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Evidence for non-uniform distribution of D-glucose within human red cells during net exit and counterflow

R.J. Naftalin, P.M. Smith and S.E. Roselaar

Department of Physiology, King's College London, Strand, London WC2R 2LS (U.K.)

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The kinetic parameters of net exit of D-glucose from human red blood cells have been measured after the cells were loaded to 18 mM, 75 mM and 120 mM at 2°C and 75 mM and 120 mM at 20°C. Reducing the temperature, or raising the loading concentration raises the apparent K_m for net exit. Deoxygenation also reduces the K_m for D-glucose exit from red blood cells loaded initially to 120 mM at 20°C from 32.9 ± 2.3 mM (13) with oxygenated blood to 20.5 ± 1.3 mM (17) ($P < 0.01$). Deoxygenation increases the ratio V_{max}/K_m from 5.29 ± 0.26 min^{-1} (13) for oxygenated blood to 7.13 ± 0.29 min^{-1} (17) for deoxygenated blood ($P < 0.001$). The counterflow of D-glucose from solutions containing 1 mM ^{14}C-labelled D-glucose was measured at 2°C and 20°C. Reduction in temperature, reduced the maximal level to which labelled D-glucose was accumulated and altered the course of equilibration of the specific activity of intracellular D-glucose from a single exponential to a more complex form. Raising the internal concentration from 18 mM to 90 mM at 2°C also alters the course of equilibration of labelled D-glucose within the cell to a complex form. The apparent asymmetry of the transport system may be estimated from the intracellular concentrations of labelled and unlabelled sugar at the turning point of the counterflow transient. The estimates of asymmetry obtained from this approach indicate that there is no significant asymmetry at 20°C and at 2°C asymmetry is between 3 and 6. This is at least 20-fold less than predicted from the kinetic parameter asymmetries for net exit and entry. None of the above results fit a kinetic scheme in which the asymmetry of the transport system is controlled by intrinsic differences in the kinetic parameters at the inner and outer membrane surface. These results are consistent with a model for sugar transport in which movement between sugar within bound and free intracellular compartments can become the rate-limiting step in controlling net movement into, or out of the cell.

Introduction

A number of problems concerning transport of monosaccharides by human red blood cells remain unresolved. Amongst these is the wide difference in apparent Michaelis-Menten parameters, K_m and V_{max} for sugar transfer from the inside of the cell membrane to the outside, when obtained by different methods.

Karlish et al. [1] using an integrated rate equation approach, observed that during net exit of D-glucose from cells loaded initially with 80 mM into large volumes of medium containing virtually zero glucose that the K_m was around 25 mM at 20°C. This finding has been confirmed several times since using radioactive tracers [2–4] and an optical method [5]. Using a rapid filtration method Brahm [6] has shown by using initial rates of exit from cells loaded to different levels that the K_m at 25°C is 5.6 mM. There is also a wide discrepancy

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between the K_m of 38 mM for equilibrium exchange measured by tracer methods [7,8] and the fast sampling approach adopted by Brahm [6] which gives a K_m of 8.1 mM.

Whilst improved resolution can be offered as an explanation for the difference between the results of Brahm [6] and Eilam and Stein [8], or Challis et al. [4], it cannot be realistically used to explain the difference between the data of Brahm [6] and of Carruthers and Melchior [5] who find with a stopped-flow spectrophotometric technique that the K_m for net efflux from whole red blood cells loaded initially to 70 mM at 20°C, is 32 mM.

A possible explanation for the difference is that the initial-rate method gives weighting to the early phase of exit, whereas the integrated-rate approach is more influenced by data from the middle period of the transient.

If, glucose is uniformly distributed within the cytosol, during the whole course of exit, there should be no gross difference between the estimates of K_m for exit using initial-rate data and data obtained from the integrated-rate equation. That large differences do exist suggests that the sugar is not homogeneously distributed within the cytosol during next exit.

Inhomogeneity of D-glucose distribution has been suggested previously by Miller [9] and Lieb and Stein [10] as a reason for the non-conformity of the observed patterns of uphill counterflow to predicted kinetics. Both Miller and Lieb and Stein deduced that labelled D-glucose entering the cells during uphill counterflow was not equilibrating fully with all the unlabelled D-glucose within the cells as the inflow of labelled sugar slowed too early.

Baker and Widdas [11] used the counterflow transients to match their asymmetric transport model. They assumed that the K_m at the external site for D-glucose was 1.1 mM at 16°C and that the K_m at the internal site was 10-times higher. However, they could only achieve a good fit of their model to observed transients by assuming that the activity of D-glucose inside the cell was 0.95. This assumption is operationally similar to the compartmental hypothesis, which was abandoned by Miller [7,9].

The apparent asymmetric behaviour of the human red blood cell sugar transport system has also been ascribed to D-glucose forming a loose association with an intracellular component, like haemoglobin [12,3]. With this model net exit of D-glucose has two serial components, sugar transfer across the symmetrical membrane transport system from the intracellular free sugar compartment and transferance of sugar bound to the haemoglobin to the free compartment.

One prediction of this model was that the K_m obtained from the initial rate of labelled D-glucose exit into external solutions containing a high concentration of unlabelled sugar (infinite trans exit) should be lower than the rate obtained from D-glucose exit using the integrated-rate equation approach. This was found to be the case at 2°C [3]. Challis et al. [4] using rapid sampling techniques confirmed this finding at 20°C.

Another prediction is that removal of haemoglobin should reduce the apparent K_m for zero-trans exit of D-glucose from the haemoglobin-free ghosts. This prediction was at first not confirmed [4], but then later confirmed [5].

The lower K_m for zero-trans net exit obtained from initial rates [6] is consistent with a serial compartmental model because the initial exit rate is unaffected by delays due to dissociation of ligand from internal sites as it comes directly from the 'free' compartment.

However, Carruthers and Melchior [5] have suggested that the extrinsic asymmetry of the sugar transport system is not due to compartmentation between bound and free form of sugar, but rather arises from a low molecular weight inhibitor acting at the inner membrane sites. This inhibition is unlikely to be caused by a metabolic product of D-glucose, as the asymmetry characteristics have also been noted with non-metabolizable sugars [13].

We have devised two further tests to determine whether sugar transport asymmetry is due to intrinsic or extrinsic factors. First, the effect of altering the loading concentration of D-glucose on the K_m and V_{max} of zero-trans exit has been measured at 20°C and 2°C. Second, the effect of varying temperature and loading concentration on the counterflow transients has been determined. The results of these experiments confirm the view that there is kinetic compartmentation of D-glucose within the human red cell which becomes

more pronounced as temperature is reduced and as the loading concentration raised. The results are inconsistent with the view that asymmetry results from a cytosolic inhibitor affecting the symmetry of the sugar transporter within the membrane or that the membrane transport system is intrinsically asymmetric.

Methods

The following solutions were used.

Phosphate-buffered saline: 147 mM NaCl, 20 mM Na_2HPO_4, pH was adjusted to 7.4 with HCl. The total osmolarity was 310 mosmol/l. In addition when the solutions were to be made isotonic with the glucose-loaded cells mannitol was added to the phosphate-buffered saline to bring it to the same concentration as the intracellular sugar.

