported substrate. Diffusion pathways of Types I and II can be distinguished by using both inhibitors that do and inhibitors that do not satisfy conditions a or b.

Quantitative treatment

Notation. ϕ, Proportion of transport apparatus functioning at any instant in the presence of inhibitor; T, T/ϕ, resistance of transport apparatus to substrate flux in the control and inhibited situations, respectively; d, resistance of the diffusion pathway to substrate flux; ΔS, drop in concentration of substrate across the whole transport system, including the diffusion pathway; ΔS_T, drop in concentration of substrate across the transport apparatus; ΔS_D, drop in concentration of substrate

across the diffusion pathway. If part of the diffusion pathway is inside the cell and part outside, ΔS_D is the sum of the concentration differences across the two parts: v/v_0, inhibited substrate flux divided by control flux; I, concentration of competitive inhibitor; K_i, inhibition constant of competitive inhibitor.

Measurement of the resistance due to diffusion, d. The influence of a diffusion step on a transport system can be measured by the resistance to substrate flux due to diffusion, d, defined as

$$\text{flux, } v_0 = \frac{\Delta S}{d} \tag{1}$$

In work on red cells, fluxes are usually expressed in cell units, mmoles · (l cell water)$^{-1}$ · min^{-1}, so that d would be in min. More generally, flux across a membrane would be expressed in mmoles · cm^{-2} · min^{-1} and d would then be in min · cm^{-1}, with ΔS in mmoles · cm^{-3}. $d = 1$ min in cell units is equivalent to $d = 2.5 \cdot 10^4$ min · cm^{-1} in c.g.s. units [21].
By definition,

$$\Delta S_T + \Delta S_D = \Delta S \tag{2}$$

In the steady state,

$$\text{substrate flux, } v_0 = \frac{\Delta S_D}{d} = \frac{\Delta S_T}{T} = \frac{\Delta S}{d+T} \tag{3}$$

In both types of diffusion pathway, I and II in Fig. 1, when conditions a or b apply,

$$\text{inhibited substrate flux, } v = \frac{\phi \Delta S'_T}{T} = \frac{\Delta S'_D}{d} \tag{4}$$

This equation will also hold for other types of inhibition in Case I of Fig. 1 if the thickness of the unstirred layer is very large compared to the distance between the transport sites (except in this limiting case the inhibition of flux of a rapidly transported substrate by an irreversible inhibitor will depend on the ratio of the distance between the sites to the thickness of the layer, because this will determine to what extent the flux through one transport site is influenced by the flux through neighbouring sites).

Eliminating ΔS_T and ΔS_D in Eqns 3 and 4 using Eqn 2, solving for the fluxes, and deriving the observed degree of inhibition, v/v_0,

$$v/v_0 = \frac{T+d}{T/\phi + d} \tag{5}$$

If the diffusion step has no significant effect on the rate of transport, as in the absence of diffusion or when the flux of a slowly transported substrate is measured, $d \ll T$ and Eqn 5 becomes

$$(v/v_0)_{d \ll T} = \phi \tag{6}$$

So ϕ can be measured and, providing ϕ does not change, d/T can be determined for other substrates from Eqn 5, and d follows from $v_0/\Delta S$ using Eqn 3.

Applying the test to the red cell glucose transport system

Glucose was used as the rapidly transported substrate, sorbose was the slowly transported substrate. Transport was inhibited with the competitive inhibitor maltose [15], which does not cross the red cell membrane [16]. It was not possible to measure efflux of glucose at concentrations less than about 0.8 mM because too much of the intracellular [^{14}C]glucose was metabolised. v_0, the control flux of the rapidly transported substrate glucose at a tracer concentration, was therefore replaced by a measurement of tracer glucose flux in the presence of 0.8 mM glucose, which was inhibiting tracer flux to some degree ϕ_0. In order to apply the test to these results it was necessary to compare this flux, v'_0, with the flux of sorbose, the slowly transported substrate, in the presence of 0.8 mM glucose and it was necessary to assume that 0.8 mM glucose increased the resistance of the transport site to glucose and sorbose fluxes by the same factor, ϕ_0. This would not necessarily be true of recent models of transport [8, 17, 18], but it holds for the carrier model so the results are only discussed with respect to the carrier model. The calculation of d is the same except that v_0 and T in the control are replaced by v'_0 and T/ϕ_0, ϕ is replaced in Eqn 6 by ϕ/ϕ_0, and $v'_0/\Delta S = 1/(T/\phi_0+d)$.

