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REJECTION CRITERIA FOR THE ASYMMETRIC CARRIER AND THEIR APPLICATION TO GLUCOSE TRANSPORT IN THE HUMAN RED BLOOD CELL

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SUMMARY

1. We have derived two criteria, either of which is sufficient to permit the rejection of the conventional, asymmetric carrier model for facilitated diffusion for any given membrane transport system. These criteria involve only measurable parameters of transport.

2. We have measured the half-saturation concentration of glucose at the inner face of the human red cell membrane at 20 °C using the infinite-*cis* procedure and found it to be 2.8 mM, with a standard deviation of 1.5 mM (eight observations).

3. Using this result and previously determined experimental transport parameters, both of our rejection criteria force us to rule out the conventional, asymmetric carrier as a model for the glucose transport system of the human red blood cell.

4. Our results are consistent with the tetramer model for sugar transport.

INTRODUCTION

Some time ago we developed a rejection criterion for the conventional, symmetric carrier model[1]. Since then, we have used the criterion in an experimental test of the validity of this model for glucose transport in the human red blood cell; the criterion enabled us to unambiguously reject this symmetric model[2]. Recently, however, it has been suggested that the conventional model may nonetheless be applicable, if only the constraint of symmetry is removed[3,20]. To investigate this possibility, in the present paper we extend our treatment to the more general case of the conventional, asymmetric carrier model. We derive two separate rejection criteria, either of which is sufficient in itself to allow for the rejection of this asymmetric model for any facilitated-diffusion transport system. We then describe experiments designed to test for possible asymmetric transport of glucose in human red blood cells and show that the results of these experiments, when used in either of our rejection criteria, force us to reject the asymmetric model.

THE REJECTION CRITERIA DERIVED

Fig. 1 depicts the kinetic scheme for the conventional, asymmetric carrier model. This scheme embodies most of the assumptions which have been made for glucose transport in human red cells by carrier mechanisms. As before[1], the kinetic scheme includes the possibility of substrate-facilitated diffusion of the carrier[4–7] as well as the possibility that substrate and carrier are not in equilibrium at the membrane–water interfaces[8]. In addition, the scheme now allows for the carrier to be asymmetric in the most general sense. That is, the rates of movement of carrier (loaded and unloaded) can be different in the two directions, and the rate constants for the association and the dissociation of the carrier–substrate complex can be different at the two faces of the membrane. The scheme, therefore, includes both the asymmetric scheme of Geck[3] and that of Miller[20].

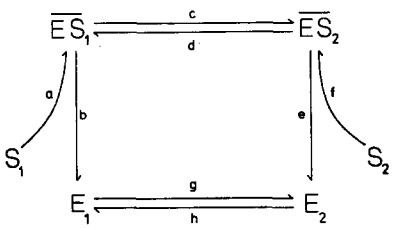

Fig. 1. Kinetic scheme for the asymmetric carrier model. S_1 and S_2 denote substrate concentrations in the solutions bathing sides 1 and 2 of the membrane, respectively. The symbols with E refer to free carriers, while those with ES refer to loaded (complexed) carriers. The terms a–h are rate constants.

Our rejection criteria will involve observable parameters measured in three different procedures. Two of these procedures involve net flows of sugar only, while the third involves the exchange of labeled sugar with unlabeled sugar under equilibrium conditions. We will discuss first (Cases 1 and 2) the net flow procedures and later (Case 3) the procedure involving exchange but no net flow of sugar. The solution of the kinetic scheme of Fig. 1 is derived readily from an excellent but much-neglected analysis by Britton[9]. Collecting terms and cancelling out common factors in his Eqn 1, using his Eqn 2 for the special case of uncharged substrate (or for charged substrate in the absence of a transmembrane electrical potential), leads to

$$\mathrm{NET}_{1\to 2} = \frac{T\,a\,c\,e\,h\,(S_1 - S_2)}{a[h(d+e)+c(e+h)]S_1 + f[g(b+c)+d(b+g)]S_2 + af(c+d)S_1S_2 + (h+g)[e(b+c)+bd]} \quad (1)$$

where $\mathrm{NET}_{1\to 2}$ is the net flux from solution 1 to solution 2, S_1 is the substrate concentration in solution 1 and S_2 that in solution 2, T is the total concentration of carrier, and the terms a through h are the rate constants for the kinetic scheme of Fig. 1. For uncharged substrate or for charged substrate in the absence of a transmembrane electrical potential, the Second Law of Thermodynamics requires that[9]

$$a\,c\,e\,h = b\,d\,f\,g \quad (2)$$

Case 1: Zero-trans procedure

In this procedure the concentration of substrate at the *trans* face of the membrane is kept at zero while the concentration at the *cis* face is varied. With face 2 *trans*, Eqn 1 reduces to

$$\mathrm{NET}_{1\to 2} = \frac{T\,a\,c\,e\,h\,S_1}{a[h(d+e)+c(e+h)]S_1 + (h+g)\,[e(b+c)+bd]} \tag{3}$$