Stopping solution: 1 μM $HgCl_2$, 1.25 mM KI, 0.1 mM phloretin added to phosphate-buffered saline 220 mM. This hypertonic solution prevents haemolysis by the $HgCl_2$.

Red cell extraction solution: a 5% w/v trichloroacetic acid was used to precipitate the cell protein and extract the radiolabelled D-glucose.

Red cells: Transfusion blood used before the expiry date was washed several times in phosphate buffer at a haematocrit of 10% at 24°C. In some experiments freshly drawn heparinized blood was used.

Zero-trans exit

Packed washed red cells were resuspended to a haematocrit of 20% in phosphate-buffered saline solution at 24°C containing 10 mM, 100 mM or 200 mM D-glucose. The solutions were washed and resuspended in the same solutions after an hour to avoid dilution of the glucose. After 3 h the cells were centrifuged at $1500 \times g$ for 5 min and the lightly packed cells were exposed to ^{14}C-labelled D-glucose for 15 min, during this time the cells were shaken gently to distribute the radioactive tracer homogeneously, (1 μCi/ml of cells) and cooled to the working temperature 2°C or 20°C for 15 min before flux measurements. The cells were then centrifuged and the supernatant removed.

A volume of loaded and labelled packed cells was taken into a Gilson automatic pipette and at zero-time projected into a beaker containing 50 ml of phosphate-buffered saline which was held at either 2 ± 0.5°C or 20 ± 0.5°C. and stirred continuously with a magnetic stirring bar at 600 r.p.m. At intervals, 1 ml of the cell suspension was removed and injected into centrifuge tubes containing 3 ml of ice-cold stopping solution. The tubes were centrifuged at $6000 \times g$ for 2 min, the supernatant removed and the cell pellet rewashed in 2 ml of stopping solution. The cell pellets obtained after the second wash were dispersed by mechanical agitation and extracted in 1.5 ml of trichloroacetic acid. The precipitates were packed by centrifugation for 5 min at $6000 \times g$ and 1 ml aliquots of the extract were added to 4 ml of scintillation fluid in 5-ml polythene vials and counted in a Packard Scintillation counter.

To avoid backflux of glucose into the cells, the final concentration in the external medium must be maintained at low values. The volumes of cells injected were calculated to ensure that the final concentration never rose above 0.1 mM. Thus for cells loaded with 200 mM D-glucose, (initial concentration after dilution with Ringer = 120 mM D-glucose (see Eqn. 2), 25 μl of packed cells were injected into 50 ml of phosphate-buffered saline. The efficacy of this protocol was checked by showing that further dilution did not affect the estimates of K_m and V_{max}.

Each kinetic run was done in triplicate and data points within each run were normalized to the zero-time reading before averaging. The estimates of K_m and V_{max} were obtained from the following relationship:

$$\frac{\ln(G_0/G_t)}{(G_t - G_0)} = \frac{V_{max}}{K_m} \cdot \frac{t}{(G_t - G_0)} - (1/K_m + 1/P) \qquad (1)$$

where G_0 is the amount of sugar contained in 1 litre of cell water at zero-time, G_t is the amount contained in the cell water of the same number of cells at time t, K_m and V_{max} are the Michaelis parameters. P is the osmolarity of impermeant salts (310 mosmol/litre cell water) and of mannitol when present. This relationship is a form of the integrated-rate equation transformed as previously suggested [3].

By plotting $+\ln(G_0/G_t)/(G_t - G_0)$ on the ordinate versus $t/(G_t - G_0)$ on the abscissa K_m

and V_{max} are obtained from the linear least-square fit of the slope and intercept of the line. When cells are added to Ringer without compensating for the extra osmolarity of the cell glucose, P (mosM) is the osmolarity of the Ringer without added glucose. The integrated rate equation, (Eqn. 1) accounts for the effects of volume changes due to the change in amount of sugar within the cell with time on intracellular sugar concentration assuming that it is uniformly distributed with the cell water.

Effect of deoxygenation

The effects of deoxygenation on the kinetic parameters of zero-trans exit were examined at 20°C. Deoxygenated blood was obtained by bubbling of O_2-free nitrogen into a 20% haematocrit cell suspension, 95% O_2 5% CO_2 into control cell suspensions. 10 seconds before a kinetic run 10 mM sodium dithionite was added to the buffer prepared for deoxygenated cells, or 10 mM Na_2SO_4 for O_2 loaded cells.

Sodium dithionite effects were tested on the stopping solution. No decrease in the efficacy of the stopping solution was observed. As the sugar fluxes were all determined in open solutions, it was impractical to control the level of cell oxygenation further. The efficacy of deoxygenation was simply checked using the spectral shift resulting from deoxygenation. This was evident and maintained throughout the flux period.

Counterflow experiments

This is a two part experiment.

(A) Uphill counterflow. The cells were loaded with unlabelled sugar, as above and the sugar and temperature equilibrated cells were added to 50 ml of external solution containing 1 mM ^{14}C-labelled D-glucose diluted from the supernatant of the batch used in (B) (see below), to a final haematocrit of 1%. 1 ml aliquots of cell suspension were taken at intervals and added to stopping solution as described above. The cells were washed twice in stopping solution before extraction in trichloroacetic acid, as described above. This avoids error due to trapping of label in the extracellular fluid.

(B) Net exit of labelled D-glucose into 1 mM labelled D-glucose. The same batch of glucose-loaded cells as in (A) above was labelled with ^{14}C-labelled D-glucose as described in the Methods for zero-trans exit. After equilibration of the label at a known concentration, e.g. 100 mM the cells were centrifuged out of suspension and the supernatant containing 100 mM D-glucose at the same specific activity of ^{14}C-glucose as was present in the cells was removed. This supernatant was added to phosphate-buffered saline to bring the concentration to 1 mM ^{14}C-labelled D-glucose.

The time-course of net exit of ^{14}C-labelled D-glucose from cells into 50 ml of external solution containing 1 mM ^{14}C-labelled D-glucose at the same specific activity as present in the cells was obtained as described above.

At 2°C, D-glucose equilibration across the cell membrane takes a long time, so after 2 h incubation, to obtain the equilibrium values of intracellular counts the cells were brought to 20°C and incubated for a further 30 min at the raised temperature.

Estimation of operational asymmetry using counterflow data

The conventional criterion for asymmetry of the red cell membrane sugar transport system is the difference between the K_m and V_{max} for zero-trans net entry and exit of D-glucose.

The data derived from uphill counterflow gives an alternative method of determining the degree of asymmetry existing in the transport system.

It is assumed that at all times, sugar is uniformly distributed within the cell interior and that the inside and outside of the cell membrane have a single type of transport site characterized by V_i, K_i and V_o, K_o. Here, subscripts to V and K, i and o, refer to inside and outside V_{max} and K_m respectively. A non-accumulating transport system requires [12,13]:

$$V_o/K_o = V_i/K_i$$

Hence, $K_i/K_o = V_i/V_o = a$; where a is the asymmetry ratio.

During the counterflow transient, the uphill movement of radioisotope S reaches a maximum and then declines (see Fig. 5). The maximal concentration to which the radioactive sugar is accumulated, S_{max} can be calculated, if the cell volume at time t_{max} and the equilibrium concentration of

labelled sugar at infinite time are known.

The concentration of unlabelled sugar, C_{max} present within the cell at the turning point of the counterflow transient time, 't_{max}', may be estimated by subtracting the concentration of radioactive sugar from the total amount of sugar within the cell at t_{max} estimated from the transient obtained by method (B).

