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Glucose transport kinetics in human red blood cells

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D-[^{14}C]Glucose self exchange and unidirectional efflux from human red blood cells were studied at 20°C (pH 7.2) by means of the Millipore-Swinnex filtering technique whose time resolution is > 1 s and the continuous flow-tube method with a time resolution of > 2 ms. The unidirectional efflux data were analyzed using both the method of initial rates and the integrated rate equation. Simple Michaelis-Menten kinetics apply to the results obtained under both experimental conditions. In self-exchange mode, the half-saturation constant, $K_{1/2ex}$, was 10 (S.E. ± 1) mM. In unidirectional efflux mode $K_{1/2ue}$ was 6.6 (S.E. ± 0.5) mM (initial rates) or by the method of integrated rates 7.7 mM, with a range of 2.7–12.1 mM, $K_{1/2ue}$ increasing with an increased initial intracellular glucose concentration. Our results of $K_{1/2ex}$ oppose previous published values of 32 mM for self exchange (Eilam and Stein (1972) Biochim. Biophys. Acta 266, 161–173) and 25 mM for unidirectional efflux (Karlish et al. (1972) Biochim. Biophys. Acta 255, 126–132) that have been used extensively in kinetic considerations of glucose transport models. Under self-exchange conditions J_{ex}^{max} was $1.8 \cdot 10^{-10}$ mol cm^{-2} s^{-1}, and in unidirectional efflux mode J_{ue}^{max} was $8.3 \cdot 10^{-11}$ mol cm^{-2} s^{-1} (initial rates) and $8.6 \cdot 10^{-11}$ mol cm^{-2} s^{-1} (integrated rates). We suggest that the previous high values of J^{max} and in particular $K_{1/2}$ are due to the use of methods with insufficient time resolution. Our results indicate that the transport system is less asymmetric than was generally accepted, and that complicated transport models developed to account for the great difference between the determined $K_{1/2}$ and J^{max} values are redundant.

Introduction

For over 40 years it has repeatedly been demonstrated that glucose transport in human red cells almost completely is mediated by a transport system. The transport shows the classical signs of facilitated diffusion: saturation kinetics, competitive and noncompetitive inhibition, *trans*-stimulation that is an increase of the net transport rate by the sugar when present (at a lower concentration) on the side to which the sugar is transported. A temperature dependence of the transport process different from that of simple diffusion further supports the concept of the involvement of a transport system. This transport system, now structurally characterized [1] also shows a high degree of specificity as the apparent permeability (40 mM, 20°C (pH 7.2)) for D-glucose is $3.0 \cdot 10^{-6}$ cm/s, i.e. approx. 4000-times larger than the apparent permeability for L-glucose of $7.6 \cdot 10^{-10}$ cm/s [2], the latter being within the range of simple diffusion permeability coefficients of glucose in lipid bilayer membranes ($2.6 \cdot 10^{-8}$–$2.0 \cdot 10^{-10}$ cm/s [3,4]). The saturation kinetics of D-glucose transport have been treated in terms of Michaelis-Menten like kinetics, characterized by a maximum flux, J^{max}, and a half-saturation constant, $K_{1/2}$. Though intensively studied, it has for several years been a puzzle why $K_{1/2}$ for self exchange and for unidirectional efflux are high while $K_{1/2}$ for glucose uptake is very low, as the reported values do not fit to any simple symmetric or asymmetric transport model (for reviews, see Refs. 5–11). The puzzle apparently now is so well-established that even an extraordinary high, and to date the highest, $K_{1/2}$ under conditions of self exchange (63 mM (0°C); [12]) has been published with no comments.

We are concerned about the continuous opinion that "the biggest discrepancy is between the values of K derived from the zero trans [unidirectional efflux] and equilibrium [self] exchange experiments, on the one hand, and the infinite cis uptake (net influx) experiment on the other" [10], because our study of glucose transport kinetics [13] indicates that this great discrepancy may not be real. The study shows that values of $K_{1/2}$ under conditions of self-exchange and unidirectional efflux are much lower when determined by means of methods with high time resolutions. In the present study

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we reevaluate the methods, including the integrated rate method, used by others and compare their previous results with results obtained under similar experimental conditions by means of the high performance methods.