The maximal velocity of transport is thus

$$V^{zt}_{1\to 2} = \frac{T\,c\,e\,h}{h(d+e)+c(e+h)} = \frac{T}{1/c + 1/e + 1/h + d/ce} \tag{4}$$

while that substrate concentration (the Michaelis constant or half-saturation concentration) which gives one-half this maximal velocity is

$$K^{zt}_{1\to 2} = \frac{(h+g)\,[e(b+c)+bd]}{a[h(d+e)+c(e+h)]} \tag{5}$$

Case 2: Infinite-cis procedure

In this procedure the system is saturated with substrate at the *cis* face of the membrane, while the concentration at the *trans* face is varied. With face 2 *trans*, Eqn 1 reduces to

$$\mathrm{NET}_{1\to 2} = \frac{T\,c\,e\,h}{h(d+e)+c(e+h)+f(c+d)S_2} \tag{6}$$

The maximal velocity of net transport $V^{ic}_{1\to 2}$ is of course equal to $V^{zt}_{1\to 2}$ as given in Eqn 4, while that substrate concentration in the *trans* solution which reduces the net flux to one-half this maximal velocity is

$$K^{ic}_{1\to 2} = \frac{h(d+e)+c(e+h)}{f(c+d)} = \frac{1/c + 1/e + 1/h + d/ce}{f/eh + a/bg} \tag{7}$$

For both Cases 1 and 2 the observable parameters for net fluxes in the direction $2\to 1$ can be obtained from the corresponding formulae applicable to the direction $1\to 2$ by making the substitutions $a \leftrightarrow f$, $b \leftrightarrow e$, $c \leftrightarrow d$, $g \leftrightarrow h$, and $S_1 \leftrightarrow S_2$. The results are collected in Table I.

Using the results collected in Table I, it is easy to verify that Eqn 1 can be expressed in terms of observable parameters as

$$\mathrm{NET}_{1\to 2} = \frac{V^{zt}_{1\to 2}(S_1 - S_2)}{(S_1 + K^{zt}_{1\to 2}) + \dfrac{S_2}{K^{ic}_{1\to 2}}(S_1 + K^{ic}_{2\to 1})} \tag{8}$$

Since Eqn 8 is a general solution to the kinetic scheme of Fig. 1, it follows that a knowledge of only four experimental parameters is sufficient to completely characterize all net movements of a given substrate by a conventional, asymmetric carrier for any concentrations S_1 and S_2 and for any experimental situation. Furthermore,

TABLE I

THE ASYMMETRIC CARRIER: INTERPRETATION OF EXPERIMENTAL PARAMETERS IN TERMS OF RATE CONSTANTS

The rate constants *a–h* are those of Fig. 1. T is the total concentration of carrier. The superscripts are zt for the zero-*trans*, ic for the infinite-*cis*, and ee for the equilibrium exchange procedure. The subscripts are $1 \rightarrow 2$ for an experiment in which transport is measured from face 1 to face 2 of the membrane and $2 \rightarrow 1$ for the opposite direction. V refers to a maximal velocity of transport and K (a Michaelis constant) to that substrate concentration which gives one-half of this maximal velocity. Q is an experimental index of the degree of asymmetric behavior shown by the system. The expressions are derived in the text.

