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Kinetics of glucose transport in human erythrocytes: zero-*trans* efflux and infinite-*trans* efflux at 0°C

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The kinetic features of glucose transport in human erythrocytes have been the subject of many studies, but no model is consistent with both the kinetic observations and the characteristics of the purified transporter. In order to reevaluate some of the kinetic features, initial rate measurements were performed at 0°C. The following kinetic parameters were obtained for fresh blood: zero-*trans* efflux $K_m = 3.4$ mM, $V_{max} = 5.5$ mM/min; infinite-*trans* efflux $K_m = 8.7$ mM, $V_{max} = 28$ mM/min. For outdated blood, somewhat different parameters were obtained: zero-*trans* efflux $K_m = 2.7$ mM, $V_{max} = 2.4$ mM/min; infinite-*trans* efflux $K_m = 19$ mM, $V_{max} = 23$ mM/min. The K_m values for fresh blood differ from the previously reported values of 16 mM and 3.4 mM for zero-*trans* and infinite-*trans* efflux, respectively (Baker, G.F. and Naftalin, R.J. (1979) Biochim. Biophys. Acta 550, 474–484). The use of 50 mM galactose rather than 100 mM glucose as the infinite-*trans* sugar produced no change in the infinite-*trans* efflux K_m values but somewhat lower V_{max} values. Simulations indicate that initial rates were closely approximated by the experimental conditions. The observed time courses of efflux are inconsistent with a model involving rate-limiting dissociation of glucose from hemoglobin (Naftalin, R.J., Smith, P.M. and Roselaar, S.E. (1985) Biochim. Biophys. Acta 820, 235–249). The results presented here support the adequacy of the carrier model to account for the kinetics.

Introduction

The kinetic features of glucose transport in human erythrocytes have been studied extensively but remain controversial (see Refs. 1–5 for reviews). While some aspects of the kinetics are consistent with a carrier model in which no assumptions are made concerning symmetry or equal mobility of loaded and unloaded carriers [6], other observations are inconsistent with this model. A number of other models (see, for example, Refs. 2, 7–10) have been proposed to account for the kinetic data obtained using erythrocytes.

In recent years more information has become available concerning the transport protein itself. The transporter has been purified and reconstituted into liposomes [11]. The protein, which spans the membrane, appears to be a single polypeptide of about 45 kDa after removal of most of the carbohydrate [12]. Antibody cross-reactivity and cytochalasin B photolabeling studies show that the erythrocyte transporter is closely related to the passive glucose transporters of a number of other tissues [5]; one of these has recently been sequenced [13]. Each 45 kDa polypeptide of the erythrocyte glucose transporter appears to contain one binding site for cytochalasin B [14], and bind-

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ing studies of glucose and various inhibitors indicate that only one ligand binds at a time [15,16] (however, recent fluorescence studies of binding have been interpreted as supporting a two-site model [10]).

The results obtained with the purified transporter argue against transport models involving different types of subunits and multiple binding sites [2,7–9]. They are consistent with a model involving a conformation change which exposes a binding site alternately to the two sides of the membrane, which is kinetically equivalent to the carrier model [17]. Direct evidence for such a conformational change has been obtained using the purified protein [18].

In light of these findings, it seems more reasonable to question the validity of some of the kinetic data obtained using erythrocytes than to propose complex but less probable models to explain that data. One possible source of error is the use of integrated rate equations to analyze transport time courses; such analyses will not be valid unless special conditions are satisfied. Accordingly, we began an investigation of the kinetics relying solely on initial rate data. This paper presents some findings which disagree with those in the literature, and which support the adequacy of the carrier model.

Materials and Methods

Materials. Fresh blood was obtained from healthy volunteers; outdated erythrocytes were provided by the American Red Cross, Louisville, KY. Each of the types of experiments reported here was performed using blood from at least two different donors. Outdated blood was used at 37 to 50 days after donation. D-[U-^{14}C]Glucose (99.5% purity) was obtained from ICN and phloretin from Sigma Chemical Co.

Cell washing and glucose equilibration. Aliquots of cells were washed four times with phosphate-buffered saline (PBS) (150 mM NaCl/5 mM sodium phosphate (pH 7.4)) containing the desired concentrations of nonradioactive glucose, incubating 10 min at room temperature for each wash. In the case of infinite-*trans* efflux experiments, the solutions also contained sufficient NaCl to equal the osmolarity of the efflux solution for the assays (phosphate-buffered saline plus 100 mM glucose or 50 mM galactose). For efflux experiments, after the incubations were completed the cells were left at room temperature until about 10 min before assay, at which time an aliquot of radioactive glucose was added. After 10 min of equilibration of the radioactive glucose at room temperature the cells were placed on ice. For uptake and exchange experiments, the cells were placed on ice after the washes.