The relative cell volume, V_r with respect to the isotonic volume may be estimated from run B as follows

$$V_r = \frac{G_t + \text{OsM}}{\text{OsM}} \tag{2}$$

where G_t is the total amount of cell sugar (mosmol/liter cell water) at time t and OsM is the osmolarity of non-diffusible solute within the cell = 310 mosmol/liter cell water in isotonic Ringer.

When the labelled sugar concentration within the cell is at the counterflow maximum, inflow of labelled sugar = outflow of labelled sugar.

Inflow = (probability of sugar occupying a binding site at the cis surface) × (the probability of the sugar moving to an unoccupied site on the trans (inner) surface).

Probability of sugar occupying a binding site at the cis surface is;

$$S_o/(K_o + S_o).$$

Probability of an empty site at the trans surface is;

$$1 - S_i/(K_i + S_i + C_i) = (K_i + C_i)/(K_i + S_i + C_i).$$

Hence:

$$\text{inflow sugar } v = \frac{V_o \cdot S_o}{K_o + S_o} \cdot \frac{a \cdot K_o + C_i}{a \cdot K_o + S_i + C_i}$$

and since $a \cdot V_o = V_i$ and $a \cdot K_o = K_i$

$$\text{outflow of sugar } v = \frac{a \cdot V_o \cdot S_i}{a \cdot K_o + S_i + C_i} \cdot \frac{K_o}{K_o + S_i}$$

Thus at the time of peak label concentration, t_{max}

$$\frac{S_o}{S_o + K_o} \cdot \frac{a \cdot K_o + C_{max}}{a \cdot K_o + S_{max} + C_{max}}$$

$$= \frac{a \cdot S_{max}}{a \cdot K_o + S_{max} + C_{max}} \cdot \frac{K_o}{K_o + S_o}$$

Hence, when $S_o = 1$ mM

$$a = \frac{C_{max}}{K_o(S_{max} - 1)}$$

The value of the asymmetry factor, a, is fully determined, since the K_m of the external site is known. (From the infinite-cis Sen-Widdas exits it is approximately 0.7 mM [14,15]); S_o = the initial concentration of sugar in the external solution, S_{max} and C_{max} are readily determined from counterflow experiments (A) and (B) (see above).

Providing that intracellular D-glucose is uniformly distributed, the asymmetry factor a [11,13] estimated at the counterflow maximum should be the same as that estimated from the ratio of K_i/K_o for zero-trans exit/entry.

However, as will be shown in Results, this is not the case, hence an alternative model for sugar transport must be considered.

A model of red cell sugar transport

A model for sugar transport which suggested that intracellular glucose was loosely bound to haemoglobin and movement between free and bound glucose was slow enough to limit the rate of net sugar exit across the cell membrane has been described previously [12,3].

As no analytical solution is available for the flow equations, numerical solutions to the set of differential equations defining sugar flow have been obtained using fourth-order Runge-Kutta numerical integration using a BBC microcomputer equipped with a 6502 second processor and output displayed graphically on a dot-matrix printer.

The system is considered to consist of three compartments. Compartment 1, contains the bound sugars S_1 and R_1. S and R are considered to be isotopes of the same sugar with the same rate constants and affinities in all parts of the system. Compartment 2 contains the free intracellular sugar and compartment 3 contains the extracellular sugar, which is uniformly distributed.

S and R refer to the concentrations of sugar isotopes in their appropriate compartments (suffixed number).

Flow of sugars between compartments 1 and 2 is considered to be related to the concentration difference between the compartments. Thus

$$H_1 = L_1 \cdot (S_2 - S_1) \quad \text{(M1)}$$

$$G_1 = L_1 \cdot (R_2 - R_1) \quad \text{(M2)}$$

where H and G are the rates of flow between compartments 2 and 1 of sugar isotopes S and R, respectively.

In order to simulate accelerated-exchange transfer across the membrane, it was assumed that the maximal rate of transferance is proportional to the fractional saturation of sugar binding sites at both the inside and outside of the membrane sugar transport system so that sugar binding at one side opens one of the two 'gates' on either side of the membrane.

Thus, when net movement across the membrane transport system is rapid, as with zero-trans exit the cell behaves as a two compartment system as the rate of sugar transfer between bound and free forms becomes rate limiting to net exit. However, when net transport across the cell membrane is the rate-limiting step, as with inhibited systems e.g. infinite-cis exit or inhibitor studies of exit, the transport system behaves as a one compartment quasi-symmetrical process. The rate of sugar movement across the membrane between 2 and 3 is considered to depend on two factors: (1) the difference in fractional saturation of sites at the inner and outer surface of the membrane and (2) the rate of movement between the sites.

It is assumed that the transport sites are in quasi-equilibrium with the solutions in the adjacent solutions. Hence, the fractional saturations of S and R at the inner surface of the membrane, x_{S2} and x_{R2}, respective, are

$$x_{S2} = S_2/(K_2 + S_2 + R_2) \quad \text{(M3)}$$

$$x_{R2} = R_2/(K_2 + S_2 + R_2) \quad \text{(M4)}$$

The fractional saturations of S and R at the outer surface of the membrane, x_{S3} and x_{R3} are

$$x_{S3} = S_3/(K_3 + S_3 + R_3) \quad \text{(M5)}$$

$$x_{R3} = R_3/(K_3 + S_3 + R_3) \quad \text{(M6)}$$

where K_2 and K_3 are the dissociation constants of both S and R at the inner and outer sites, respectively.

Hence, the rate of net movement of S and R between the inner and outer sites is a function of:

$$x_{S2} - x_{S3} \quad \text{(M7)}$$

and

$$x_{R2} - x_{R3} \quad \text{(M8)}$$

Additionally, the rate of ligand movement across the membrane is considered to be accelerated by the fractional occupancy of all the ligand binding sites on both sides of the membrane.

Hence:

$$H_2 = (1 + x_{S3} + x_{R3}) \cdot (x_{S2} - x_{S3}) \cdot L_2 \quad \text{(M9)}$$

$$G_2 = (1 + x_{S3} + x_{R3}) \cdot (x_{R2} - x_{R3}) \cdot L_2 \quad \text{(M10)}$$

$$H_3 = (1 + x_{S2} + x_{R2}) \cdot (x_{S2} - x_{S3}) \cdot L_3 \quad \text{(M11)}$$

$$G_3 = (1 + x_{S2} + x_{R2}) \cdot (x_{R2} - x_{R3}) \cdot L_3 \quad \text{(M12)}$$

where H_2 and G_2 are the transport rates of S and R from compartments 2 to 3, respectively, and H_3 and G_3 are the rates of transfer of sugars from compartments 3 to 2 of S and R, respectively.

Since the limit of $x_{S3} + x_{R3} = 1$ and the limit of $x_{R2} + x_{S2} = 1$, it follows from Eqns. M9–M12 that exchange rate can only be accelerated by a factor of 2 above net flux. This could be adjusted for other models in which accelerated exchange is much greater than 2-fold stimulation.

The amounts of sugars S and R in compartment 1, AS_1 and AR_1 are the products of concentrations, S_1 and R_1 at any time t and the volume of compartment 1, V_1; similarly the amounts of S and R in compartment 2 are AS_2 and AR_2 ($V_2 \cdot S_2$ and $V_2 \cdot R_2$), respectively.