$$V^{zt}_{1\rightarrow 2} = V^{ic}_{1\rightarrow 2} = \frac{T\,c\,e\,h}{h(d+e)+c(e+h)}$$

$$V^{zt}_{2\rightarrow 1} = V^{ic}_{2\rightarrow 1} = \frac{T\,b\,d\,g}{g(b+c)+d(b+g)}$$

$$V^{ee} = V^{ee}_{1\rightarrow 2} = V^{ee}_{2\rightarrow 1} = \frac{T\,b\,c\,d\,e}{(c+d)\,[e(b+c)+bd]}$$

$$K^{zt}_{1\rightarrow 2} = \frac{(h+g)\,[e(b+c)+bd]}{a[h(d+e)+c(e+h)]}$$

$$K^{zt}_{2\rightarrow 1} = \frac{(h+g)\,[e(b+c)+bd]}{f\,[g(b+c)+d(b+g)]}$$

$$K^{ic}_{1\rightarrow 2} = \frac{h(d+e)+c(e+h)}{f(c+d)}$$

$$K^{ic}_{2\rightarrow 1} = \frac{g(b+c)+d(b+g)}{a(c+d)}$$

$$K^{ee} = K^{ee}_{1\rightarrow 2} = K^{ee}_{2\rightarrow 1} = \left(\frac{b}{a}\right)\left(\frac{d}{c+d}\right)\left(\frac{g+h}{h}\right) = \left(\frac{e}{f}\right)\left(\frac{c}{c+d}\right)\left(\frac{g+h}{g}\right)$$

$$Q = \frac{K^{ic}_{2\rightarrow 1}}{K^{ic}_{1\rightarrow 2}} = \frac{K^{zt}_{1\rightarrow 2}}{K^{zt}_{2\rightarrow 1}} = \frac{V^{zt}_{1\rightarrow 2}}{V^{zt}_{2\rightarrow 1}} = \frac{f\,[g(b+c)+d(b+g)]}{a[h(d+e)+c(e+h)]}$$

$$a\,c\,e\,h = b\,d\,f\,g$$

these four observable parameters can be obtained using only the zero-*trans* and the infinite-*cis* procedures. The reader may check for himself that, under limiting conditions (for instance S_1 or S_2 equal to zero or to infinity), the familiar forms of the zero-*trans* and infinite-*cis* procedures are obtained.

Case 3: Equilibrium-exchange procedure

In this procedure the cells are pre-equilibrated with substrate at various concentrations and the unidirectional velocity of transport is then determined using radioactive tracer. For this procedure, the solution to the kinetic scheme of Fig. 1 has been given by Regen and Morgan[10].

$$J_{1\to 2} = J_{2\to 1} = \frac{F_s B_s S}{S + B_s} \tag{9}$$

where $J_{1\to 2}$ is the unidirectional flux from solutions 1 to 2 and $J_{2\to 1}$ that from solutions 2 to 1, $S = S_1 = S_2$ is the equilibrium concentration of substrate, and F_s and B_s in our nomenclature are

$$F_s = \frac{T\ b\ d\ f\ g}{(g + h)[e(b + c) + bd]} \tag{10}$$

and

$$B_s = \frac{bd}{ah}\frac{(g + h)}{(c + d)} = \frac{ce}{fg}\frac{(g + h)}{(c + d)} \tag{11}$$

Therefore the maximal velocity, which must of course be the same in either direction, is

$$V^{ee} = F_s B_s = \frac{T\ b\ c\ d\ e}{(c + d)\ [e(b + c) + bd]} = \frac{T}{1/b + 1/c + 1/d + 1/e + d/ce + c/bd} \tag{12}$$

while that equilibrium concentration of substrate which gives one-half of this maximal velocity is

$$K^{ee} = B_s = \frac{bd}{ah}\frac{(g + h)}{(c + d)} = \frac{ce}{fg}\frac{(g + h)}{(c + d)} = \frac{c\ e\ h}{f(c + d)}(1/g + 1/h) = \frac{b\ d\ g}{a(c + d)}(1/g + 1/h) \tag{13}$$

The asymmetry factor Q

In Table I we have also defined an observable parameter Q, which we will call the asymmetry factor. This is a measure of the degree to which the membrane transport system behaves asymmetrically and is determined by measuring the ratio of the Michaelis constants or maximal velocities in the two directions for the net transport experiments discussed above. For a system which behaves symmetrically, $Q = 1$. For an asymmetric system Q can have any positive value.

The rejection criteria

It is important to stress that although a transport system may behave symmetrically, it by no means follows that the system is symmetric at the molecular level. Therefore, to reject the asymmetric carrier model it is not sufficient to show that Q does not differ significantly from unity. Conversely, if a transport system does behave asymmetrically, it of course does not follow that it is an asymmetric carrier. For these reasons we have derived unambiguous rejection criteria, which can be applied to transport systems which behave either symmetrically or asymmetrically.