Assay procedure. Each assay used 10 μl of cell suspension in a test tube (13 × 100 mm). At time zero, 1 ml of phosphate-buffered saline containing the appropriate concentration of extracellular sugar was added while mixing on a vortex mixer. The assay was stopped by the addition, also while mixing, of 3 ml of a stopping solution containing 171 mM NaCl, 1 μM HCl_2, 1.25 mM KI, and 0.1 mM phloretin in 1% ethanol [19]. For zero-time points, stopping solution was added first. All tubes and solutions were kept on ice except while mixing, and after initiation tubes were returned to ice until about 1 s before stopping. After stopping, cells were centrifuged 15 s in a clinical centrifuge, and the supernatant removed. The cells were resuspended in 3 ml of stopping solution, centrifuged again, and lysed by resuspension in 3 ml H_2O. Preliminary experiments showed that the content of intracellular radioactive glucose does not decrease during an incubation of at least 5 min in stopping solution; therefore sets of 3 to 6 individual assays were performed and then carried through the centrifugation steps together. Assays were done in triplicate or quadruplicate at each time point. For most experiments, the entire set of assays was performed in a period of 60–70 min. In order to minimize systematic errors, in most experiments the various glucose concentrations were tested in a random order.

Under the conditions for efflux experiments, about 2/3 of the radioactive glucose is extracellular. However, about 95% of this is removed in the first centrifugation, with most of the remainder being removed in the wash. Thus the radioactivity that is initially extracellular makes a negligible contribution to the total radioactivity being measured. In one experiment, determinations of the efflux using no wash, one wash, and two washes gave results that were in good agreement. This

demonstrates the effectiveness not only of the initial dilution with stopping solution in removing extracellular glucose, but also of the ability of the stopping solution to prevent loss of intracellular glucose.

After all assays were completed, the cells were centrifuged 1 min. A 1.5 ml aliquot of each supernatant was treated with 0.3 ml 50% (w/v) trichloroacetic acid, centrifuged, and 1 ml of the supernatant counted. The absorbance at 540 nm of the lysate before acid addition was determined. This allowed normalization of each assay to the number of cells recovered and calculation of the intracellular volume (using a concentration of 5 mM for intracellular hemoglobin [2] and an extinction coefficient of 14.27 for hemoglobin monomer [20]).

Calculations. Comparing the radioactive glucose associated with the cells at a given time to that at time zero gives the amount of glucose that has entered or left the cells in that time interval. Kinetic parameters were determined using a nonlinear regression program [21], with points being weighted according to $1/(S.D.)^2$.

Results

Preliminary initial rate experiments at 0°C

An investigation of the kinetics of glucose transport in human erythrocytes at 0°C was undertaken, using initial rate determinations. For preliminary experiments, procedures similar to those described in Materials and Methods were used, with appropriate modifications. These procedures use a vortex mixer to mix a small volume of cells with a relatively large volume of extracellular medium. Assays are stopped with an even larger volume of inhibitor-containing stopping solution. An advantage to this method is that all of the standard transport experiments can be performed using a single basic procedure. Previous studies have often used much different methodologies for the various experiments, making comparisons of kinetic parameters less reliable.

Using such procedures, preliminary experiments with outdated blood were performed for the zero-*trans* uptake and efflux, infinite-*trans* uptake and efflux, and equilibrium exchange procedures (nomenclature according to Ref. 22). For zero-*trans* uptake, infinite-*trans* uptake, and equilibrium exchange, the kinetic parameters obtained were similar to those reported in the literature for fresh blood. For zero-*trans* efflux and infinite-*trans* efflux, however, results much different than those in the literature were obtained. Therefore these procedures were investigated in more detail.

Time-courses of zero-trans efflux and infinite-trans efflux from outdated erythrocytes

Fig. 1A shows an experiment in which outdated erythrocytes equilibrated at various glucose concentrations from 0.5 to 4 mM were diluted into 100 vol. of glucose-free buffer, and the decrease of intracellular glucose measured at various times. In order to facilitate the comparison the efflux is expressed as glucose space rather than in absolute amounts. The decrease in the slopes of the efflux curves with increasing glucose concentrations indicates saturation of the transporter in the concentration range tested.

Fig. 1B shows a similar experiment, except that in this case the transporter was exposed to a saturating concentration (100 mM) of nonradioac-

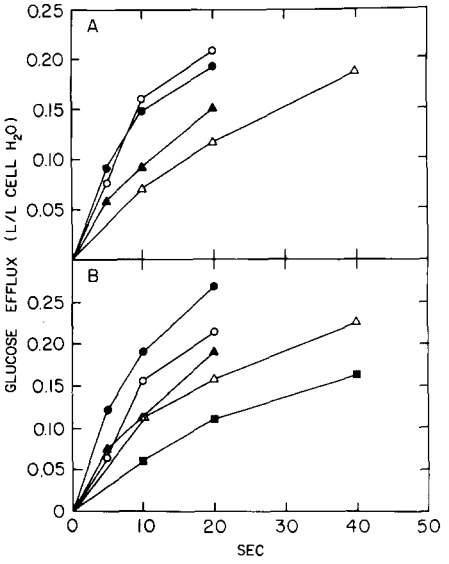

Fig. 1. Time-course of glucose efflux from outdated erythrocytes at 0°C. Efflux is expressed as volume of intracellular water containing the amount of glucose lost, or glucose space; multiplying by the concentration of glucose gives the amount in mmol/l intracellular water. A, zero-*trans* efflux of 0.5 (●), 1 (○), 2 (▲), and 4 (△) mM glucose; B, infinite-*trans* efflux of 5 (●), 10 (○), 20 (▲), 30 (△), and 50 (■) mM glucose into buffer containing 100 mM glucose.

tive glucose in the efflux medium, and the intracellular concentration ranged from 5 to 50 mM. Again, the slopes of the curves decrease with increasing intracellular glucose concentrations in this range, indicating saturation of the transporter from the intracellular side of the membrane.

