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EVIDENCE OF MULTIPLE OPERATIONAL AFFINITIES FOR D-GLUCOSE INSIDE THE HUMAN ERYTHROCYTE MEMBRANE

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Summary

1. The Michaelis-Menten parameters of labelled D-glucose exit from human erythrocytes at 2°C into external solution containing 50 mM D-galactose were obtained. The K_m is 3.4 ± 0.4 mM, V 17.3 ± 1.4 mmol · l^{-1} cell water · min^{-1} for this infinite-trans exit procedure.

2. The kinetic parameters of equilibrium exchange of D-glucose at 2°C are K_m = 25 ± 3.4 mM, V 30 ± 4.1 mmol · l^{-1} cell water · min^{-1}.

3. The K_m for net exit of D-glucose into solutions containing zero sugar is 15.8 ± 1.7 mM, V 9.3 ± 3.3 mmol · l^{-1} cell water · min^{-1}.

4. This experimental evidence corroborates the previous finding of Hankin, B.L., Lieb, W.R. and Stein, W.D. [(1972) Biochim. Biophys. Acta 255, 126—132] that there are sites with both high and low operational affinities for D-glucose at the inner surface of the human erythrocyte membrane. This result is inconsistent with current asymmetric carrier models of sugar transport.

Introduction

Several papers recently have suggested that an asymmetric form of the mobile carrier model for sugar transport is consistent with the observed asymmetric transport of D-glucose across the human erythrocyte membrane [1—6]. The transport site at the inner surface is commonly considered to have a 10-fold lower affinity, but a 10-fold higher rate of transference to the other side of the membrane than the sites at the outer surface. This straightforward adaptation of the mobile-carrier model explains the apparent asymmetries in net entry and exit fluxes as well as the differences in operational affinity between the net entry, net exit and exchange transport systems for sugars whose transport is facilitated across the erythrocyte membrane.

The observation of Hankin et al. [7] and Ginsburg and Stein [8] that there is

at the inner membrane surface a site with a high affinity for sugar, in addition to a site with low affinity, is at variance with the predictions of the mobile-asymmetric-carrier-hypothesis and if correct is sufficient reason for rejection of this transport model.

At present there is no clear consensus as to the existance of a high affinity site for D-glucose at the inner surface of the erythrocyte membrane. Karlish et al. [9] were first to show that there is a low affinity site at the inner membrane surface. Later Hankin et al. [7] found by analyzing the change of rate of net D-glucose uptake into human erythrocytes at 20° C from external solutions containing high concentrations of D-glucose that another site with high affinity, $K_m \approx 3$ mM, exists at the inner surface. As glucose entry is rapid, the estimate of the high affinity K_m, using the integrated rate equation is subject to error. Ginsburg and Stein [8] therefore repeated the experiment using D-galactose, which because it has a lower affinity for the transport system than D-glucose gives an estimate of the high affinity K_m which is less subject to error. The high affinity K_m at the inner membrane surface for D-galactose was found to be 25 mM, which coexists with a low affinity site for D-galactose at the same surface, $K_m = 250$ mM. This newer estimate of the high affinity site K_m was corroborated using an alternative technique, in which initial rates of labelled D-galactose uptake were measured into cells containing varying initial concentrations of labelled D-galactose (infinite-cis entry).

The earlier data of Hankin et al. [7] has been criticised on the basis that the integrated rate equation method of solution is too unstable [10]. Lieb and Stein [11] have since refuted this argument and have shown that a K_m of 6.3 mM for the high affinity of the internal site for D-glucose is within a confidence interval of 1 : 100 for the estimate of internal K_m from the previous data [7].

Nonetheless, it must be admitted that the integrated rate equation is of limited accuracy for several reasons; because it is assumed that the cell volume changes which occur during the course of sugar uptake are ideal yet they may not be [12]; at the earliest sampling time, when the rate of change of influx is maximal, hence particularly important for accurate determination of the high affinity K_m, maximal correction for volume change must be made; furthermore the number of counts above background is necessarily small; and the error coefficient of the estimate of elapsed time before stoppage of influx is maximal. In other words, the most pertinent data are least reliable.