The effect of a diffusion step on K_i and K_m, according to the carrier model

It is necessary to derive the expression for apparent K_i values and the K_m of exchange predicted by the carrier model, since the expression given by Wilbrandt (Line 2, p. 94 of ref. 3) is incorrect. Let v represent the flux of some substrate S at a constant tracer concentration, in the presence of I, a competing substrate present on both sides of the membrane at equilibrium or a non-transported competitive inhibitor. T will be given by

$$T = K_s/V \tag{7}$$

where K_s is the K_m of exchange of the substrate S that would be observed if there were no diffusion step, and V is the V of exchange. In the absence of a diffusion step inhibition by I would obey the classical equation of competitive inhibition

$$T/\phi = K_s(1+I/K_i)/V \tag{8}$$

The diffusion step cannot change the concentration at the transport site of either a non-transported competitive inhibitor or a substrate present on both sides of the membrane at equal concentrations, so inhibition will also obey Eqn 8 in the presence of a diffusion step.
From Eqns 5, 7 and 8

$$v/v_0 = \frac{K_s/V+d}{K_s(1+I/K_i)/V+d} \tag{9}$$

$$= \frac{1}{1+I/K_i^*} \tag{10}$$

where $K_i^* = K_i(1+dV/K_s)$ (11)

So inhibition will show the same sort of concentration dependence as in the absence of a diffusion step, but the apparent K_i will be larger by a factor dependent

on V/K_s for the tracer substrate, V/K_s being a measure of how fast individual molecules of that substrate are transported through the specific transport site. So for sorbose, with a very low V/K_s, K_i will not be altered by a diffusion step. When glucose is the tracer sugar and unlabelled glucose the inhibitor, i.e. when glucose exchange is measured, K_m for glucose exchange will be K_s+dV, where K_s is the K_m of glucose exchange that would be seen if the diffusion step were removed. The carrier model prodicts that in the absence of a diffusion step K_i for glucose inhibition of sorbose flux will be equal to the K_m of exchange (K_s) [12], so in the presence of a diffusion step,

$$\text{observed } K_m \text{ of exchange} = K_i + dV \tag{12}$$

where V is the V of exchange, which is not altered by the presence of a diffusion step.

METHODS AND MATERIALS

Solutions: phosphate-buffered saline (pH 7.4) according to Miller [19]. Stopper solution according to Karlish et al. [20]: 1% (w/v) NaCl, 1 μM $HgCl_2$, 1.25 mM KI, 0.1 mM phloretin, 1% (v/v) ethanol. Glucose and sorbose solutions were hypertonic; maltose solutions were made isotonic by diluting the saline.

Human red cells from bank blood 2–11-days-old were washed five times in at least 8 vol. of saline.

Efflux of glucose

For glucose efflux experiments the cells were starved to reduce their ability to phosphorylate glucose. They were suspended at about 2% hematocrit in sterile saline containing 0.01% (w/v) penicillin and incubated overnight at room temperature in bottles rotated about a horizontal axis at 8 rev./min. To load cells with 0.8 mM glucose, they were resuspended at 5% hematocrit in 0.8 mM unlabelled glucose, incubated at room temperature for 5 min,centrifuged and resuspended in 0.8 mM glucose for 2 min, centrifuged and packed cells added to an equal volume of saline containing 0.8 mM [^{14}C]glucose. After 45 s at 20 °C, the suspension was cooled on ice, centrifuged and kept on ice at about 80% hematocrit. When loading cells with 8 or 50 mM glucose, the incubations with unlabelled glucose were increased, respectively, to 15 and 5 min or 30 min and 5 min and the incubations with labelled glucose to 1 or 10 min.

Glucose efflux was measured by blowing 50 μl of this suspension into 4 ml of vigorously vortexed saline containing glucose at the same concentration with or without maltose, at 19.9–20.4$\pm$0.1 °C, incubating for 2.4 to 75 s, and dropping the suspension into 50 ml of vigorously stirred ice-cold stopper solution. All samples were vortexed for at least 1.4 s at the start of the incubations, so the cells should have been dispersed adequately [21, 29]. The manual operations were timed by listening to the ticking of a clock. After centrifuging for 1.5 min at 2500$\times g$, the supernatant was decanted not more than 10 min after stopping the incubation, and the sides of the tube were wiped. The radioactivity in the remaining supernatant was less than 5% of the radioactivity in the cell pellet (it was measured by suspending 50 μl cells not loaded with glucose in 50 ml stopper solution, adding [^{14}C]glucose, centrifuging and decanting). The cell pellet was resuspended in 1 ml saline, 1 ml 5% (w/v) tri-

chloroacetic acid containing 1 mM glucose was added and, after centrifuging, 1 ml of supernatant was taken for scintillation counting as previously described [11].