Determination of the kinetic parameters for zero-trans efflux and infinite-trans efflux

The kinetic parameters for zero-*trans* and infinite-*trans* efflux were estimated from the time course data shown in Fig. 1, using a weighted nonlinear regression program [21]. No significant differences in K_m values were observed using the total efflux at the first, second or third nonzero time point; using all 10 s points; using the differences between the first and second nonzero time point; or using the differences between the second and third nonzero time points (as expected, the V_{max} values were somewhat lower when later time points were used because of the gradual decrease in the rates). Similar conclusions apply to time-course data obtained using fresh blood (see below). This validates the procedure employed in most of the experiments described here, which was to use a single time point at each of six different glucose concentrations. The glucose concentrations were chosen to include values both below and above the K_m, while the time points (10 s for outdated blood and 5 s for fresh blood) were chosen to give both measurable levels of efflux and good approximations of the initial rates.

TABLE I

KINETIC PARAMETERS FOR ZERO-*TRANS* EFFLUX AT 0°C

Parameters were determined by weighted nonlinear regression [21]. Results are expressed as mean ± S.D.

Expt.	K_m (mM)	V_{max} (mM/min)
Outdated blood		
1	1.9 ± 0.4	1.6 ± 0.7
2	2.0 ± 0.8	1.2 ± 0.9
3	4.8 ± 1.5	4.3 ± 2.0
4	2.0 ± 0.9	2.5 ± 0.7
Mean	2.7 ± 1.4	2.4 ± 1.4
Fresh blood		
1	6.2 ± 1.8	9.5 ± 2.2
2	3.0 ± 1.5	5.0 ± 1.6
3	2.5 ± 1.5	3.7 ± 1.5
4	2.0 ± 0.5	3.8 ± 0.7
Mean	3.4 ± 1.9	5.5 ± 2.7

TABLE II

KINETIC PARAMETERS FOR INFINITE-*TRANS* EFFLUX AT 0°C

Trans sugar	Expt.	K_m (mM)	V_{max} (mM/min)
Outdated blood			
100 mM glucose	1	8.9 ± 4.6	13.4 ± 3.6
	2	30.7 ± 18.4	28.1 ± 12.5
	3	23.6 ± 13.3	23.1 ± 9.7
	4	17.6 ± 5.5	26.5 ± 4.1
	5	15.8 ± 0.3	23.2 ± 0.3
	mean	19.3 ± 8.2	22.9 ± 5.7
50 mM galactose	1	23.6 ± 3.9	20.9 ± 2.3
	2	18.4 ± 12.2	15.5 ± 6.3
	3	8.5 ± 8.1	11.1 ± 4.9
	4	12.6 ± 7.4	14.1 ± 5.2
	mean	15.8 ± 6.6	15.4 ± 4.1
Fresh blood			
100 mM glucose	1	6.1 ± 2.5	20.1 ± 2.7
	2	9.3 ± 2.4	34.4 ± 3.8
	3	10.6 ± 3.0	28.0 ± 5.3
	mean	8.7 ± 2.3	27.5 ± 7.2
50 mM galactose	1	8.3 ± 2.4	18.3 ± 2.9
	2	6.0 ± 1.0	17.0 ± 1.3
	mean	7.2 ± 1.6	17.6 ± 0.9

The kinetic parameters obtained in several experiments of each type are listed in Tables I and II, while the rate data are plotted in Fig. 2. The data show greater scatter at high glucose concentrations. This is to be expected because as the transporter becomes saturated the fraction of intracellular sugar leaving in 10 s becomes smaller (Fig. 1), and thus small differences in relatively large numbers of counts are being determined.

Zero-trans efflux and infinite-trans efflux from fresh erythrocytes

The K_m observed for zero-*trans* efflux in the experiments described above (2.7 mM) was much lower than the value of 16 mM reported previously [23], while the K_m for infinite-*trans* efflux was much higher (19 mM vs. 3.8 mM). However, two experimental conditions were different for the two sets of experiments. First, Baker and Naftalin [23] used fresh rather than outdated blood. Weiser