Materials, Methods and Calculations

Materials

The electrolyte media, all made from grade chemicals (pro analysi), were (mM): Medium A (= wash medium, and flux medium in self-exchange experiments): 150 KCl or NaCl, 2 KH_2PO_4, 1–120 glucose; Medium B (= flux medium in unidirectional efflux experiments): 150–222 KCl or NaCl, 2 KH_2PO_4. Both media were titrated at 20°C to pH 7.2 with 1 M KOH. The osmolarity of all media were measured by use of the freezing point depression method (Roebling Micro Osmometer, Messtechnik, Berlin, Germany). The glucose concentration of the media and in the cells were measured using the GOD-Perid method (Boehringer Mannheim GmbH, Mannheim, F.R.G.). The principle of the test is that glucose is oxidized to gluconate and hydrogen peroxide (catalyzed by glucose oxidase = GOD), which is complexed with ABTS (di-ammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] (catalyzed by peroxidase = Perid). The complex is colored and can be measured spectrophotometrically at 610 nm. The glucose tracer was D-[U-^{14}C]glucose suspended in 90% ethanol (NEN-Dupont, Boston, MA, U.S.A.).

Preparation of red blood cells

Freshly drawn, heparinized blood samples from the two healthy authors were used. The cells were immediately isolated from the plasma by centrifugation and the white cells were removed. The red cells were washed one time in medium A, titrated to pH 7.2 at 20°C, and washed three more times in medium A. The duration of the washing procedure ensured that glucose was at equilibrium across the membrane. Next the cells were loaded at a cytocrit of approx. 50% with [^{14}C]glucose, 0.5 μCi per ml cell suspension (final ethanol content approx. 1%), packed (see Refs. 14,15) and stored on ice until use for glucose efflux experiments the same day (see below).

Determination of radioactivity, intracellular glucose concentration and cell water volume

The radioactivity was determined by β-liquid scintillation spectrometry of the samples suspended in Optifluor (Tricarb, Packard Instrument, Downers Grove, IL) in: (1) the cell samples and the supernates after precipitation with 7% perchloric acid, and (2) the cell-free filtrates from the efflux experiments, including equilibrium samples.

The intracellular glucose concentration of the packed cell samples, $C^{(i)}$, was calculated, assuming that the distribution ratio of ^{14}C across the cell membrane equals the distribution ratio of unlabeled glucose. $C^{(i)}$ was also determined by use of the GOD-Perid method.

The ratio of cell volume to surface area, $V_c^{(i)}/A_c$, was determined in each experiment by drying a cell sample at 104°C for more than 24 h to constant weight, and assuming that 1 g of cell solids equals $3.1 \cdot 10^{10}$ normal erythrocytes with a total membrane area of $4.4 \cdot 10^4$ cm^2 ($1.42 \cdot 10^{-6}$ cm^2/cell, see Ref. 16). The cell water volume was corrected for contribution from extracellular trapped volume in the cell samples. The trapped volume, as determined by means of the extracellular marker [^{14}C]inulin, was approx. 2%. At 120 mM glucose in the medium, the cell solids content was increased by approx. 4% that was not corrected for, except in experiments to determine the dependence of the relative changes of cell water volume on glucose concentration.

Determination of the rate of efflux

All experiments were carried out at pH 7.2, 20°C under conditions of either: (1) self exchange with equal glucose concentrations on the two sides of the membrane, or (2) unidirectional efflux of cell glucose into a glucose-free flux medium. Under both conditions the packed, isolated and radioactive labeled red blood cells were resuspended in a large volume of flux medium (hematocrit < 1%). Series of cell-free samples, that were used for determination of the extracellular radioactivities used in Eqn. 1 (see below), were obtained by means of either the Millipore-Swinnex filtering method with a time resolution of approx. 1 s or the continuous flow tube method with a time resolution of 2–3 ms (for details, see Refs. 14,15). The Millipore-Swinnex filtering method was used at glucose concentrations ≧ 5 mM, and the continuous flow tube method was used at $C^{(i)} \leqq 5$ mM (unidirectional efflux) and < 5 mM (self exchange). In all experiments equilibrium samples for scintillation counting were obtained from the suspension by centrifugation after isotopic equilibrium was attained (> 7 half-times).

Self exchange

In self-exchange experiments the intracellular and extracellular chemical concentrations of glucose remain constant during the experiments, and one can assume a closed two-compartment model with constant volumes. The tracer efflux rate coefficient, k, was obtained by a fitting of Eqn. 1 to the experimental data by linear regression:

$$\ln[1-(a_t/a_\infty)] = -k \cdot t + \ln[1-(a_0/a_\infty)] \quad (1)$$

where a_0, a_t and a_∞, respectively, are the extracellular radioactivities at time zero, t and after isotopic equilibrium is reached. The left side of Eqn. 1 represents the intracellular fraction of tracer at time t. In plots of

$\ln[1-(a_t/a_\infty]$ vs. t the interception with the ordinate ($\ln[1-(a_0/a_\infty)]$) depends on the amount of extracellular [^{14}C]glucose trapped between the cells during the packing procedure.