Cells incubated for more than 10 times the initial half-time of efflux should have had about 1% of their initial [^{14}C]glucose left inside them. The radioactivity remaining was more than this and it did not change between 10 and 20 half-times, so apart from the 1% it presumably represented [^{14}C]glucose that had been metabolised. The proportion of the original radioactivity remaining was always less than 5% in cells which had contained 8 or 50 mM glucose. However, in cells freshly loaded with 0.8 mM glucose it was about 10% and by the time a whole set of efflux incubations had been made (about 1.5 h, 25 incubations) it had risen to up to 20%. At least every sixth efflux incubation was, therefore, for more than then half-times so that the amount of radioactivity not taking part in the efflux was known throughout the experiment. The radioactivity in the cells when they were added to the incubation medium was measured by blowing 50 μl of cells directly into a mixture of 50 ml of ice-cold stopper solution and 4 ml of saline. Neither the initial radioactivity nor the residual radioactivity were altered by the presence of maltose in the incubation medium.

At low hematocrit, the rate constant of efflux of glucose at osmotically insignificant concentrations can be defined as

$$-\frac{1}{(S^*-S^*_\infty)}\frac{d(S^*-S^*_\infty)}{dt} = -\frac{d(\ln(S^*-S^*_\infty))}{dt} \tag{13}$$

where S^* is the concentration of [^{14}C]glucose inside the cell at time t, S^*_∞ is S^* at equilibrium. Provided the amount of non-glucose radioactivity does not change during the incubation,

$$S^*-S^*_\infty = a(C-C_\infty) \tag{14}$$

where C is the radioactivity in cells incubated for a time t, C_∞ is C when the incubation was for 10 to 15 half-times, and a is a constant. The rate constant of efflux was therefore taken to be the slope of

$$-\ln(C-C_\infty) \text{ versus } t \tag{15}$$

This graph should be a straight line for equilibrium-exchange efflux.

Hankin and Stein [29] found that when cells containing [^{14}C]glucose were suspended in stopper solution mixed with saline (3 : 1, v/v), they retained 100±5% of their radioactivity during 15 min at 0 °C. In any case, it was not essential that transport be stopped instantly by the stopper solution. Suppose that a certain fraction of the [^{14}C]glucose in the cells when they were dropped into the stopper came out before the stopper was decanted. Since all the samples were treated similarly, they would all have lost this same fraction of their radioactivity and the slope of Graph 15 would not have been altered.

Efflux of sorbose

Equilibrium-exchange efflux of [^{14}C]sorbose was determined by measuring the appearance of labelled sorbose in the medium during about 20 min at 19.9–20.6±0.1 °C as previously described [10]. The rate constant of efflux was obtained

from the slope of $\ln(1-C'/C'_T)$ against time, where C' was the supernatant radioactivity at time t, C'_T is the total radioactivity of the suspension.

Materials

[^{14}C]Sorbose and [^{14}C]glucose were obtained from the Radiochemical Centre, Amersham. Unlabelled sorbose was from Koch-Light Laboratories; maltose was from Sigma Chemical Co., and phloretin was from Fluorochem, Glossop, England.

RESULTS

The test for diffusion

Fig. 2 shows an experiment in which exchange efflux of 0.8 mM [^{14}C]glucose was measured in the presence and absence of 33.3 mM maltose. The time = 0 points do not lie on the straight line through the remaining points, because the stopping solution does not stop efflux instantaneously in the incubations while efflux is never initiated in the time = 0 determinations. The time = 0 points were, therefore, ignored in calculating the results, and later experiments concentrated on the longest and

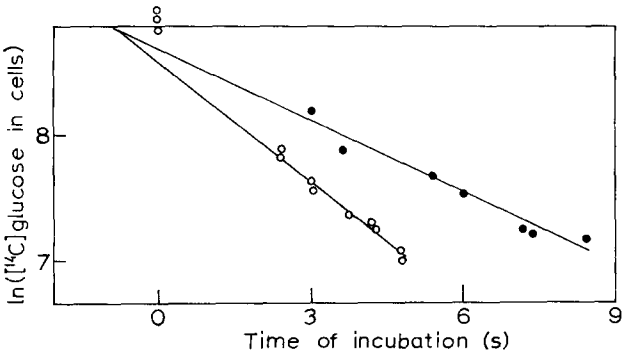

Fig. 2. Equilibrium-exchange efflux of 0.8 mM glucose with (●–●) and without (○–○) isotonic 33 mM maltose outside the cell. One experiment; inhibition by maltose was 40.5 %.