In order to obtain a satisfactory answer to what is now a question of critical importance to the interpretation of sugar transport across the erythrocyte membrane namely, the existence of a high affinity site inside the cell membrane, we have adopted an alternative approach to that of Stein and co-workers [7,8,11]. We have measured the rate of exit of D-glucose at different initial concentrations into solutions containing near saturating concentrations of D-galactose at 2° C. The reasons for adopting this approach are that sugar exit kinetics are more accurately determined than uptake kinetics because there is a much lower background at all times and initially the counts are highest, when most accuracy is required. The fluxes measured at 2° C, so were slow enough, (even at the lowest internal loading concentrations) to measure over a period of 40 s. D-Galactose was chosen as the external unlabelled cotransported sugar, as

it has a lower affinity than D-glucose and hence its uptake does not interfere grossly with labelled D-glucose exit.

Our results indicate that there is a high affinity site at the inner membrane surface for D-glucose, K_m = 3.8 mM.

In order to measure the infinite-trans exit kinetics we used unlabelled D-galactose in the external solution in preference to unlabelled D-glucose, for the following reason. D-Galactose has a 10-fold lower affinity for the sugar transport system than D-glucose. In the infinite-trans exit experiment to be described the cells are preloaded with D-glucose over a range of concentrations. During the initial flux period D-glucose emerging from the cell will exchange with sugar entering the cell. The uptake of unlabelled sugar will reduce the exit rate of labelled sugar by competition. To avoid having to correct for this effect unlabelled D-galactose was used.

Methods

The procedures were essentially the same for each set of experiments. The following solutions were used. Tris-buffered saline consisting of 150 mM NaCl, 20 mM Tris methylamine buffered to pH 7.4 with HCl. Stopping solution: 1 mM $HgCl_2$, 1.25 mM KI, 0.1 mM phloretin in 2% saline for small samples. Where larger samples of cell suspension were taken, stopping solution was concentrated appropriately. Scintillation fluid: 500 ml toluene, 500 ml Metapol HC 100 (Durham Chemicals Birtle, Tyne-and-Wear), 4 g PPO.

Erythrocytes obtained from heparinized blood, freshly drawn, were washed three times in Tris-buffered saline and were preincubated in saline solutions containing D-glucose at the required concentrations at a haematocrit of 20%, for 2 h, at room temperature for the lower concentrations and at 35° C for the higher concentrations. 10 min before the start of the kinetic runs D-[^{14}C]-glucose tracer was added to the cell suspensions. The cells were then concentrated to 80% haematocrit by centrifugation and then cooled to 2° C. The required volumes of packed cells were taken into a Hamilton microsyringe and at zero time injected into a beaker containing the appropriate bathing solution held in an ice bath and placed on a magnetic stirrer. This arrangement gives a steady solution temperature of 2 ± 0.5° C.

At suitable intervals and at 'infinite' time samples were withdrawn from the stirred suspension using a calibrated syringe and injected into tubes containing ice-cold stopping solution. These were then centrifuged at approx. 3000 × *g* for 4 min, the supernatant discarded and the cell pellet rewashed if necessary with stopping solution. The washed pellets of cells were broken up with a mechanical agitator and 2 ml of 50% w/v trichloroacetic acid added. The precipitates were packed by centrifuging for 1—2 min and then aliquots of trichloroacetic acid extract added to scintillation fluid for counting either in a Beckman or Packard Tricarb scintillation counter.

Individual procedures

Infinite-trans exit. Packed cells (0.5 ml) incubated with varying concentrations of D-glucose containing tracer were suspended in 9.5 ml of 50 mM galactose in saline solution. 1 ml samples were taken at 10—15-s intervals and

a zero time point determined by adding 0.5 ml of cells to 9.5 ml of stopping solution.

The exit rate was determined from the slope of the linear regression line to the equation: $\ln(C_t - C_\infty) = kt$ (where t is elapsed time, C is counts per min of sample and k is the rate constant (slope)).

Equilibrium exchange. This procedure is identical to that described above except that 50 mM galactose is the external bathing solution is replaced by unlabelled D-glucose at the same concentration as that to which the cells have been preequilibrated.