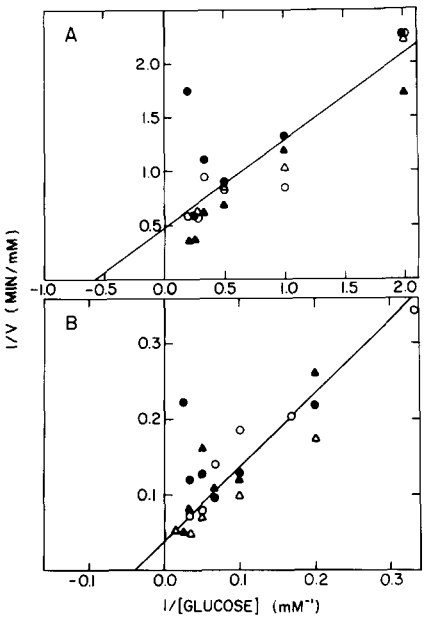

Fig. 2. Double-reciprocal plots of initial rate data for outdated erythrocytes. For each procedure, four experiments were performed and the initial rates of efflux estimated from 10-s points. The experiments are those designated 1 (●), 2 (○), 3 (▲), and in 4 (△) in Tables I and II, which list the kinetic parameters obtained from the data. Experiment 4 of each type is the same as shown in Fig. 1. The lines are drawn according to a weighted nonlinear regression analysis [21] of the pooled data; the kinetic parameters were K_m 1.7 mM and V_{max} = 2.1 mM/min for zero-*trans* efflux and K_m = 28 mM and V_{max} = 28 mM/min for infinite-*trans* efflux. A, zero-*trans* efflux; B, infinite-*trans* efflux into buffer containing 100 mM glucose.

et al. [24] reported significant differences in kinetic properties of glucose transport in fresh vs. outdated erythrocytes, with outdated cells showing about a 2-fold higher K_m for equilibrium exchange at 30°C. Second, the infinite-*trans* experiments in Ref. 23 used 50 mM galactose rather than 100 mM glucose as the *trans* sugar. The effects of these two factors on the kinetic parameters at 0°C were therefore investigated.

Fig. 3 shows time-course data for zero-*trans* efflux and infinite-*trans* efflux from fresh erythrocytes, while Fig. 4 shows initial rate data. Kinetic parameters obtained from these experiments are listed in Tables I and II. For zero-*trans* efflux, the V_{max} was significantly greater ($P < 0.05$) when fresh erythrocytes were used, but it was still 1.7-fold lower than that reported in Ref. 23. The

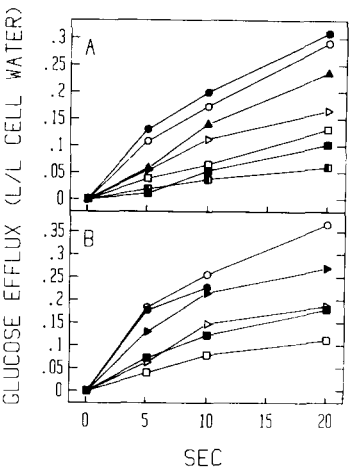

Fig. 3. Time-course of glucose efflux from fresh erythrocytes at 0°C. A, zero-*trans* efflux of 0.5 (●), 1 (○), 2 (▲), 4 (△), 8 (■), 12 (□), and 16 (half-filled squares) mM glucose. B, infinite-*trans* efflux of 3 (●), 5 (○), 10 (▲), 20 (△), 30 (■), and 50 (□) mM glucose into buffer containing 100 mM glucose. The infinite-*trans* data are composites of results from three separate experiments.

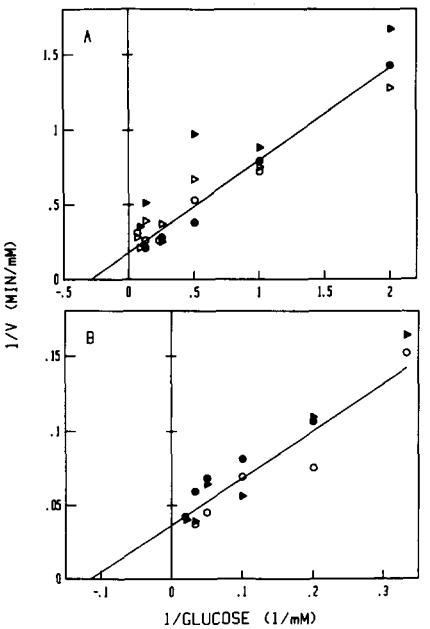

Fig. 4. Double-reciprocal plot of initial rate data for fresh erythrocytes. Initial rates of efflux were estimated from 5-s points in most cases, otherwise from 10-s points. The experiments are those designated 1 (●), 2 (○), 3 (▲), and 4 (△) in Tables I and II, which list the kinetic parameters obtained from the data. Zero-*trans* efflux experiment 4 is the same as shown in Fig. 3A. The lines are drawn according to the mean values of the kinetic parameters listed in the tables. A, zero-*trans* efflux; B, infinite-*trans* efflux into buffer containing 100 mM glucose.

K_m was not significantly different from fresh and outdated blood; the value for fresh blood was 4.6-fold lower than in Ref. 23 (the time-course data in Fig. 3A is clearly inconsistent with a K_m of 16 mM). In the case of infinite-*trans* efflux, the V_{max} values for fresh and outdated blood were not significantly different. The K_m was significantly lower for fresh blood ($P < 0.05$), but was still 2.3-fold higher than in Ref. 23.