There are a number of ways in which rejection criteria could be formulated. In

particular, the observable net parameters (other than Q) appearing in them could be expressed for either direction of net transport. We have chosen forms in which Q is expressed in terms of those observable net parameters measured in the direction $1\to2$, as the observable net parameters currently available in the glucose transport field have been measured for a single direction only.

The first rejection criterion is derived as follows. Notice the use of the reciprocals of the rate constants to simplify the calculations. From the expression for Q given in Table I and from Eqn 2, it is readily seen that the asymmetry factor can be expressed as

$$Q = \frac{1/b + 1/d + 1/g + c/bd}{1/c + 1/e + 1/h + d/ce} \tag{14}$$

so that

$$Q + 1 = \frac{1/b + 1/c + 1/d + 1/e + 1/g + 1/h + c/bd + d/ce}{1/c + 1/e + 1/h + d/ce} \tag{15}$$

Using those forms of Eqns 7 and 13, involving reciprocals of rate constants, and Eqn 2, it is apparent that

$$\frac{K^{ee}}{K^{ic}_{1\to2}} = \frac{1/g + 1/h}{1/c + 1/e + 1/h + d/ce} \tag{16}$$

Also, it follows directly from Eqn 5 and from the first and third expressions of Eqn 13 that

$$\frac{K^{zt}_{1\to2}}{K^{ee}} = \frac{h}{bd}\,\frac{(c+d)\,[e(b+c)+bd]}{h(d+e)+c(e+h)}$$

$$= \frac{1/b + 1/c + 1/d + 1/e + c/bd + d/ce}{1/c + 1/e + 1/h + d/ce} \tag{17}$$

(Notice from those forms of Eqns 4 and 12 involving reciprocals of rate constants that the right-hand sides of Eqn 17 are also equal to $V^{zt}_{1\to2}/V^{ee}$.)

From a direct comparison of Eqns 15, 16, and 17 it is seen that

$$Q + 1 = \frac{K^{ee}}{K^{ic}_{1\to2}} + \frac{K^{zt}_{1\to2}}{K^{ee}} \tag{18}$$

Note that Eqn 18 contains only observable parameters and can thus be tested experimentally. This equation must hold for any transport system which behaves according to the conventional, asymmetric carrier model of Fig. 1 and forms the basis of our first rejection criterion. To apply the criterion, it is first necessary to measure the four observable transport parameters Q, K^{ee}, $K^{ic}_{1\to2}$, and $K^{zt}_{1\to2}$ for the transport system being investigated. One is then in a position to apply the following test.

First Rejection Criterion: Calculate Q' from the defining equation

$$Q' + 1 = \frac{K^{ee}}{K^{ic}_{1\to2}} + \frac{K^{zt}_{1\to2}}{K^{ee}}$$

and compare this with the value of the asymmetry factor, measured directly as the ratio of the Michaelis constants or maximal velocities in the two directions. If Q and Q' differ significantly from each other, then the asymmetric carrier model of Fig. 1 must be rejected.

To obtain a second rejection criterion, we transform Eqn 18 into a quadratic equation in K^{ee}:

$$\frac{(K^{ee})^2}{K^{ic}_{1\to 2}} - (Q+1)K^{ee} + K^{zt}_{1\to 2} = 0 \tag{19}$$

Eqn 19 yields real solutions for the observable parameter K^{ee} only when

$$(Q+1)^2 \geqq 4\,\frac{K^{zt}_{1\to 2}}{K^{ic}_{1\to 2}} \tag{20}$$

or, since all three parameters are positive, when

$$Q + 1 \geqq 2\left[\frac{K^{zt}_{1\to 2}}{K^{ic}_{1\to 2}}\right]^{\frac{1}{2}} \tag{21}$$

Since K^{ee} must be real, we have the following test.

Second Rejection Criterion: Calculate Q'' from the defining equation

$$Q'' + 1 = 2\left[\frac{K^{zt}_{1\to 2}}{K^{ic}_{1\to 2}}\right]^{\frac{1}{2}}$$

and compare this with the directly measured value of the asymmetry factor Q. If Q is significantly less than Q'', then the asymmetric carrier model of Fig. 1 must be rejected.