The total intracellular volume V_t consists of 3 components, V_1, V_2 the bound and free water and B inaccessible space.

$$F = V_2/V_1 \quad \text{(M13)}$$

For simplicity, it is assumed that the osmotic pressure within the cell is proportional to the total amount of bound sugar confined within V_1 and that the ratio of $V_2/V_1 = F$ is a constant.

Hence the cell volume relative to isotonic volume V_r is

$$V_r = ((C + (R_1 + S_1) \cdot (1 - F)) - (S_3 + R_3)) \cdot (1 - B)/(C + B) \quad \text{(M14)}$$

where C is the osmolarity of non-diffusible solute in isotonic solution.

From Eqns. M13 and M14

$$V_1 = (V_r - B)/(1 - F) \quad \text{(M15)}$$

and

$$V_2 = V_1 \cdot F$$

The time-dependent changes in the amounts of sugar and the concentrations of sugars in compartments 1 and 2 can be obtained by integration of the differential equations M1, and M2, M9–M15.

The model is simplified by assuming that the membrane has symmetrical transport parameters, i.e. $K_2 = K_3$ and $L_2 = L_3$.

Results

Effects of changing the loading concentration of D-glucose at 2°C and 20°C on the kinetic parameters of zero-trans exit

Figs. 1a and 1b are typical sets of data showing glucose exit from cells loaded to initial concentrations of 120 mM and 75 mM and the transformed lines from which the kinetic parameters are derived. The lines to which the points are fitted in Fig. 1a are the best-fit lines obtained from solution of the integrated-rate equation lines to the later period of exit. It is not possible to fit both the early and late points on the same lines, as initially sugar exit is faster than would be predicted from the equation parameters fitting the later points.

Table I shows that both the K_m and V_{max} are significantly lower in exits from deoxygenated blood than from oxygenated controls. The ratio V_{max}/K_m is 35% greater for exits from deoxygenated blood than control cells. As this ratio is assessed directly from the slope of the transformed lines it is inherently more reliable than the estimates of either K_m, or V_{max}, which are estimated from the intercept and slope. Table I also shows results obtained from zero-trans exit at 20°C from normally oxygenated cells loaded initially to 75 mM. It can be seen that the K_m is significantly lower and the ratio V_{max}/K_m significantly higher in cells loaded with 75 mM than in cells loaded to 120 mM. The estimated V_{max} does not differ significantly between the test groups.

The results obtained from zero-trans exit at 2°C

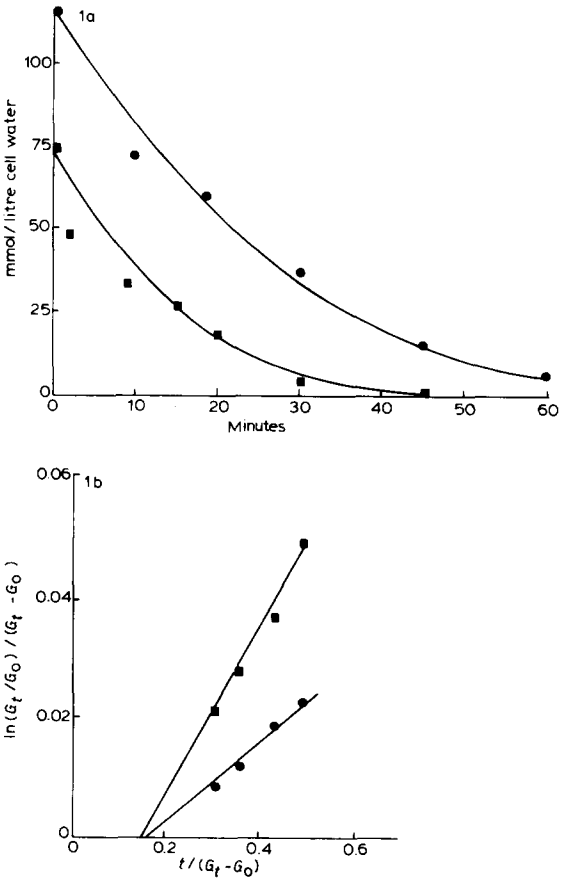

Fig. 1. (a) Time-course of loss of D-glucose from human red cells at 2°C. ●———●, initial concentration 120 mmol/litre cell water; ■———■, initial concentration 75 mmol/litre cell water. The lines fitted to the points were generated from the integrated-rate equation with $K_m = 150$ mM; $V_{max} = 9$ μmol/ml cells per min for the cells loaded to 120 mM and for cells loaded to 75 mM: $K_m = 75$ mM; $V_{max} = 8$ μmol/ml cells per min. Each point is the mean from three independent runs. Errors are within the areas of the points. (b) Transformed data of the time-course of loss of D-glucose from cells. K_m and V_{max} are obtained from the intercept and slope of the lines (see Methods).

from normally oxygenated blood, loaded to initial concentrations of 7.5 mM, 75 mM and 120 mM D-glucose are shown in Table II. The apparent V_{max} falls significantly and K_m rises as the loading concentration of D-glucose is raised. The ratio V_{max}/K_m decreases significantly as the loading concentration is increased. Comparison of the results in Tables I and II shows that reducing temperature from 20°C to 2°C reduces V_{max} by an order of magnitude, the ratio V_{max}/K_m by two orders of magnitude and increases K_m by 4–5-fold.