Infinite-cis exit (Sen-Widdas)

0.1 ml of packed cells incubated with labelled glucose solution 100 mM was resuspended in 100 ml of saline containing supernatant from the preincubated cells. This gives an external solution containing glucose at any concentration required with the same specific activity as that present within the cell. 9-ml samples were added to 2 ml of concentrated stopping solution and the exit rate determined as the time taken for all the glucose to leave the cells at the initial rate (exit time).

Zero-trans exit

Similar procedure to that above, but cells were loaded with varying concentrations of D-glucose and no glucose was present initially in the external solution. However the loading concentration was usually kept low (5—10 mM) in order to avoid backflux from the external solution which complicates analysis. The V and K_m were obtained from the integrated equation of Karlish et al. [9] in the form suggested by Ginsburg and Ram [13]:

$$\frac{-\ln S_t/S_0}{(S_0 - S_t)} = \frac{V}{K_m} \cdot \frac{t}{(S_0 - S_t)} - (1/K_m + 1/P)$$

where S_0 is the quantity of sugar contained in 1 l of cells at the start of the experiment, S_t is the amount contained in the cells at the time t and P is the tonicity of the cell content.

Results

Previously Lacko et al. [14], showed that the infinite-trans entry procedure gives an estimate of the K_m of the external membrane surface for D-glucose at 20°C and 0°C which are similar to those found using the infinite-cis net exit procedure. Consequently we decided that infinite-trans exit of labelled D-glucose into external solutions containing high concentration of an unlabelled transport sugar would be a satisfactory means of estimating the K_m of the high affinity site at the inner surface of the cell membrane. In order to prevent the unlabelled sugar which enters the cells from inhibiting labelled sugar exit, D-galactose was used in the external solution, as it has a 10-fold lower affinity for the transport system than D-glucose [8].

Effect of varying concentrations of D-galactose on labelled D-glucose exit from erythrocytes at 2°C

Fig. 1 shows the effect of varying the concentration of D-galactose contained

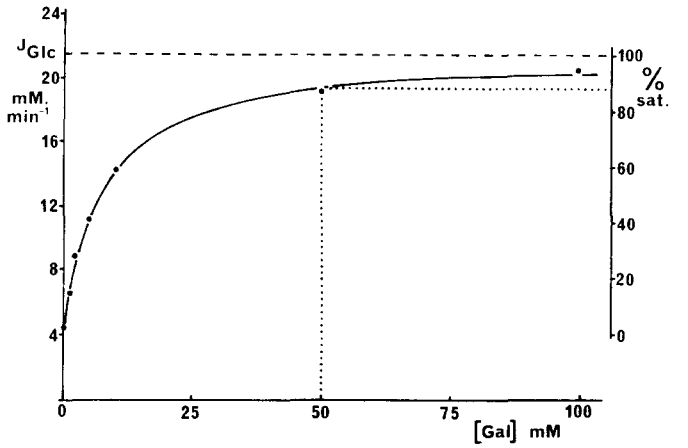

Fig. 1. Effect of external D-galactose on the efflux of D-[^{14}C]glucose from cells at 2° C. The K_m for the enhancement of efflux is determined as 7.5 mM and with 50 mM galactose as used in the experiments, the system is nearly 90% saturated.

in the external bathing solution (0—100 mM) on the rate of D-glucose exit from human erythrocytes at 2° C (see Methods). The K_m for D-galactose-dependent activation of D-glucose exit is 7.5 mM, and it can be seen that with 50 mM galactose in the external solution, the initial rate of D-glucose exit is approx. 90% maximal. 50 mM galactose was the external concentration used in all subsequent infinite-trans exit experiments.

Infinite-trans exit of D-glucose into 50 mM D-galactose at 2° C

Fig. 2 shows the effect of different initial concentrations of D-glucose on the loss of D-glucose into solutions containing 50 mM D-galactose. It can be seen that the plots of the data are linear. The exit rate decreases markedly when the