A strength of this second rejection criterion is that it involves only data from net flux experiments, avoiding any complications arising from the interpretation of tracer experiments. In the symmetric case, where $Q = 1$, this criterion reduces to rejection if the half-saturation concentration for the zero-*trans* procedure is found to be significantly greater than that for the infinite-*cis* procedure, which is the result we derived previously[1]. However, it is now clear that this previously derived rejection criterion is more powerful than we stated in our original paper[1], in that it allows for the rejection of the conventional carrier in not only its symmetric form but also in its asymmetric form for any transport system which behaves symmetrically.

METHODOLOGICAL CONSIDERATIONS

In what follows we designate as 1 that face of the membrane which faces the cell interior. It can be seen from Table I that at least three methods are available for determining the asymmetry factor Q. Two of these involve performing a zero-*trans* experiment with the cell interior maintained at zero concentration of glucose; because of the rapid initial rate of glucose entry such an experiment would be technically very difficult. For this reason we have chosen the third method, that of estimating the ratio of the Michaelis constants for the two infinite-*cis* procedures. An advantage of

this approach is that it is only necessary for us to determine $K_{1\to2}^{ic}$, since $K_{2\to1}^{ic}$ (the parameter in the reverse direction) for glucose at 20 °C has been determined in numerous laboratories with very consistent results. Thus Sen and Widdas[11] obtained $K_{1\to2}^{ic} = 1.7$ mM, Harris[12] found 1.9 mM, and Miller[13] reported 1.8 mM.

We have estimated $K_{2\to1}^{ic}$ by measuring the time course of net glucose uptake into cells and then subjecting the results to an integrated rate equation analysis. This approach is both necessary and convenient. Necessary because the size of the cellular compartment is such that the internal sugar concentration cannot be maintained constant during even the shortest feasible initial rate experiment. And convenient because a single time course of uptake curve is itself equivalent to a set of infinite-*cis* experiments performed over a range of intracellular glucose concentrations. For the derivation of the integrated rate equations, we introduce the following symbols:

$S = S_1$ = cellular glucose concentration (mM) at time t,
N = cellular glucose concentration (mmoles/cell unit) at time t,
$C_0 = S_2$ = constant extracellular glucose concentration (mM),
P = osmolarity of non-penetrating salts in both the extracellular solution and in the isotonic cells,
V_{rel} = liters of cell water per cell unit at time t,
$v = V_{2\to1}^{ic} = V_{2\to1}^{zt}$,
$K = K_{2\to1}^{ic}$,

where a cell unit is that number of cells whose solvent water volume is 1 l under isotonic conditions.

Under truly infinite-*cis* conditions, the net flux in the direction $2\to1$, obtained by making the appropriate substitutions in Eqn 6, is

$$NET_{2\to1} = \frac{T\,b\,d\,g}{g(b+c) + d(b+g) + a(c+d)S_1} \tag{22}$$

which is equivalent to

$$NET_{2\to1} = \frac{dN}{dt} = \frac{vK}{K+S} = \frac{vK}{K + N/V_{rel}} \tag{23}$$

To obtain an expression for V_{rel} we assume that the cell is always in osmotic equilibrium with the extracellular solution, so that extracellular osmolarity = intracellular osmolarity or

$$P + C_0 = \frac{P+N}{V_{rel}} \tag{24}$$

whence

$$\frac{1}{V_{rel}} = \frac{P+C_0}{P+N} \tag{25}$$

Substituting from Eqn 25 into Eqn 23 gives

$$\frac{dN}{dt} = \frac{vK}{K + \dfrac{N(P+C_0)}{P+N}} \tag{26}$$

On integrating Eqn 26, with the boundary conditions that $N = 0$ when $t = 0$ and $N = N$ when $t = t$, we arrive at

$$KN + (P + C_0)[N - P\ln(1 + N/P)] = vKt \tag{27}$$

For plotting the data, it is convenient to put Eqn 27 into the form

$$\frac{\ln(1 + N/P)}{t} = \frac{K + P + C_0}{P(P + C_0)} \cdot \frac{N}{t} - \frac{vK}{P(P + C_0)} \tag{28}$$

Thus, if $\ln(1 + N/P)/t$ is plotted against N/t, K can be calculated from the slope and then v determined from the intercept on the ordinate axis (when $N/t = 0$).