Effects of galactose as trans sugar

Fig. 5 shows the time-courses of efflux of 1, 10, and 50 mM glucose into 100 mM glucose and 50 mM galactose. The curves are similar in shape, but the extent of efflux was 15–46% higher at each point when glucose was the *trans* sugar. Additional infinite-*trans* efflux experiments were performed using 50 mM galactose as the *trans* sugar; the results are listed in Table II. For both fresh and outdated blood, the V_{max} was about 50% higher when 100 mM glucose was used, in agreement with the faster time-courses shown in Fig. 5. In the case of outdated blood, where more data was obtained, the difference is statistically significant ($P < 0.05$). The K_m values were not significantly different.

The V_{max} for infinite-*trans* flux should be the same as that for equilibrium exchange, since in each case exchange at saturating sugar is being measured. The V_{max} for infinite-*trans* efflux reported here for fresh blood using 100 mM glucose as the *trans* sugar (28 mM/min) is in excellent agreement with the equilibrium exchange V_{max} of 30 mM/min reported by Baker and Naftalin [23], while we observed nearly identical values (17.6 mM/min and 17.3 mM/min) for the infinite-*trans* V_{max} when 50 mM galactose was used as the *trans* sugar. Apparently 50 mM galactose is insufficient to saturate the transporter under conditions when the exchange rate is high.

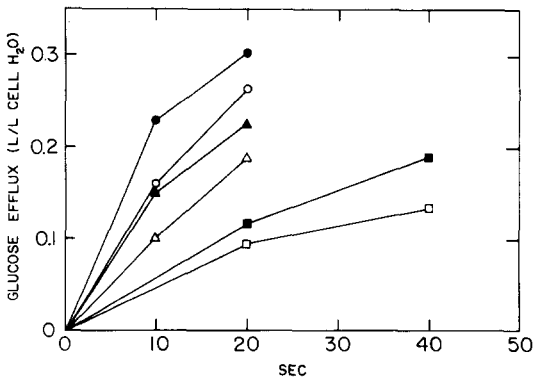

Fig. 5. Comparison of glucose and galactose as *trans* sugar. Efflux of 1 (●, ○), 5 (▲, △) and 50 (■, □) mM glucose from outdated erythrocytes into 100 mM glucose (solid symbols) or 50 mM galactose (open symbols) was determined. Results are means of two experiments. The kinetic parameters obtained from the data are listed in Table II (experiment 5 for glucose and experiment 1 for galactose).

Discussion

The results presented here gave a lower value for the K_m of zero-*trans* efflux and a higher value for the K_m of infinite-*trans* efflux than reported previously [23]. These values had been cited as evidence for "sites with both high and low operational affinities for D-glucose at the inner surface of the human erythrocyte membrane" [23], but the ratio of the K_m values observed here (0.4 ± 0.2) is much different than the ratio in Ref. 23 (4.2 ± 0.6). The new values are in much better agreement with the carrier model, as discussed below. Zero-*trans* efflux was measured in Ref. 23 by following the time-course of efflux of 5 mM glucose and using an integrated rate equation to determine the kinetic parameters (thus the concentration range near the K_m was not actually tested). For such an equation to be applicable, the transport must be uniform in the cell population and behave ideally for the time range to which the equation is applied, but these conditions may not be satisfied. Previous studies have indicated differences between zero-*trans* efflux parameters determined from initial rates and those determined from integrated rate equations. Miller [25] observed a K_m of 7.4 mM for glucose efflux at 20°C using initial rates, whereas Karlish et al. [19] obtained a value of 25 mM using an integrated rate equation. Outdated blood was used in the latter study, but in the experiments reported here fresh and outdated blood gave similar K_m values for zero-*trans* efflux at 0°C. More recently, Brahm [26], using rapid kinetic techniques, reported a value of 5.8 mM for the K_m at 25°C. Ginsburg, measuring galactose efflux, obtained K_m values of 75 mM [27] and 241 mM [28] using initial rates and an integrated rate equation, re-

spectively. Thus these results, as well as those reported here, indicate that the integrated rate equation overestimates the value of K_m.

In addition, if the use of such an equation is valid, the same kinetic parameters should be obtained using a variety of initial intracellular glucose concentrations. However, Naftalin et al. [29], using an integrated rate equation, reported apparent K_m values of 18 and 33 mM for cells loaded at 75 and 120 mM glucose, respectively, at 20°C and values of 40 to 122 mM for cells loaded at 7.5 to 120 mM, respectively, at 2°C. It was also noted that "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". These results indicate that the integrated rate equation is inappropriate for determining the kinetic parameters for zero-*trans* efflux.

The cause of the deviation from ideal behavior is unknown. One possibility is cell heterogeneity. Blood contains erythrocytes of various ages, and comparisons of fresh and cold-stored blood indicate that cell aging might affect the kinetics of glucose transport (Ref. 24; Tables I and II). In a study of leucine transport in human erythrocytes, the time-course of exchange efflux could not be fit adequately without taking into account cell heterogeneity [30]. However, glucose exchange appeared to behave ideally for more than one half-time in studies at 20°C [24] and 38°C [26]. A second possibility, proposed by Naftalin and others [2,29], is that much of the intracellular glucose is complexed with hemoglobin in a reaction which can be rate limiting for transport under some conditions. This will be discussed below. Another possibility which has been proposed is differential transport of the α and β anomers of glucose (discussed in Ref. 5). However, this appears to be ruled out by recent experiments showing that the two anomers are transported with no differences and that they inhibit transport of radioactive glucose equally well [31].