TABLE I

INHIBITION OF THE EQUILIBRIUM-EXCHANGE EFFLUX OF GLUCOSE AND SORBOSE BY MALTOSE

Each result represents a separate experiment. The sorbose results are averages of duplicates or (§) triplicates.

	% inhibition by 33.3 mM maltose	Mean ±S.E.
0.8 mM [^{14}C]glucose	46, 40.5, 50.5, 32.5, 45, 44*	43.1 ± 2.7
5 or 11 mM [^{14}C]sorbose in the presence of 0.8 mM glucose	45.6, 52.4§, 53.0	50.6 ± 1.4
5 or 11 mM [^{14}C]sorbose	51.5§, 52.5, 54.9, 50.9§, 54.5	52.6 ± 0.5

* In this experiment inhibition was calculated using time = 0 points, assuming that the timing difference between these and the efflux samples was average, i.e. 0.7 s.

shortest possible incubations; for example 4.8 and 2.4 s for control effluxes.

The rate constant of efflux of 0.8 mM glucose was about 0.35 s^{-1}. The inhibition by maltose is shown in Table I with the inhibition of exchange efflux of 5 or 11 mM [^{14}C]sorbose. 5 and 11 mM are negligible compared to the K_m of sorbose flux, estimates of which vary between 0.6 and 3 M [22, 23], but 0.8 mM glucose might compete slightly with the maltose. To avoid having to allow for any competition, I measured the inhibition of [^{14}C]sorbose flux by maltose in the presence, in both the control and the inhibited samples, of 0.8 mM glucose (Table I). The experiment then compares tracer glucose and sorbose flux either slightly inhibited by 0.8 mM unlabelled glucose or inhibited by a combination of glucose and maltose. The only difference between the conditions for inhibition by maltose was that in the experiments on glucose efflux exposure to maltose was for a few seconds but in the sorbose experiments exposure was for about 20 min.

These results suggest that if glucose transport has a carrier mechanism, then there is a small apparent diffusion step. The probability P, as judged by the one-tailed Student's t test, that maltose inhibits sorbose (in the presence of glucose) and glucose fluxes equally, so that there is no apparent diffusion step, is 0.01. The probable size of the hypothetical diffusion step can be measured by the resistance d it would offer to substrate flux (Eqn 1). The best estimate of d, obtained from the average inhibition of glucose flux, 43%, and the average inhibition of sorbose flux in the presence of 0.8 mM glucose, 50.6%, is 0.012 min in cell units, or 300 min/cm in c.g.s. units, plus and minus one standard error giving 0.016 and 0.007 min.

Comparison of K_i for inhibition by glucose of sorbose flux and the K_m of glucose exchange

An experiment measuring equilibrium-exchange of 0.8 and 8 mM [^{14}C]glucose is shown in Fig. 3. Table II lists the rate constants of efflux from this experiment and six others, together with the inhibition of efflux of 5 or 11 mM [^{14}C]sorbose by 0, 0.8 or 8 mM glucose. The apparent K_m for glucose exchange deduced from the rate constants of 0.8 and 8 mM glucose flux was 10, 13, 14, 15 and 19 mM in five experiments, while the K_i for inhibition of sorbose flux deduced from the sorbose fluxes in the presence of 0.8 and 8 mM glucose was 9.1, 10.0 and 9.3 mM in three

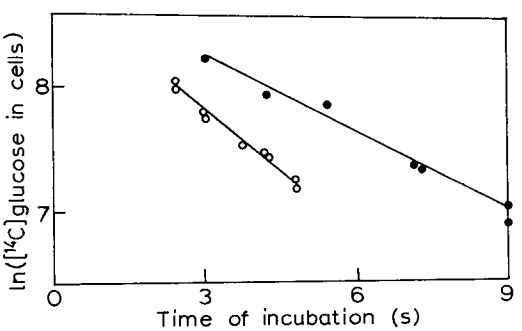

Fig. 3. Equilibrium-exchange efflux of 0.8 and 8 mM glucose. One experiment. ○–○, 0.8 mM; ●–●, 8 mM.