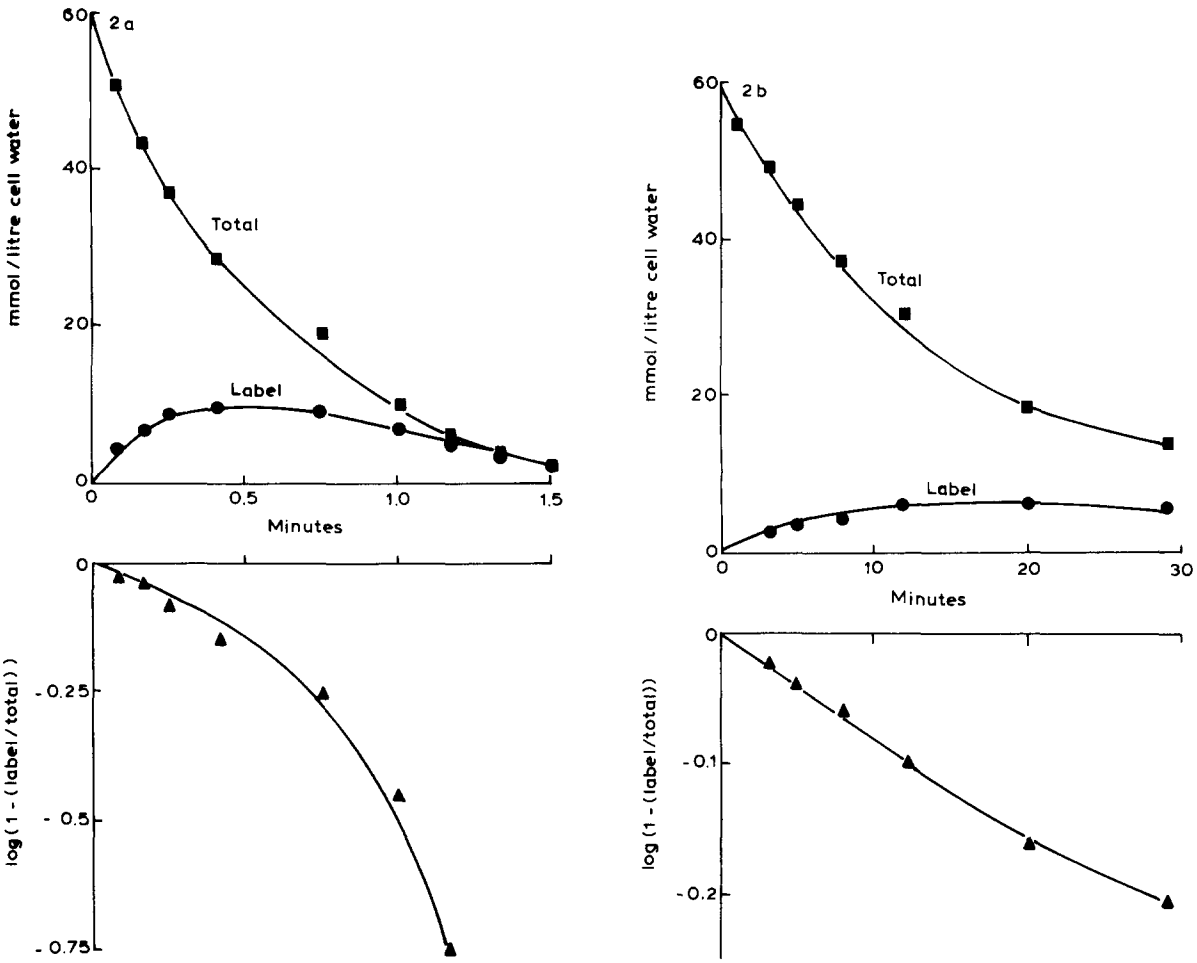

Fig. 2. (a) The time-course of the counterflow transients of ^{14}C-labelled D-glucose entry into cells preloaded to 60 mM unlabelled D-glucose at 20°C. Upper line (■———■) is the time-course of the change in total sugar within the cells (labelled + unlabelled sugar). The lower trace (●———●) is the time-course of labelled sugar entry from the external solution. Each point is the mean of four independent estimates of intracellular sugar concentration. Errors lie within the spots. The lines are fitted by eye. The lower trace (▲———▲) plots the specific activity increase in intracellular D-glucose. At 20°C this is a single exponential for at least 70% of the time-course. (b) The time-course of the counterflow transients of ^{14}C-labelled D-glucose entry into cells preloaded to 60 mM with unlabelled D-glucose at 2°C. Upper line (■———■) shows total sugar, lower line (●———●) the uptake of label from the external solution. The line in the lower panel (▲———▲) is the time-course of specific activity change. The line tends to flatten beyond 12 min ($n = 4$ for each point).

Counterflow

Figs. 2a and 2b show the transient changes in total cell D-glucose and uptake of label from the external solution containing 1 mM ^{14}C-labelled D-glucose into cells containing an initial concentration of 60 mM of unlabelled D-glucose at 20°C and 2°C. Reducing the temperature from 20°C to 2°C has the following effects; it slows both the rate of net exit and of uptake of label; it delays the time when labelled sugar uptake reaches its peak; it reduces the maximal accumulation of label by approximately 3-fold and reduces the specific activity of the intracellular label at the turning point, as estimated from the ratio of labelled sugar/total sugar at the turning point 0.46 ± 0.07 ($n = 4$) to 0.35 ± 0.05 ($n = 4$) of the equilibrium value.

Fig. 3a shows counterflow data of uptake into

TABLE IA

LOADING TO 120 mM D-GLUCOSE AT 20°C

Units: K_m in mM; V_{max} in mmol/litre cell water per min; V_{max}/K_m in min^{-1}.

Deoxygenated blood			Oxygenated blood			
K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	
13.56	123.22	9.07	44.22	191.1	4.32	
19.67	122.03	6.2	30.87	157.22	5.09	
22.39	150.12	6.7	41.47	167.31	3.99	
28.82	189.91	6.57	18.45	130.7	7.08	
14.54	120.24	8.27	25.61	140.73	5.49	
23.62	151.18	6.4	31.85	175.33	5.5	
18.53	141.07	7.61	40.48	208.42	5.15	
21.52	126.81	5.89	30.98	183.51	5.76	
21.46	146.09	6.8	31.15	179.51	5.76	
16.67	127.51	7.65	20.5	140.99	6.87	
16.00	121.91	7.62	33.19	162.24	4.88	
25.21	154.17	6.11	44.62	197.20	4.41	
20.84	147.4	7.07	34.25	155.28	4.53	
32.15	162.43	5.05				
24.56	152.32	6.20				
15.35	127.73	8.32				
12.96	125.41	9.67				
20.46 *	140.56 *	7.13 *	32.93	168.43	5.29	mean
±1.3	±4.56	±0.29	±2.31	±6.5	±0.26	±S.E.
(17)	(17)	(17)	(13)	(13)	(13)	(*n*)

* $P < 0.001$, deoxygenated blood vs. oxygenated blood.

TABLE IB

LOADING TO 75 mM D-GLUCOSE AT 20°C

Units as in Table IA.

K_m	V_{max}	V_{max}/K_m	
17.6	83.5	4.7	
19.9	170.9	8.6	
18.9	167.5	3.6	
18.1	160.2	8.8	
18.4	159.1	8.6	
14.2	147.1	10.4	
12.0	130.7	10.9	
22.8	177.3	7.8	
17.7 **	149.5	7.9 **	mean
±1.1	±10.7	±1.0	±S.E.
(8)	(8)	(8)	(*n*)

** $P < 0.001$, 75 mM vs. 120 mM D-glucose loading.

cells initially loaded to 18 mM and Fig. 3b uptake into cells initially loaded to 90 mM. Apart from the difference in rate of equilibration, there is a surprisingly small difference in maximal accumulation of label. In cells loaded initially to 18 mM D-glucose, the uptake of label is maximal after 4 to 5 minutes incubation. At this time, the amount of sugar remaining within the cells is 5.8 ± 0.3 mM and labelled sugar is present at 2.5 ± 0.2 mM. Thus the specific activity of label within the cell at the turning point is 43% of maximum (the specific activity in the external solution). In cells loaded initially to 90 mM, the maximal accumulation of label is attained between 30 and 40 minutes after the start of uptake. At this time the total sugar concentration within the cell was 36 ± 4.1 mM ($n = 4$) and of labelled sugar was 9 ± 1 mM ($n = 4$). This gives a specific activity ratio at the turning point of 20 ± 3% of maximal.

From the counterflow data shown in Figs. 2 and 3, it is possible to estimate the asymmetry factory '*a*' for each condition. The maximum estimated asymmetry factor obtained with a loading concentration of 90 mM at 2°C is not more than 6.4 ± 0.9. The asymmetry factor is 3.1 ± 0.7 and 3.1 ± 0.4 at loading concentrations of 60 and 18 mM, respectively. At 20°C the asymmetry factor is 0.9 ± 0.2. These asymmetries are markedly different from those estimated from the ratio of K_m(zero-trans exit)/K_m (infinite cis) which at 2°C is approx. 100 and at 20°C 10 (Table III).