It was of interest to see if the new parameters

TABLE III

KINETIC PARAMETERS FOR GLUCOSE TRANSPORT IN HUMAN ERYTHROCYTES NEAR 0°C

For fresh blood, experimental parameters are from the literature or from this study (mean values, Tables I and II). The fit to the carrier model was performed as described in the text, resulting in the model parameters $R_{12} = 3.09$, $R_{21} = 0.142$, $R_{ee} = 0.038$, $R_{oo} = 3.19$, and $K = 0.168$. For outdated blood, the zero-*trans* efflux and infinite-*trans* efflux values are from Tables I and II, while the other parameters are means from two preliminary experiments of each type. Here the model parameters were $R_{12} = 4.17$, $R_{21} = 0.417$, $R_{ee} = 0.044$, $R_{oo} = 4.54$, and $K = 0.28$. Units of K and K_m, mM; of R, min/mM; of V_{max}, mM/min.

Procedure	Experimental		Fit to carrier model	
	K_m	V_{max}	K_m	V_{max}
Fresh blood				
Zero-*trans* uptake	0.20	0.21 [32]	0.17	0.32
Zero-*trans* efflux	15.8	9.3 [23]	3.8	7.0
	3.4	5.5 (this study)		
Infinite-*trans* uptake	0.65	12.6 [32]	0.63	27
Infinite-*trans* efflux	3.8	17.3 [23]	14	27
	8.7	27.5 (this study)		
Infinite-*cis* uptake	–	–	14	0.32
Infinite-*cis* efflux	0.39	8.6 [23]	0.63	7.0
Equilibrium exchange	20	22 [32]	14	27
	25	30 [23]		
Outdated blood				
Zero-*trans* uptake	0.52	0.24	0.31	0.24
Zero-*trans* efflux	2.7	2.4	3.1	2.4
Infinite-*trans* uptake	0.80	14.8	2.7	23
Infinite-*trans* efflux	19.3	22.9	27	23
Equilibrium exchange	42	31	29	23

obtained in this study and others in the literature are consistent with the carrier model. Table III lists sets of parameters for the model, and the values of K_m and V_{max} predicted for the various transport experiments. The fit to the carrier model was performed as follows, using the equations in Ref. 22. For fresh blood, the zero-*trans* efflux (this study) and infinite-*cis* efflux [23] V_{max} values were averaged, and the reciprocal was used for R_{21}. The two values for the equilibrium exchange V_{max} [23,32] and the infinite-*trans* efflux V_{max} (this study) were averaged, and the reciprocal used for R_{ee}; literature values for infinite-*trans* uptake [32] and efflux [23] should also yield this parameter, but these appear to have underestimated V_{max}. R_{12} was decreased somewhat from the value indicated by the zero-*trans* uptake V_{max} [32] in order to make the ratios of V_{max} and K_m values equal to the two directions of zero-*trans* flux. R_{oo} is then determined from the following relationship

$$R_{oo} = R_{12} + R_{21} - R_{ee}$$

The final parameter, K, was chosen to optimize the agreement between the predicted parameters and the observed K_m values for infinite-*trans* efflux and equilibrium exchange.

The parameters calculated from the model are in reasonable agreement with the experimental values with the exception of the zero-*trans* efflux and infinite-*trans* efflux parameters of Ref. 23, which were redetermined in this study, and the infinite-*trans* uptake V_{max}, which appears low as noted above. The other parameters (including the infinite-*cis* efflux K_m [23]) agree within a factor of 1.6, which is not unreasonable considering that the measurements were made in different laboratories using different techniques. The results thus support the adequacy of the carrier model. The fact that the initial rate measurements reported here have yielded kinetic parameters which are somewhat different than reported previously and which are consistent with the carrier model suggests that possibly other discrepancies between observation and the model could be accounted for by difficulties in determining the kinetic parameters rather than an inherent inadequacy of the model. Further studies on the kinetics at both 0°C and other temperatures will be required to confirm this idea.

One result that has been considered inconsistent with the carrier model is the infinite *cis* uptake K_m of 2.8 mM at 20°C [33], which is much lower than the value of 25 mM [19] for the zero-*trans* efflux K_m. However, as noted above, the latter value, determined using an integrated rate equation, seems too high; initial rates gave values of 7.4 mM [25] and 5.8 mM [26]. It seems possible that the infinite-*cis* uptake K_m, which was also determined using an integrated rate equation, could also be in error. If the rate of glucose entry decreased with time for reasons (such as cell heterogeneity or slow equilibration between intracellular compartments) other than the accumulation of intracellular glucose in an ideal manner, the method would underestimate the K_m. The V_{max} (85 mM/min) was also much greater than that determined for zero-*trans* uptake [32], but these numbers should be the same. The infinite-*cis* parameters should therefore be redetermined using initial rate measurements (the parameters should also be determined at 0°C as a further test of the carrier model). In another study [34], similar values of the infinite-*cis* uptake K_m for galactose were obtained using an integrated rate equation and initial rates, but the values (21 ± 17 and 25 ± 18 mM, respectively) had large standard errors. An analysis of the remaining kinetic parameters for 20–25°C (using the K_m values of 5.8 mM for zero-*trans* efflux and 8.1 mM for exchange reported by Brahm [26]) indicates that they are in reasonable agreement with the carrier model, and predicts an infinite-*cis* uptake K_m of about 9 mM (Table IV).