TABLE II

INHIBITION OF TRACER GLUCOSE AND SORBOSE FLUXES BY UNLABELLED GLUCOSE

Each line represents a separate experiment. The sorbose results are averages of duplicates or triplicates. The apparent K_m or K_i was calculated from the rates in the presence of 8 mM and 0.8 mM glucose; the rate of sorbose efflux in the absence of glucose was neglected (see text).

Substrate	Rate constants of efflux (s^{-1})		% inhibition by 8 mM relative to 0.8 mM	Apparent K_m or K_i (mM)
	0.8 mM glucose	8 mM glucose		
Glucose	0.332			
	0.393	0.263	33	13.8
	0.321	0.210	34.5	12.9
		0.250		
	0.350	0.223	27	18.7
	0.379	0.261	31	15.2
	0.392	0.238	39	10.3
	% inhibition relative to no glucose by:			
	0.8 mM glucose	8 mM glucose		
Sorbose	4	45	42.1	9.1
	12	47	39.8	10.1
	7	45	41.7	9.3
	8			
	10			

experiments. The sorbose flux in the absence of glucose was ignored in this comparison, because the analogous glucose flux, the flux of glucose at a vanishingly low concentration, could not be measured.

The rate of efflux of a vanishingly low concentration of glucose

From the apparent K_m of glucose exchange, it seems that the rate constant of efflux of a vanishingly low concentration of glucose, V/K_m for a flux that obeys Michaelis kinetics, would be about 6% more than for 0.8 mM glucose, i.e. 0.37 s^{-1} (range 0.32–0.42). V/K_m must be the same for exchange, zero-trans influx and zero-trans efflux, if the fluxes obey Michaelis kinetics. This value of V/K_m for exchange agrees with the value of 0.38 s^{-1} obtained for influx by Lacko et al. [13] but it disagrees in particular with published V/K_m values for zero-trans efflux of glucose, 0.08 to 0.15 s^{-1} [20, 24] [Fuhrmann, F. and Wilbrandt, W. (1972) unpublished results cited in ref. 8]. To clarify this disagreement, some exploratory measurements of zero-trans flux were made. There was no significant difference between the rate of efflux of 0.8 mM [^{14}C]glucose into glucose-free buffer and into 0.8 mM glucose, implying that V/K_m for zero-trans flux is also about 0.37 s^{-1}. In two experiments, efflux from 8 mM [^{14}C]glucose into glucose-free solution was measured. Incubation was terminated after 6 s, giving a true period of efflux of about 6.7 s (see discussion of Fig. 2). There was 2.03 and 2.06 mM glucose, respectively, left inside the cells (averages of duplicates), so the average rate constant of efflux over the 6.7 s was about 0.20 s^{-1}.

DISCUSSION

Diffusion steps

This test should be applicable to any transport system, and it should not be necessary to assume a mechanism of transport if sufficiently low concentrations of substrate can be used. If no evidence of a diffusion step is found, the interpretation is unambiguous, but if differences are found in the inhibition of different substrates, one can only conclude that the transport has more than one rate-determining step with different sensitivities to inhibitor. So the test would be positive not only when there was a diffusion step but also, for example, when there were two transport systems, either in parallel or in series, with different specificities for the substrates tested.

To apply the test to the glucose transport system it was assumed that tracer glucose and sorbose fluxes are competitively inhibited by 0.8 mM glucose. This would be true of a carrier mechanism but might not be true of more recent theories of transport [8, 17, 18].

It is unlikely that an unstirred layer could give a significant diffusion step in glucose transport because the thickness or viscosity of the layer would have to be very high [21]. But it is quite possible that glucose diffuses slowly to the transport site because it has to cross an activation-energy barrier, which might be, for example, the loss of water of hydration.