Specific activity changes

The time-dependent changes in intracellular specific activity of D-glucose during counterflow are shown in Figs. 2 and 3 (lower panels). At 2°C when the cells are loaded to 60 mM D-glucose, or 90 mM D-glucose, the increase in intracellular specific activity does not resemble a single exponential.

Discussion

Zero-trans exit

The kinetic parameters for zero-trans net exit derived using the integrated rate equation approach must be treated with caution. Because the lines obtained from the transformed data are very steep, small deviations in the slopes give large changes in the estimates of the transport parameters. Nevertheless, it can be seen in Tables I and II that there is a statistically significant increase in

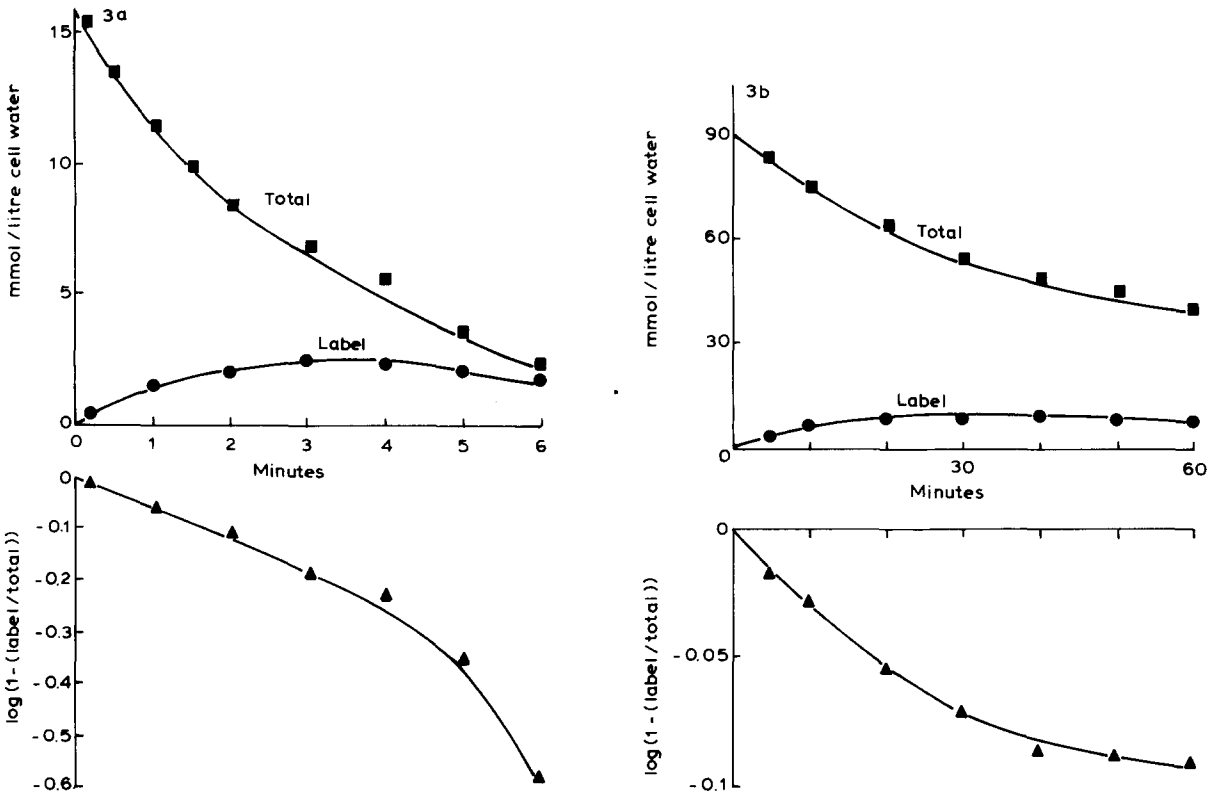

FIg. 3. (a) The time-course of counterflow transients of ^{14}C-labelled D-glucose entry into cells preloaded with 18 mM D-glucose at 2°C ($n = 4$ for each point). (b) The time-course of counterflow transients of ^{14}C-labelled D-glucose entry into cells preloaded with 90 mM unlabelled D-glucose.

the apparent K_m on increasing the loading concentration at both 2°C and 20°C. There is also a small, but significant effect of deoxygenation. Perhaps more important than the effect on the individual parameters of V_{max} and K_m, are the effects of deoxygenation on the ratio V_{max}/K_m, which is

TABLE II

EFFECT OF VARYING LOADING CONCENTRATION ON KINETIC PARAMETERS OF ZERO-TRANS EXIT AT 2°C

For units see Table IA.

7.5 mM			75 mM			120 mM			
K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	
52.2	20.2	0.38	100.2	13.6	0.13	100	5.98	0.060	
36.3	11.7	0.32	61.2	8.4	0.13	143.7	8.6	0.06	
35.4	11.05	0.31	52.1	8.3	0.16				
39.81	17.18	0.43	71.8	8.65	0.12				
37.75	12.64	0.33	46.2	6.1	0.13				
			99.8	12.5	0.12				
40.3 *	14.6	0.35 *	71.9	9.6	0.13	121.9	7.3	0.06	mean
±2.7	±1.8	±0.02	±9.6	±1.2	±0.01	±21.8	±1.3		±S.E.
(5)			(6)			(2)			(*n*)

* $P < 0.001$, 7.5 mM vs. 75 mM glucose loading.

TABLE III

THE CONCENTRATIONS AT THE COUNTERFLOW MAXIMA OF TOTAL, LABELLED AND UNLABELLED SUGAR WITHIN THE CELL WATER

Loading concn. (mM)	Total sugar (mM)	Labelled sugar (mM)	Unlabelled sugar (mM)	Label/Total	'a'
2°C					
18 ($n = 4$)	6.8 ± 0.3	2.5 ± 0.2	4.3 ± 0.4	0.36 ± 0.03	3.1 ± 0.4
60 ($n = 4$)	18.8 ± 2.5	6.6 ± 0.6	12.2 ± 2.6	0.35 ± 0.05	3.1 ± 0.7
90 ($n = 4$)	45 ± 4	9 ± 1	36 ± 4.1	0.2 ± 0.03	6.4 ± 0.9
20°C					
60 ($n = 4$)	20 ± 3	9 ± 0.5	10.7 ± 3.0	0.46 ± 0.07	0.9 ± 0.2

K_o at 20°C = 1.5 mM; K_o at 2°C = 0.7 mM (Refs. 14 and 15).

determined from the slope alone. This indicates the deoxygenation leads to more rapid loss of D-glucose from the cells. As considerable precautions were taken to avoid error due to sugar in the external solution, it is unlikely that errors due to this source can materially affect the interpretation of the main findings, namely that raising the loading concentration or reducing temperature raises K_m.