EXPERIMENTAL METHODS

We used the following solutions: (1) Saline buffer. This is composed of 147 mM NaCl; 20 mM Na_2HPO_4; adjusted to pH 7.4 with HCl. The total osmolarity was 310 mosmoles/l. (2) Glucose solution in saline buffer. In most of the experiments, the solution was prepared so as to give a final concentration of 60 mM glucose after dilution by the red blood cells. Two experiments were performed at a final concentration of 120 mM glucose as a check on possible effects on the infinite-*cis* constants due to the use of necessarily finite glucose concentrations. The glucose solution was labeled with insignificantly small quantities of D-[^{14}C]glucose dissolved in distilled water. (3) Stopper solution. This contains 1 % (w/v) NaCl; 10^{-6} M $HgCl_2$; 1.25 mM KI; phloretin, dissolved in ethanol, to give final concentrations of 10^{-4} M phloretin and 1 % (v/v) ethanol. The stopper solution was maintained at 0 °C.

The human red blood cells were prepared from out-dated transfusion blood by washing twice in at least 10 vol. of isotonic saline. To insure removal of all intracellular glucose, a final washing in at least 10 vol. of saline buffer was performed immediately before the experiment. The washings were followed by refrigerated centrifugatio at 2100 × *g* for 15 min. The cells were then resuspended in saline buffer to about 75 % hematocrit and stored on ice until required.

The time course of net glucose uptake was determined by the following procedure. A small flask with magnetic stirrer was equilibrated at 20 °C and 0.2 ml of the cell suspension was added. The system was rewarmed to 20 °C. 2 ml of the radioactive glucose solution, also at 20 °C, were added rapidly with magnetic stirring at time zero. At timed intervals thereafter, 0.25-ml aliquots of the reaction mixture were removed and blown into 80 ml of the ice-cold stopper solution. The duration of a given run was less than 1 min. After two to three consecutive runs, the suspensions of cells in stopper solution were centrifuged at 2100 × *g* for 15 min at 0 °C.

As the presence of small amounts of trapped extracellular glucose in the cell pellet could cause large and irreproducible errors, it was necessary to exert special precautions. As a first step, we suspended the reaction aliquots in large volumes of stopper solution; this provided good rinsing of the cells and high dilution of extracellular glucose. After centrifugation, the stopper was immediately removed by thorough aspiration. Comparison of subsequent treatments on identical samples indicated that the following method yielded lowest residual counts without causing cell hemolysis: the centrifuge tubes were inverted for about 5 min, after which the region

surrounding the cell pellet and the sides of the tube were blotted dry with filter paper.

The red cell pellet was then suspended in 0.6 ml saline buffer with vigorous mixing. These suspensions were stored at 0 °C while aliquots were being taken for the subsequent analyses. To determine the radioactivity of the glucose present in the cells, 0.3 ml of the suspension was added to 0.1 ml of 40 % trichloroacetic acid solution and mixed vigorously. The cell debris was removed by centrifugation, and 0.3 ml of the clear supernatant was added to 10 ml of scintillation fluid (68 % (v/v) of a solution of 0.4 % (w/v) 2,5-diphenyloxazole, 0.04 % (w/v) 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in toluene; 32 % (v/v) ethanol). Radioactive counting was performed using the Packard liquid scintillation system. The relative number of cells present in each pellet was determined by measurement of the hemoglobin content using the standard Drabkin procedure[14]; 0.25-ml aliquots of the suspension were added with rapid mixing to 2 ml of the Drabkin reagent, and the resulting absorbance at 540 nm was read in a Gilford spectrophotometer.

For the determination of absolute internal glucose concentrations, we required a measurement of the radioactivity present in the cells after effective equilibration with the glucose. This was obtained by allowing influx to proceed for at least 1 h in each reaction mixture before removing the usual 0.25-ml aliquot for further processing. It was also necessary to make a small correction for the radioactivity due to residual entrapped extracellular glucose. This residual radioactivity was determined by adding 0.125 ml of the original cell suspension to an ice-cold mixture of 400 ml stopper solution and 1.25 ml of the radioactive glucose solution. After thorough mixing, five aliquots of 80 ml each were taken for immediate centrifugation and processed subsequently in the usual manner.

Reagents were Analar. Phloretin was from Fluka A.G. D-[^{14}C]glucose was from the Radiochemical Centre. Reagents for liquid scintillation counting were from Packard Instrument Co.