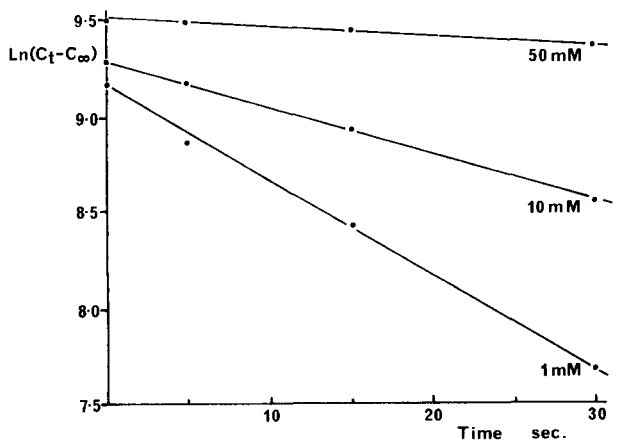

Fig. 2. Data from three experiments chosen to show the effect of increasing the loading concentration on the rate of D-glucose efflux into 50 mM galactose at 2° C.

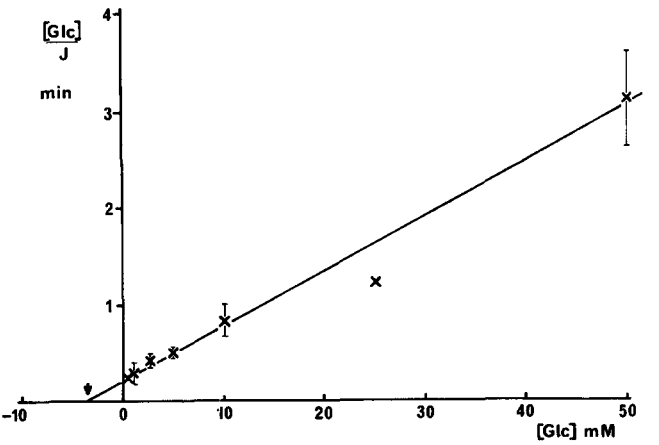

Fig. 3. Plot of results of infinite-trans exit of D-glucose into 50 mM D-galactose at 2° C. Linear regression line gives values: K_m = 3.8 ± 0.4 mM, V = 17.3 ± 1.4 mmol · l^{-1} cell water · min^{-1} (n = 19).

initial concentration of D-glucose is raised from 1 to 10 mM which shows qualitatively that there must be a high affinity site at the inner surface which can bind D-glucose.

A transformed plot of D-glucose infinite-trans exit at 2° C is shown in Fig. 3. K_m = 3.8 ± 0.4 mM; V = 17.3 ± 1.4 mmol · l^{-1} cell water · min^{-1}. This result shows that there is a high affinity site at the inner membrane surface. In order to demonstrate conclusively that there are multiple affinities for D-glucose at the inner membrane surface, we have redetermined the transport parameters of equilibrium exchange of D-glucose at 2° C.

Equilibrium-exchange of D-glucose at 2° C

Fig. 4 shows a plot of exchange rate as a function of the equilibrium glucose concentration. The K_m determined from the linear regression line of the data

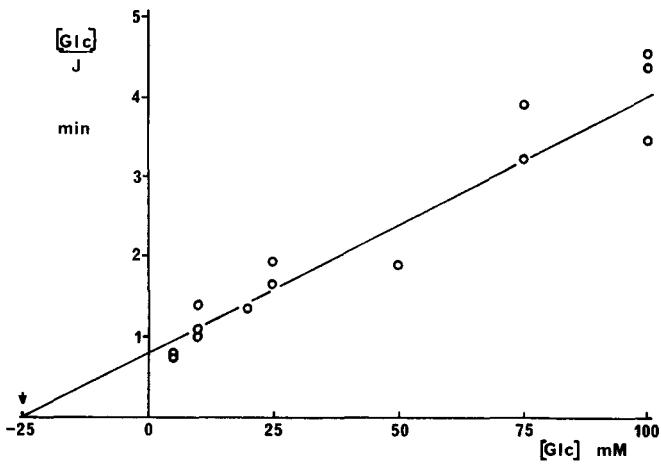

Fig. 4. Linear plot of rate of D-glucose exit into external D-glucose at the same concentration (equilibrium exchange) at 2° C. K_m = 25 ± 3.4 mM, V = 30 ± 4.1 mmol · l^{-1} cell water · min^{-1} (n = 14).

is 25 ± 3.4 mM; V = 30 ± 4.1 mmol · l^{-1} cell water · min^{-1}. As the only difference between this experiment and the infinite-trans exit experiment described in the previous section is that varied concentrations of D-glucose are present in the external solution, instead of a fixed concentration of galactose, these findings demonstrate clearly that variation in the conditions at the external surface alters both the affinity and the transfer rate of sugar at the inner surface. This indicates some form of linkage between the events occurring at either side of the membrane (see Discussion).