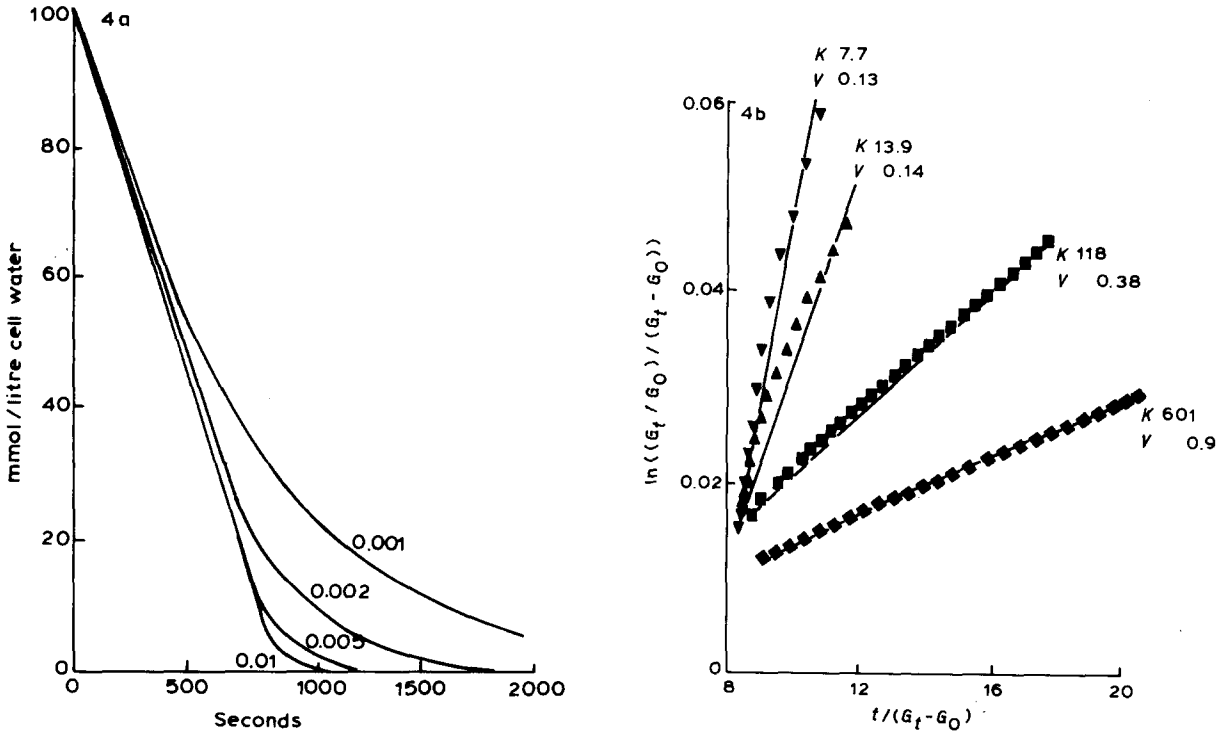

Fig. 4. (a) Simulation of zero-trans net exit from cells loaded initially with 100 mmol/litre cell water. Ordinate shows the net loss from both bound and free compartments. The lines show the effects of changing the rate constant of sugar movement between bound and free compartments at 0.01, 0.05, 0.002 and 0.001 s^{-1}. In all cases the ratio of volume of free compartment, V_2 to bound sugar compartment, V_1; $F = 0.1$. The K_m for the membrane transport system is 0.75 mM and symmetrical. $V_{max} = 6$ μmol/ml cells per min. The initial rates of exit are unaffected by the bound compartment. (b) The data from (a) are transformed using the standard integrated-rate equation formula. The lines shown are truncated to avoid the early non-linear portion of the transformed line (lower part of the lines).

Effects of deoxygenation and cold-storage on sugar transport

The mechanism whereby oxygenation affects the apparent kinetic parameters of net sugar exit is unknown. It is possible that alteration in quaternary structure of haemoglobin affects the rate at which glucose binds to and dissociates from the protein.

Recently Weizer, Razin and Stein [16] have reported that the K_m for equilibrium exchange rises after cold storage of the cells. We have found that the K_m for zero-trans exit from fresh cells at 20°C loaded with 120 mM D-glucose is 19.35 ± 1.45 ($n = 7$) compared with 32.0 ± 4.9 mM ($n = 5$) measured from cold-stored cells. This result would tend to confirm the earlier report [16]. Jacquez [17] has suggested, on the basis of experiments with ATP replacement, that the kinetics of zero-trans influx at 5°C are affected by loss of cell ATP. He suggests that the number of effective transport proteins can be modulated by the metabolic state of the cells. These apparent changes in kinetic parameters are also consistent with alterations in factors extrinsic to the transport system. The effects of deoxygenation shown here imply that haemoglobin is involved in this process.

Simulation of zero-trans exit from cells

Simulation of zero-trans exit with varying rates of equilibration of sugar between bound and free compartments, shows that retarding sugar equilibration between bound and free compartments increases the apparent K_m and V_{max} and reduces the ratio V_{max}/K_m (Figs. 4a and 4b). The initial rate of sugar exit is unaffected by the rate of equilibration between the bound and free compartments as the initial loss of sugar is from the free compartment. However, whenever the sugar within the free compartment becomes depleted, sugar exit is rate limited by desorption from the bound compartment. This leads to an apparent increase in K_m, as estimated by the integrated-rate equation method (Fig. 4b).

Alteration of the loading concentration has only a small effect on the apparent K_m for zero-trans exit. The large observed effect of raising the loading concentration on K_m and V_{max} is consistent with a sugar concentration dependent effect on the rate of equilibration of sugar between bound and free compartments. As the sugar concentration rises, the rate of equilibration falls thereby raising the K_m for zero-trans exit. This effect has not been implemented in the model here, which is used simply to illustrate the point that slow equilibration between bound and free compartments raises the apparent K_m and V_{max} for zero-trans exit when employing the integrated rate equation approach to determination of the kinetic parameters. The model indicates that the effect of oxygenation and cold storage and reduction in temperature on

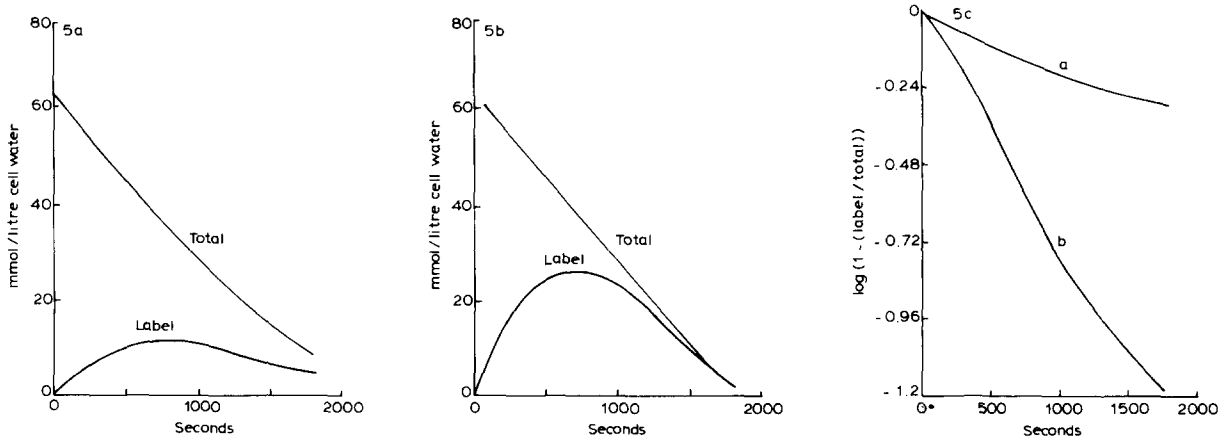

Fig. 5. Simulation of counterflow into cells with (a) slow intracellular equilibration and (b) rapid equilibration. Slow equilibration reduces the height of the counterflow peak and changes the plot of apparent equilibration of intracellular specific activity from a single-exponential to a more complex form (c).