Carruthers [10] has recently reported a model in which the transporter is inherently symmetric, is capable of binding two molecules of glucose simultaneously, and has its kinetic properties modulated by ATP. The experiments presented here do not address this model except that insofar as they support the carrier model they argue against the need for a more complex model. It should also be noted that the experiments which support the symmetry of the transporter and the ATP effects [10,41,42] used a turbidometric method to measure net sugar fluxes, and these results should be confirmed using isotopic measurements of stereospecific glucose transport. We had previously seen no effects of ATP on the

TABLE IV

KINETIC PARAMETERS FOR GLUCOSE TRANSPORT IN HUMAN ERYTHROCYTES AT 20–25°C

The model parameters used to calculate K_m and V_{max} values were: $R_{12} = 0.0278$, $R_{21} = 0.0083$, $R_{ee} = 0.0032$, $R_{oo} = 0.0329$, and $K = 0.99$. Units are as in Table III.

Procedure	Experimental		Fit to carrier model	
	K_m	V_{max}	K_m	V_{max}
Zero-*trans* uptake [32]	1.6	36	1.2	36
Zero-*trans* efflux	5.8 [26]	120 [a]	3.9	120
Infinite-*trans* uptake [32]	1.7	174	2.6	310
Infinite-*cis* uptake [33]	2.8	85	8.6	36
Infinite-*cis* efflux [b]	1.8	132	2.6	120
Equilibrium exchange	8.1 [26]	310 [c]	10	310

[a] Mean from Refs. 19 and 25.
[b] Means from Refs. 35–37.
[c] Mean from Refs. 32, 38–40.

purified reconstituted transporter in preliminary experiments (Wheeler, T.J. and Haugh, L.M., unpublished observations). In addition, we have presented evidence that effects of ATP [43] appear not to be due to a covalent modification of the transporter but might be indirect effects due to changes in the structure of the membrane [44].

Table III also includes parameters for outdated blood. In this case the experimental values from Tables I and II are listed along with preliminary values for the zero-*trans* uptake, infinite-*trans* uptake, and equilibrium exchange procedures. The zero-*trans* uptake and efflux V_{max} values were used to determine R_{12} and R_{21}, respectively. R_{ee} was determined from the average of the V_{max} values for exchange and infinite-*trans* uptake and efflux. Using the R values, K was the calculated from each of the five K_m values and the average used as the model value. The calculated values for outdated blood are also in reasonable agreement with the experimental values, except for the preliminary determinations of the infinite-*trans* uptake K_m, which was lower than the calculated value.

Although the model parameters listed for outdated blood are somewhat preliminary, it is of interest to compare them to the parameters for fresh blood. All of the R values were lower in the case of fresh blood (by 14 to 66%), indicating faster rate constants for the steps in the transport process. The value for K, which can be considered "the operative affinity of the system for the substrate ... at limitingly low levels of substrate" [22] was also lower (by 40%). These results suggest that with aging of the erythrocytes the transporter operates less rapidly and with reduced affinity for glucose. These changes could be due to changes in the protein itself, in its membrane environment, or in intracellular factors which might influence its activity. We have previously performed kinetic studies of the reconstituted transporter purified from outdated blood [45]; a similar study using the protein purified from fresh blood might indicate whether the kinetic changes are due to changes in the protein itself. Alternatively, such studies might be done using the transporter reconstituted directly from erythrocyte ghosts [44]. In another study which compared fresh and outdated blood [24], a 2-fold increase in the exchange K_m was observed in the latter at 20°C, which is in good agreement with the results at 0°C (Table III).

Rapid assay techniques have been developed for measuring glucose transport in human erythrocytes; however, these are limited to either uptake [46] or efflux [26] studies. One objective of the work described here was to develop an experimental procedure for measuring initial rates for both types of experiments and thus avoid possible errors from comparing parameters obtained using much different techniques.

In the experiments described here, the initial rates were estimated from single relatively early

time points (usually 5 or 10 s). Since the time-courses are not linear, even in the ranges studied (Figs. 1 and 3), the use of multiple time points and regression analysis is not appropriate. When the data was transformed according to an exponential function [23,24,26], the data generally was also nonlinear (though in the case of the zero-*trans* efflux experiment with fresh blood shown in Fig. 3A, fairly linear curves were obtained, which yielded initial rates and parameters in good agreement with those determined from the early time points). However, as noted above (Results), the derived kinetic parameters were relatively insensitive to the choice of the time points, which supports the validity of the method. Since changes in substrate concentration occur during the time intervals used, it is important to estimate how closely the measurements approximate the true initial rates. For typical experiments of each type, the time course of efflux for each glucose concentration was simulated; comparing the calculated efflux over the time interval to that predicted from the calculated initial rates gives factors which can be used to correct the observed apparent initial rates and to generate revised sets of kinetic parameters. These simulations did not take into account changes in cell volume, which would be very small under the conditions of the experiments.