Since it is merely an assumption of the asymmetric carrier model that the high K_m for exchange is primarily determined by the low affinity of transport site at the inner surface [1—6], we decided that a more direct and unambiguous determination of the low affinity site K_m at the inner surface was required.

Zero-trans exit determination of the K_m for D-glucose at the inner membrane surface at 2° C

It has been shown by Sen and Widdas [15] that reduction in temperature increases the affinity of the external site for sugars. Since interpretation of the zero-trans exit procedure requires that the external binding sites are unsaturated, we first determined the affinity of the external surface for D-glucose with an infinite-cis exit procedure (see Methods).

Infinite-cis exit

We measured the initial rate of net exit of D-glucose from cells containing 100 mM D-glucose. The external solution contained varying concentrations of glucose at the same specific activity of [^{14}C]glucose as was present within the cells. Fig. 5 shows a plot of the effects of variation of the external glucose concentration on the initial rates of exit. From this plot we obtain a K_m for D-glucose at the external surface of 0.39 ± 0.06 mM and the V for infinite-cis net exit 8.6 ± 1.7 mmol · l^{-1} cell water · min^{-1} at 2° C. These results are consistent with the values found by Sen and Widdas [15] who used an optical method to

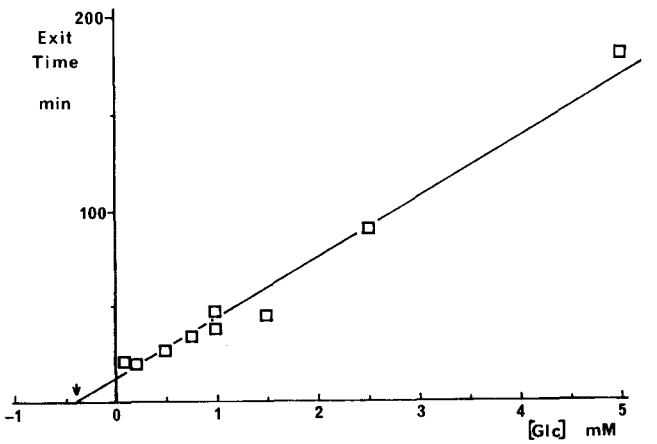

Fig. 5. Plot of the exit times of 100 mM D-[^{14}C]glucose into D-glucose of the same specific activity at 2° C. K_m = 0.39 ± 0.6 mM, V = 8.6 ± 1.7 mmol · l^{-1} cell water · min^{-1} (n = 17).

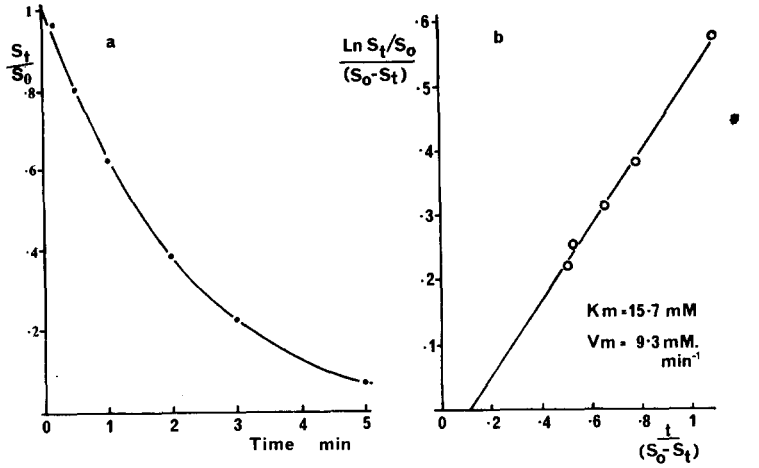

Fig. 6. (a) Fraction of D-glucose remaining in cells after resuspending cells loaded with 5 mM in a sugar-free medium at 2°C. (b) Points in Fig. 6a plotted according to the equation suggested by Ginsburg and Ram [13] to give transfer parameters. K_m = 16.26 mM, V = 9.56 mmol · l^{-1} cell water · min^{-1}. Mean values for n = 8. K_m = 15.8 mM, V = 9.3 mmol · l^{-1} cell water · min^{-1}.

determine effects of temperature on the operational parameters of infinite-cis exit.