K_m are also likely to be due to a decreased rate of equilibration of sugar between bound and free compartments.

Counterflow

Previous attempts to model counterflow on the basis of uniform intracellular concentrations of sugars have failed to simulate the early turning point in the uphill counterflow [9–11]. During counterflow, the specific activity of entering labelled sugar should increase exponentially until the specific activity of sugar within the cell is the same as outside the cell according to the following relationship.

$$\ln(1-(C_{label}/C_{total})) = -kt$$

where C_{label} and C_{total} are the concentrations or amounts of labelled and total = (unlabelled + labelled sugar) within the cell at any time t.

At 2°C, the specific activity of labelled D-glucose within the cells does not increase as a single-exponential function as would be predicted with equilibration into a single compartment (Fig. 3, lower panel).

Simulation of counterflow with a single intracellular compartment (Fig. 5b or two intracellular compartments (Fig. 5a), with slow equilibration of sugar between the two compartments shows that the apparent premature decrease in rate of label uptake is emulated when there is a significant delay in equilibration of label between the intracellular compartments (Figs. 5a and 5b). Plots of the time-dependent change in apparent specific activity of intracellular label are shown in Fig. 5c. In the case of a single intracellular compartment (curve a), entry of label behaves as a single exponential, whereas with two compartments (curve b), the rise in apparent intracellular specific activity does not behave as a single-exponential function.

Other features of the compartmental model [12,3,17]

The model of sugar transport presented here permits all the apparent kinetic parameter variations to be simulated (Table IV) using a single set of parameters.

This means that sugar transport in human red cells may be interpretated within the framework of simple physical parameters. These kinetic features fall into two categories: (A) asymmetry problems and (B) membrane conformation problems.

(A) Asymmetry problems. Asymmetry of zero-trans entry and exit, resulting from delayed movement of sugar from bound to free compartments and vice-versa, (a) raises the apparent K_m and V_{max} for the net exit, as determined by the integrated-rate equation and to a lesser extent the initial rates of zero-trans exit from cells with varying loading concentrations. (b) Following entry, the slow rate of movement of sugar from free to bound compartments with consequent accumulation of sugar in the free compartment, retards both the uptake rate and V_{max} and accounts for the anomalously low apparent K_m for infinite cis entry and zero-trans entry of D-glucose [16,19,20].

(B) Exchange problems. Phenomena which can be specifically related to the membrane transport system are (i) accelerated exchange and (ii) the high K_m for equilibrium exchange. The current model describes these phenomena as follows: bilateral conformational change which occurs when the concentration of transported ligand is raised within equilibrium exchange protocols, raises both the rate of transfer across the membrane and fractional saturation of the ligand binding sites on both sides. The increased rate of transmembrane movement with increasing saturation of the ligand binding sites on the trans side increases the apparent K_m for equilibrium exchange and the V_{max} for exchange transfer above those of net transfer [10,12,3]. The low K_m values found in infinite-trans exchange exit or entry result from the absence of change on the trans(fully saturated) side at different fractional saturations of the cis side. In this circumstance, the rate of cis-trans ligand movement across the membrane is always at the same accelerated rate.

Counterflow involves both net flux and exchange flux over a prolonged period, thus can only be described in terms of both (A) and (B) phenomena.

The close similarity between the compartmental model and the observed data predicted for counterflow and zero-trans exit by simulation is consistent with the view that asymmetry of the glucose transport system results from an extrinsic cause.

TABLE IV

KINETIC PARAMETERS DERIVED FROM SIMULATED D-GLUCOSE FLUXES AT 2°C

The model parameters used to simulate these operational parameters are:
$K_m = 0.5$ mM
$V_{max} = L_2 = L_3 = 6$ μmol/ml cells per min.
L_1 = rate of equilibration between bound and free compartments = 0.002 s^{-1}
$F = V_2/V_1$ = ratio of free compartment volume to bound compartment volume = 0.1
B = the inaccessible intracellular space = 0.25 ml cell water.

	K_m (mM)	V_{max} (μmol/ml cell water per min)
Equilibrium exchange (measured during initial 60 s)	127.0	24.6
Infinite-trans entry (measured from 10 to 70 s)	0.8	11.8
Infinite-cis entry (initial rate measured from 10 to 70 s)	9.5	1.0
Infinite-cis exit	0.8	13.0
(initial rate determined from net loss from 12 to 72 s)	0.8	13.0
Zero-trans exit) (integrated-rate equation)	108.0	22.0

Alternative models

Internal inhibitor model. Carruthers and Melchior [5] have suggested that a low-molecular-weight inhibitor present within the cytosol reduces the affinity of the ligand binding site at the inner membrane surface. This suggestion is inconsistent with the data presented here because:

(1) An inhibitor could not retard equilibration of sugar label once it had entered the cell, i.e. reduce the maximal accumulation of label during counterflow.

(2) The inhibitor model does not adequately account for the large increase in apparent K_m for net exit in zero-trans exits as loading concentration is raised. Only if the inhibitor increased in concentration with the loading concentration would this model fit the results presented here.

(3) If it is assumed sugar is uniformly distributed within a single internal compartment as Curruthers and Melchior do [5], then the absence of any gross asymmetry at the counterflow turning point, i.e. 'a' = 1 (Table III) at 20°C, is inconsistent with the presence of a low molecular weight internal inhibitor.

Allosteric pore model. The experiments in this paper were not designed to test Holman's allosteric pore model rigorously [21]. The allosteric pore model assumes negative cooperativity between bound ligands at the inner surface and predicts that both low and high K_m should be observed at the inner surface on measuring infinite-cis entry and zero-trans exit. During counterflow, on loading with high sugar concentrations, it can be assumed that the inner sites would be mainly in the low affinity state, hence at the counterflow turning point a large asymmetry factor 'a' would be predicted from Holman's model. As this is not the case the model can be rejected on this ground alone. The allosteric pore model predicts that both high and low K_m values should be discernable in plots of the concentration dependence of sugar exchange [22]. However Weizer, Razin and Stein [16] find only a high affinity site and the inner surface during zero-trans influx of D-galactose and no evidence of multiple affinities in equilibrium exchange over a wide concentration range.

The data presented here showing that sugars are not uniformly distributed within the cytosol during the initial stages of inflow, makes interpretation of any slight non-linearity in the transformed plots of exchange data used to obtain the kinetic parameters difficult [22].

The role of intracellular binding of glucose to

haemoglobin has recently been corroborated by the demonstration [23] that the fluorescent glucose analogue 6-deoxy-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminoglucose(NBD-dGlc) accumulates in a temperature-dependent fashion within red cells by binding to haemoglobin. NBD-dGlc shares the same membrane transport system as D-glucose, since entry into cells, but not exit, which is very slow, is inhibited by D-glucose. However, cytochalasin inhibits both entry and exit of NBD-dGlc. Although the aromatic portion of the glucose analogue is mainly responsible for the binding to haemoglobin, it is still considered that the analogue binding could be an exaggerated form of the transport and binding processes which occur with unsubstituted D-glucose.

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