For the case of zero-*trans* efflux, the rates decreased slightly over the 5- or 10-s intervals due to the decreases in intracellular substrate concentration. The average decrease was 16% of the initial concentration. Since glucose is present only intracellularly, and gets diluted to a negligible level upon exit, the time course of efflux can be simulated using the Michaelis-Menten equation. Starting with the parameters shown in Table I, an iterative procedure was used in which corrected rates were used to generate new parameters. The final corrected K_m values were about 15% lower, while V_{max} values were virtually unaffected. The largest correction factor was 1.12 at the lowest glucose concentration (0.5 mM). Thus the experimental conditions appear to give good approximations of the initial rates.

For infinite-*trans* efflux, the intracellular radioactive glucose was not only decreasing in concentration (by an average of 29%) but was also subject to competition from nonradioactive glucose that enters the cells. To correct for these effects it is necessary to assume a model for the transport kinetics. In this case the carrier model was employed, using the observed parameters for each experiment, the values of K listed in Table III, and the equation for unidirectional flux (Eqn. 16 of Ref. 22). The iterative procedure gave K_m values about 20% lower and V_{max} values about 10% higher than obtained from the uncorrected data. The largest correction factor was 1.13 at the lowest glucose concentration (3 mM); at this concentration it was calculated that during the 5 s less than 1.5 mM nonradioactive glucose would enter fresh erythrocytes to compete with the radioactive glucose. Much smaller competitive effects would be expected at higher glucose concentrations. The simulations indicate that for the infinite-*trans* experiments the procedure also gives good approximations of the initial rates.

Naftalin and others [2,29] have suggested formation of a complex between glucose and hemoglobin as a source of anomalous kinetic results. According to their model, only about 10% of the intracellular glucose is free, and as this exits the dissociation of glucose from hemoglobin can become rate-determining for efflux. Simulations of the time-courses of zero-*trans* efflux were performed using a value of 0.1 for the relative volume of the free compartment to the bound compartment and a value of 0.002 s^{-1} for the rate constant for glucose equilibration between compartments (used to simulate the parameters for 2°C in Table IV of Ref. 29). These simulations indicated that for rates of efflux as large as those observed, extreme departures from linearity would occur during the early portions of the efflux time-courses, especially at low glucose concentrations; the time-courses shown in Figs. 1A and 3A are inconsistent with this. For infinite-*trans* efflux, simulations were performed using the carrier model * as

* I attempted to simulate time courses for glucose transport under the conditions of the experiments using the model of Naftalin et al. [29], but there appear to be errors in the Eqns. M9–M12 used to describe that model. These equations define quantities H_2, G_2, H_3, and G_3, "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". Considering, for example, zero-*trans* efflux, where R_2, R_3,

described above, except that the same parameters for complexation with hemoglobin were also incorporated. These simulations revealed extreme deviations from linearity during the efflux of the first few percent of the intracellular glucose, as the entering nonradioactive glucose reaches high levels in the unbound compartment, and such effects would be seen at all glucose concentrations. This is inconsistent with the time-courses shown in Fig. 1B and 3B. That the dissociation of glucose from hemoglobin proposed by Naftalin et al. [29] is too slow can easily be seen by considering the extreme situation in which the membrane offers no resistance to glucose efflux. After the immediate efflux of the 10% of the glucose which is unbound, the remainder would exit with a half-time of 5.8 min. The efflux data in Figs. 1 and 3 is clearly much faster than this. Thus the results presented here argue against the model of Naftalin et al., at least for the parameters which were used to simulate the data in Ref. 29.

While the causes of deviations from nonideal time course behavior remain an interesting area of investigation, it seems that useful information on the kinetics of the transporter itself are more likely to come from initial rate studies such as those described here, where such deviations are minimal.

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S_3, x_{R2}, x_{R3}, and x_{S3} are all equal to zero, and the symmetric model proposed by the authors where $L_2 = L_3 = V_{max}$, Eqns. M9 and M11 give, respectively:

$$H_2 = x_{S2} \cdot V_{max}$$

$$H_3 = (1 + x_{S2}) \cdot (x_{S2}) \cdot V_{max}$$

If H_2 and H_3 are taken to be net fluxes, they must be equal in magnitude but opposite in sign, but these expressions are not equal in magnitude, even allowing for a reversal of the sign of x_{S2}. They cannot be unidirectional fluxes, for with no external glucose the unidirectional flux compartment 3 (the outside) to compartment 2 must be zero. It is stated that "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"; however, in Eqns. M9–M12 the accelerating effect is only due to saturation on the side to which the sugar is moving.

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