Zero-trans net exit

To avoid significant saturation to the external surface with D-glucose from sugar emerging from the cell during the kinetic run and from sugar added initially to the external solution from extracellular source, we used a final haematocrit of 0.1% and the cells were loaded with only 5—10 mM D-glucose. Thus the final concentration of glucose in the external solution never rose above 10 μM which means that the external surface is never more than 2.5% saturated at 2°C.

Fig. 6a shows a plot of the fraction of labelled D-glucose remaining within the cells as a function of time following addition of the cells to the glucose-free external solution. Fig. 6b is a transformation of these data according to the formula of Ginsburg and Ram [13]. Using this transformation the K_m derived for zero-trans net exit is 15.8 ± 1.7 mM; V = 9.3 ± 3.3 mmol · l^{-1} cell water ·

TABLE I

Procedure	Operational parameters at 2°C
Infinite-trans exit	K_m = 3.8 ± 0.4 mM(n = 19)
	V = 17.3 ± 1.4 mmol · l^{-1} cell water · min^{-1}
Equilibrium exchange	K_m = 25 ± 3.4 mM(n = 14)
	V = 30 ± 4.1 mmol · l^{-1} cell water · min^{-1}
Infinite-cis exit [15]	K_m = 0.39 ± 0.06 mM(n = 17)
	V = 8.6 ± 1.7 mmol · l^{-1} cell water · min^{-1}
Zero-trans net exit	K_m = 15.8 ± 1.7 mM(n = 8)
	V = 9.3 ± 3.3 mmol · l^{-1} cell water · min^{-1}

min^{-1}. These results indicate that there is a significant difference between the K_m for infinite-trans exit and zero-trans net exit ($P < 0.001$) and also between the K_m for equilibrium exchange and zero-trans net exit (loading concentration 5—10 mM) ($P < 0.02$). The maximal rates of net exit measured by zero-trans or infinite-cis net exit procedures are similar, see Table I.

Discussion

The results described in this paper corroborate the previous results of Stein and coworkers [7,8,11] in that a high affinity site for transported sugar is demonstrated at the inner surface of the human erythrocyte membrane. These results clearly indicate that the main assumptions of the asymmetric mobile carrier, namely that there is a single carrier species at the inner surface of the cell with a low affinity, K_m approx. 15—30 mM, is incorrect, as it does not account for the high affinity sites observed here, or by Hankin et al. [7], and Ginsburg and Stein [8].

The asymmetrical carrier model proposed by Regen and Tarpley [5] has, in addition to the assumptions of the asymmetric mobile carrier, two additional parameters which are used to simulate the effects of unstirred layers at the inner and outer membrane surface. This model could be used to explain why the operational K_m for infinite-cis entry of D-glucose or D-galactose is lower than the K_m values for zero-trans exit. If the labelled sugar entering the cells were delayed from equilibrating with the whole of the cell fluid by the internal unstirred layer, this would reduce the operational K_m at the inner surface, since the 'real' concentration of sugar at the inner membrane surface would be higher than that estimated using the assumption that the sugar was equilibrated throughout the cell interior.

This reasoning cannot be equally applied to the K_m for infinite-trans exit, for in this case we are examining exit rates and to be consistent it would have to be argued that the unstirred layer effect should reduce the real concentration of radioactive sugar at the inner membrane surface so that the operational K_m should be raised by the unstirred layer effect. Additionally, the presence of unlabelled sugar in the microenvironment of the inner membrane surface will inhibit labelled sugar exit to some extent so will tend also to raise the operational K_m for infinite-trans exit. Hence we can conclude that the Regen and Tarpley model [5] does not adequately match the data either.

The symmetrical tetramer model proposed by Lieb and Stein [16] has both high and low affinity binding sites at the inner and outer membrane surfaces and is to this extent consistent with the observation that there are sites with both high and low affinities for D-glucose inside the cell membrane. However the tetramer model does not explain why the V for exit exceeds that for entry by a factor of 5—20 fold [8,14]. Hence this model may also be rejected.