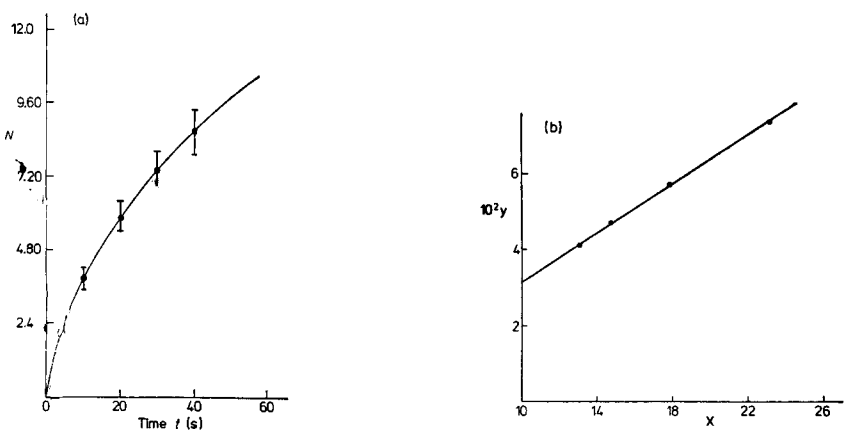

Fig. 2. Net influx of glucose into human red blood cells under infinite-*cis* conditions. (a) Time course of net glucose uptake in Expt 6 of Table II. The radioactive glucose concentration, N (mmoles/sell unit), in the cell at time t compared to that present at equilibrium is plotted against the time t of sampling. (b) The data of (a) plotted according to the integrated rate Eqn 28, using $P = 310$ mosmoles/l. The abscissa variable $x = N/t$ (mmoles/min per l isotonic cell water), while the ordinate variable $y = \ln(1 + N/P)/t$ (min^{-1}). See text for details. The bars in Fig. 2a represent $\pm$ S.D.

RESULTS

Fig. 2a shows the time course of net glucose uptake in a representative experiment. The fraction of radioactivity present in the cells, as compared to the amount present in the equilibrated cells (both corrected for residual counts), is plotted against the time of sampling. Each point is the mean of five separate observations; the average standard error in each point is 3 %, not varying significantly over the time course of the experiment. These same data have been plotted in Fig. 2b according to the integrated rate equation (Eqn 28). A straight line, calculated by the method of least squares in order to make it nonsubjective, has been drawn through the experimental points. From this line, the transport parameters are found to be $K_m = K = K_{2\to 1}^{ic} = 1.3$ mM and $v_{max} = v = V_{2\to 1}^{ic} = 66$ mmoles/min per l isotonic cell water. Table II summarizes the results of eight such experiments. The mean values (± S.E.) of the transport parameters are $K_m = 2.8 \pm 0.5$ mM and $v_{max} = 85 \pm 26$ mmoles/min per l isotonic cell water.

TABLE II

KINETIC PARAMETERS OF NET GLUCOSE INFLUX INTO HUMAN RED BLOOD CELLS UNDER INFINITE-*cis* CONDITIONS

Experiment	*Extracellular glucose concentration* C_0 *(mM)*	K_m *(mM)*	v_{max} *(mmoles/min per l isotonic cell water)*
1	60	2.2	65
2	60	0.5	253
3	60	2.8	23
4	60	3.7	59
5	60	3.6	50
6	60	1.3	66
7	120	5.2	49
8	120	3.0	112
Mean ± S.E.		2.8 ± 0.5	85 ± 26

The somewhat large variations in the derived values of the transport parameters can be attributed to experimental difficulties (*e.g.* possible timing error in the earliest time point of up to 1 s; occasional temperature variations of up to 1 °C) and their magnification by the integrated rate variation treatment. This interpretation is supported by the fact that the variation in K_m, which is derived directly from the slope of the integrated rate Eqn 28, is much smaller than the variation in v_{max}, which is derived from both the slope and the intercept on the ordinate axis. Thus our procedure is better suited for the determination K_m than for v_{max}. In the present paper, we require only the value of K_m, so that this problem does not arise.

Finally, the infinite-*cis* procedure requires, strictly, that one employ limitingly high concentrations of extracellular glucose. Since this is, of course, experimentally impossible, we have employed large but finite concentrations. We have looked for possible effects due to this necessary approximation by measuring the transport parameters at both the usual (60 mM) and at twice the usual concentration or extra-

cellular glucose. As the results were not significantly different at the two concentrations (see Table II), we conclude that the use of finite rather than infinite concentrations is justified. On the other hand, we cannot claim that a true maximum velocity is being obtained, since we have not made a complete study of the influence of external glucose concentration on the rate of glucose entry.