V/K_m for net and exchange fluxes of glucose

The estimated rate constant of efflux of a very low concentration of glucose is 0.37 s^{-1}. This rate constant must be equal to V/K_m for equilibrium-exchange, zero-trans influx and zero-trans efflux of glucose if these fluxes obey Michaelis–Menten kinetics, regardless of the mechanism of transport. V/K_m for zero-trans influx is 0.38 s^{-1} [13], but over a relatively high range of glucose concentrations the apparent V/K_m for zero-trans efflux is 0.08 to 0.15 s^{-1} [20, 24] [Fuhrmann, F. and Wilbrandt, W. (1972) unpublished results cited in ref. 8], so it is almost certain that zero-trans efflux deviates from Michaelis–Menten kinetics (this is in fact predicted by a carrier model with a diffusion step). Whether equlibrium-exchange of glucose deviates from Michaelis–Menten kinetics is not clear. Fig. 4 shows the data of this work with the data of Eilam and Stein [25] which were obtained by a similar technique, and the data of Miller [19], which were obtained by the less reliable method of estimating initial rates of flux by eye. To see if I could reproduce their results, I measured the rate constant of exchange efflux of 50 mM glucose. The result is also shown in Fig. 4 and agrees well with Eilam and Stein's value [25]. Remembering that at high substrate concentrations the points will be more scattered for a given percentage error in the rate constant, the combined data of this work and of Eilam and Stein [25] are compatible with a K_m of about 14 mM and a V of 300 mmoles/l cell water per min, compared to 32 mM and 360 mmoles/l cell water per min obtained from the data of Eilam and Stein [25] alone, which gave a V/K_m of 0.19 s^{-1}. The data might fit even better a curve concave toward the abscissa but the data are not yet adequate to demonstrate non-Michaelis–Menten behaviour.

The apparent K_i for the inhibition of sorbose flux by low concentrations of glucose is about 9 mM (this work, which agrees with Levine et al. [14]). Miller [19]

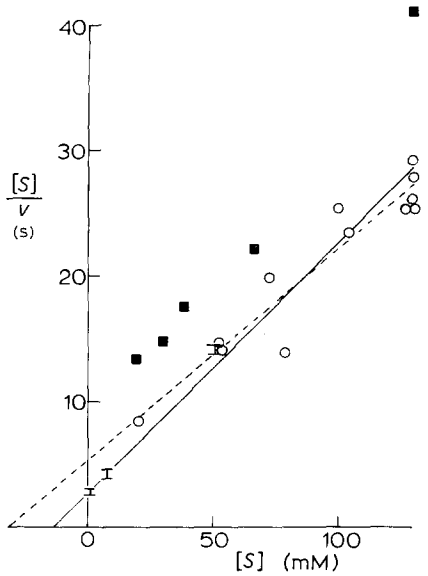

Fig. 4. Comparison of the glucose exchange data of Eilam and Stein [25] (○) and of Miller [19] (■) with the data of this work given as mean $\pm 2 \times$ S.E. (I). The line was drawn by eye to the data of Eilam and Stein and this work: it gives $K_m = 14$ mM, $V = 300$ mmoles/l cell water per min. The broken line is the line obtained by Eilam and Stein [25].

obtained less inhibition, but the concentration of sorbose used, 230 mM, may have been too high. At higher concentrations of glucose the apparent K_i seems to rise [14, 19]; this could reflect a leak flux of sorbose or be further evidence for non-Michaelis–Menten behaviour of exchange.

Can the carrier theory account for the kinetics of glucose transport?

The carrier theory is supported by studies of conformational changes in transport systems [10, 11], so the inability of the carrier models to account for the kinetics of glucose transport [8, 9] may just show that our carrier models are still oversimplified. If it is assumed that glucose exchange obeys Michaelis–Menten kinetics or that a further modification of the carrier model would explain any deviation, then it can be argued that the kinetics of glucose transport are fairly consistent with a moderately asymmetrical carrier model with an activation-energy barrier slowing glucose diffusion to the carrier.

The two major discrepancies between the carrier model and the kinetics of glucose transport at 20 °C were [8, 9, 12] that (i) the K_m of glucose exchange was much higher than the K_i for glucose inhibition of sorbose flux [12] and (ii) the K_m for exchange and probably also the K_m for zero-trans efflux [20] were very much larger than the K_m for infinite-cis efflux implying a large asymmetry of the carrier, while the K_m for infinite-cis efflux and influx were very similar implying little asymmetry [9] and other parameters suggested moderate asymmetry [9, 13].

K_i and K_m may be about 9.5 and 14 mM (Fig. 4), respectively. According to the carrier theory the K_m of glucose exchange would be equal to K_i in the absence of a diffusion step and equal to $K_i + dV$ in the presence of a diffusion step of resistance d

(Eqns 1 and 12). V, the V of exchange, is about 300 mmoles/l cells per min, so the differences between K_m and K_i could be explained by a diffusion step of resistance $d = (14-9.5)/300 = 0.015$ min, in agreement with the estimate of the diffusion resistance.