A model proposed by Eilam [6] suggests that the sugar transport system consists of paired sets of asymmetric mobile carriers α and β, the majority of carrier pairs α have the low affinity site K^L at the inner surface and the high affinity site K^H outside, however the minority set of carrier pairs β have their low affinity site outside and the high affinity site inside, when $m = K^L/K^H = n = \alpha/\beta = 10$, the model predicts operational parameters consistent with the

simultaneous presence of high and low affinity sites at the inner surface, the higher rate of maximal net exit than net entry and fits with suggestions that there is a low affinity as well as a high affinity site at the external surface [13]. In our view the evidence for this external low affinity site is unconvincing [17] and certainly there is no evidence of any low affinity transport system detectable using the infinite-trans exit studies. However Eilam [6] claims that the low affinity site at the external surface will be undetectable. She also states that where n and m are large, i.e., between 10 and 20, infinite-trans exit should be dominated by the low affinity exit system; hence the K_m for infinite-trans exit should approach that of the low affinity site = 20—30 mM and the K_m for infinite-trans entry should be low 0.5—2 mM. The former prediction clearly is not in accordance with the experimental result reported here. Eilam's model also predicts that at low internal concentrations i.e., when $S_i = K_i^H = 3.8$ mM the exit velocity should be approx. 10% of the maximal exit velocity. In fact we find that the exit velocity is 50% of maximal. In other words the kinetics of infinite-trans exit are consistent with transport via a single system and not via two simultaneous parallel systems. A model which is consistent with all the kinetic data presently available has been described previously [17].

Briefly this model suggests that the asymmetry of the sugar transport system results from factors extrinsic to the membrane. D-Glucose has been shown to bind slowly and non-specifically to haemoglobin [17]. This binding is a reversible endothermic process resulting in a conformational change in haemoglobin as indicated by an increase in the viscosity of concentrated solutions following glucose binding [12].

A consequence of intracellular glucose binding is that during rapid net exit the free sugar concentration within the cell fluid is overestimated, hence the K_m for zero-trans exit is overestimated, the V for zero-trans exit is unaffected by intracellular kinetic compartmentation. Other consequences are that during net uptake, free sugar within the cell may be underestimated and the rate of net sugar uptake will be rate limited by the rate of reaction of the sugar with haemoglobin.

This reduces the operational V for infinite-cis net entry and hence explains the asymmetry of net transport across the human erythrocyte membrane.

The membrane transport system itself is considered to be symmetrical with similar high affinity binding sites at both inner and outer sides. Binding of a transported sugar to a site appears to cause a conformational change [17]. When both sides of the transport system bind sugar, the system can be considered as a pore across which rapid exchange occurs without any intermediate conformational change. This accounts for the higher maximal rate of equilibrium exchange flux than of net flux. Intracellular compartmentation with consequent transient reduction of the specific activity of the free sugar below the specific activity of the total intracellular sugar in part accounts for the high K_m for exchange.

This latter effect is reduced in the hetero-exchange situation examined here with infinite-trans exit of D-glucose, so the K_m for this process is much less affected by intracellular compartmentation of specific activity than is equilibrium exchange between sugars of equal affinity for the transport system.

The fact that this model implies that the conformation of the transport

system is different during exchange from that which obtains during net flux distinguishes it from the carrier model where it is assumed that the sugar carrier complex moves via an identical pathway during net or exchange movement; the modulation of the rate being determined by the distribution of carrier particles within the membrane resulting from differences in the rates of movement of free carrier and loaded carrier [5]. Hence the pore model as outlined here is consistent with the lower activation energy for exchange than for net flux [17].

The pore model is also consistent with the different effects of modifiers of transport on the exchange and net flux processes [17] and Baker and Naftalin (unpublished).

However, it has been pointed out [18] that a pore model with conformational changes induced by sugar binding is indistinguishable kinetically from a mobile carrier model, hence addition of the haemoglobin-glucose reaction to a symmetrical mobile carrier could as well account for the results described in this paper as the pore model outlined above. Hence the decision as to which model is the better must be based on criteria other than the kinetic parameters of sugar transport obtained at a single temperature.

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