DISCUSSION

Our measured value (± S.E.) of $K_{2\to1}^{ic}$ for glucose at 20 °C is 2.8 ± 0.5 mM. The literature values for $K_{1\to2}^{ic}$ for glucose at 20 °C are 1.7 mM[11], 1.9 mM[12], and 1.8 mM[13], which average to 1.8 mM with an uncertainty of about 0.1 mM. The asymmetry factor Q (± S.E.), being the ratio of these values (see Table I), is thus 1.56 ± 0.29. Since Q differs from unity by less than two standard errors, it is possible that in fact $Q = 1$ and the system behaves symmetrically. Nonetheless, it is more probable that the system does behave slightly asymmetrically. Such a minor asymmetry in behavior would be expected because of the asymmetries in the environment of the transport system. For example, the solution bathing the outer face of the membrane is a dilute aqueous solution, whereas at the inner face the bathing solution is unusually concentrated in protein (as reflected in the fact that the water content (v/v) of erythrocytes in an isotonic medium is only about 70 % (ref. 15). Further, the ionic environment is very different at the two faces of the membrane, being largely NaCl outside and KCl inside the cell. Also, it has recently been proposed[16] that phospholipids are distributed asymmetrically in the red cell membrane. These and other asymmetries might be expected to modify somewhat the interactions between sugar and the membrane component involved in transport, even if this component were intrinsically symmetric, as it is on the tetramer model for sugar transport[18,19].

In any case, the measured value of Q is much too small to be consistent with the asymmetric carrier model of Fig. 1, as we proceed to discuss. To apply our rejection criteria, we require in addition to Q, values for K^{ee}, $K_{1\to2}^{zt}$, and $K_{1\to2}^{ic}$. The first two of these have been determined in our laboratory, while $K_{1\to2}^{ic}$ has been determined independently in three laboratories with remarkable agreement as discussed above, so the values should be consistent. The published values for glucose at 20 °C are $K^{ee} = 32$ mM[17], $K_{1\to2}^{zt} = 25$ mM[2], and $K_{1\to2}^{ic} = 1.8$ mM[11–13].

To apply our first rejection criterion, we calculate

$$Q' = \frac{K^{ee}}{K_{1\to2}^{ic}} + \frac{K_{1\to2}^{zt}}{K^{ee}} - 1$$

$$= 17.8 + 0.8 - 1 = 17.6$$

Notice that the only important term in Q' for glucose transport at 20 °C is $K^{ee}/K_{1\to2}^{ic}$. Interestingly, both Michaelis constants in this term have been determined independently by different investigators—$K_{1\to2}^{ic}$ by three laboratories and K^{ee} by two laboratories (Miller[13] having found $K^{ee} = 38$ mM, which would give $Q' = 20.8$)—and thus should be extremely reliable. Since Q' (at 17.6) is overwhelmingly greater than Q (at 1.6), our first rejection criterion forces us to rule out the conventional, asymmetric carrier as a model for glucose transport in human red blood cells.

It is important to stress that the two rejection criteria are each sufficient in

themselves to reject the model of Fig. 1. Thus, since the model has been rejected by the first criterion, there is no need to apply the second criterion. Nonetheless, for completeness and to illustrate the use of the second criterion, we will now consider it in detail. We first calculate

$$Q'' = 2\left[\frac{K_{1\to 2}^{zt}}{K_{1\to 2}^{ic}}\right]^{\frac{1}{2}} - 1$$

$$= 7.5 - 1 = 6.5$$

Since we know the standard errors of $K_{1\to 2}^{zt}$ and of $K_{1\to 2}^{ic}$, we can calculate the standard error of Q''. Thus $K_{1\to 2}^{zt} = 25 \pm 3$ mM[2] and $K_{1\to 2}^{ic} = 1.8 \pm 0.1$ mM[11–13] gives $Q'' = 6.5 \pm 0.5$. Since Q (at 1.6 ± 0.3) is certainly significantly less than Q'' (at 6.5 ± 0.5), the second rejection criterion also forces us to rule out the conventional, asymmetric carrier model for glucose transport in human red blood cells.

Our results are consistent with the glucose transport system being intrinsically symmetric under the conditions examined. In addition, our present result indicates the presence of a high-affinity binding site for glucose at the inner face of the human red cell membrane. A previous paper[2] demonstrated the existence of a low-affinity binding site for glucose at this same inner face. These results are among the predictions of the tetramer model for sugar transport[18,19].

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