The true dissociation constant of carrier–glucose complex measured with glucose at equal concentrations on both sides of the membrane would therefore be about 9.5 mM, in striking agreement with the value obtained by Krupka [15] from the effect of glucose on the inactivation of glucose transport by fluorodinitrobenzene, 10.5 mM.

Discrepancy (ii) still holds, but it is much smaller than has been claimed [9] and it now rests on a single measurement. K^{ee}, the intrinsic K_m of glucose exchange, that is, the K_m of exchange that would be measured if the diffusion step could be taken away, will be 9.5 mM. If all the diffusion step was inside the cell, the intrinsic and observed K_m of infinite-cis efflux K_{eff}^{ic} would be the same, 1.8 ± 0.3 [12], so K^{ee} may only be five times K_{eff}^{ic} instead of up to twenty times as previously suggested [12]. Such a difference can be explained by a moderate degree of asymmetry, Q, in the carrier, given by [9]:

$$Q = \frac{K^{ee}}{K_{eff}^{ic}} + \frac{K_{eff}^{zt}}{K^{ee}} - 1 \tag{16}$$

where K_{eff}^{zt} is the intrinsic K_m of zero-trans efflux and Q is defined by:

$$Q = \frac{K_{in}^{ic}}{K_{eff}^{ic}} = \frac{K_{eff}^{zt}}{K_{in}^{zt}} = \frac{V_{eff}^{zt}}{V_{in}^{zt}} \tag{17, 18, 19}$$

$$\text{also [9]}, \quad Q \geqslant 2\left[\frac{K_{eff}^{zt}}{K_{eff}^{ic}}\right]^{\frac{1}{2}} - 1 \tag{20}$$

The non-Michaelis–Menten behaviour of zero-trans efflux, predicted by a carrier model in the presence of a diffusion step, means that we have little idea of the intrinsic K_{eff}^{zt}. (Lieb and Stein [26] showed that zero-trans flux with a diffusion step at the trans face would give a K_m lower than the intrinsic K_m, but they did not consider the effect of a cis diffusion step). Assuming it is around 9 mM, Eqn 16 gives $Q = 5$. Eqns 18 to 20 give values not significantly different: K_{eff}^{zt} may well be about five times K_{in}^{zt}, which, if there is a diffusion step inside the cell, will be slightly greater [26] than the observed value of 1.6 mM [13]; estimates of V_{eff}^{zt} range from 100 [12] to 140 [20] and 180 [Fuhrmann, F. and Wilbrandt, W. (1972) unpublished results cited by ref. 8] mmoles/l cell water per min, and V_{in}^{zt} is about 36 mmoles/l cell water per min [13] or a little higher if this observed value is lowered by a diffusion step inside the cell; Eqn 20 requires that for $Q = 5$ and $K_{eff}^{ic} = 1.8$ mM, $K_{eff}^{zt} \leqslant 16$ mM, which is likely. Such a degree of asymmetry is also suggested by the work of Baker and Widdas [27]. The only published data that are clearly inconsistent with such an asymmetric carrier model is the value for K_{in}^{ic} obtained by Hankin et al., 2.8 ± 0.5 mM. From Eqn 17, if Q is about 5 then K_{in}^{ic} would have to be about 9 mM. The estimate of Hankin et al. [9] is probably slightly low because the concentration of glucose outside the cell in their experiments was not truly saturating but only 60 mM, so that influx would have deviated from Michaelis–Menten kinetics [28]. It can be shown graphically that in the range of internal glucose concentration used in the experiments the result-

ing tendency to underestimate K_m would probably have been greater than the tendency to overestimate K_m caused by a diffusion step [26] inside the cell of the size suggested. Nevertheless, K_{in}^{ic} appears to be too small by a factor of at least two.

In conclusion, most of the kinetic parameters of glucose transport appear to be compatible with a moderately asymmetrical carrier model in which about 30% of the resistance to glucose flux at 20 °C is due to an activation-energy barrier or other diffusion-like step inside the cell. Since a modification of this model might explain the (only slightly) too low K_m values of infinite-cis fluxes and, if confirmed, the non-Michaelis–Menten behaviour of exchange fluxes, there seems no reason to reject the carrier theory in the face of evidence from studies of conformational changes in transport systems [10, 11].

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