The unidirectional glucose tracer efflux is defined by:

$$J_{ex} = k \cdot (V_c^{(i)}/A_c) \cdot C^{(i)} \text{ (molcm}^{-2}\text{s}^{-1}) \quad (2)$$

Depictions of J_{ex} vs. $C^{(i)}$ show saturation kinetics, and J_{ex}^{max} and $K_{1/2ex}$ were obtained by assuming kinetics of Michaelis-Menten type from:

$$J_{ex} = J_{ex}^{max} \cdot C^{(i)}/(K_{1/2ex} + C^{(i)}) \quad (3)$$

Unidirectional efflux

In the unidirectional efflux experiments the flux medium (Medium B) was glucose-free, and the KCl concentration was raised to obtain the osmolarity of the wash media. Under unidirectional efflux (J_{ue}) $C^{(i)}$ approaches 0 with time, and the kinetics are also of the Michaelis-Menten type. It follows that at high glucose concentrations J_{ue} is (almost) constant and k varies considerably, while at low glucose concentrations J_{ue} decreases and k is (almost) constant as the transport system becomes increasingly unsaturated. The Michaelis-Menten parameters for unidirectional glucose efflux, J_{ue}^{max} and $K_{1/2ue}$ were determined by means of (1) the initial rate method, and (2) the integrated rate method.

Initial rate method. The principle is to determine glucose efflux at different glucose concentrations in so short observation times that the change of $C^{(i)}$ is minimal. As described above the initial concentration of glucose indicates whether the initial efflux ($dS^{(i)}(t)/dt$) or the initial efflux rate ($d\ln[S^{(i)}(t)]/dt$) of glucose should be determined (see below). Hence, convenient linear plots should be obtained by means of Eqn. 1 at low glucose concentrations, that were arbitrarily set to $\leqq 5$ mM, close to the value of $K_{1/2ue}$. J_{ue} was next calculated from Eqn. 2. For $C^{(i)} > 5$ mM at $t = 0$, the flux (in mmol/(liter isotonic cell water) per s) converted to molcm^{-2}s^{-1}, cf. Table IB.) was determined as the initial slope of plots of $S^{(i)}$ vs. time according to the equation:

$$S^{(i)}(t) = S^{(i)}(0) \cdot [1-(a_t/a_\infty)]/[1-(a_0/a_\infty)] \quad (4)$$

where $S^{(i)}(0)$ is the amount of glucose in a fixed number (N) of cells having a total cell water volume of 1 liter at time zero and $S^{(i)}(t)$ is the amount in the N cells at time t (see below)). We calculated $S^{(i)}(0)$ after determination of the distribution ratio of tracer, $r = C^{(i)}(0)/C^{(o)}(0)$. The values of $S^{(i)}(0)$ were also obtained with the GOD-Perid method and were in good agreement with the tracer data. Finally, $J_{ue} = J_{ue}^{max} \cdot C^{(i)}/(K_{1/2ue} + C^{(i)})$ were fitted to the data to obtain J_{ue}^{max} and $K_{1/2ue}$.

Integrated rate method. A sophisticated alternative approach to obtain the Michaelis-Menten parameters under conditions of unidirectional efflux is the integrated rate method. This approach incorporates the change of efflux rate as the glucose concentration decreases from the initial value to zero. The integrated rate method includes some assumptions amongst which is that the cells behave as perfect osmometers when they lose water in connection to glucose exit. One can derive:

$$\{(S^{(i)}(t) - S^{(i)}(0)) \cdot \{K_{1/2ue} + Q^{(i)}(0) + C^{(i)}(0)\} + K_{1/2ue} \cdot Q^{(i)}(0) \cdot V^{(i)}(0) \cdot \ln\{S^{(i)}(t)/S^{(i)}(0)\}\} / [Q^{(i)}(0) + C^{(i)}(0)] = -V_{ue}^{max} \cdot t \quad (5)$$

$Q^{(i)}(0)$ (osmol/l) is the concentration of 'impermeable', osmotically active solutes in the cells at $t = 0$. $K_{1/2ue}$ (M) and V_{ue}^{max} (mol/(liter isotonic cell water) per s) are the Michaelis-Menten parameters for unidirectional efflux where $V_{ue}^{max} = J_{ue}^{max} \cdot A_c \cdot N$.

Inserting $Q^{(i)}(0) = 310$ mosmol and $V^{(i)}(0) = 1$ liter, Eqn. 5 can be rearranged to Eqn. 2 in Karlish et al. [17]. Eqn. 5 can be transformed into a linear form ($x = t/\ln(f)$, $y = (1-f)/\ln(f)$) (equivalent to Eqn. 3 in Karlish et al. [17]):

$$\frac{1-f}{\ln(f)} - \frac{V_{ue}^{max} \cdot [Q^{(i)}(0) + C^{(i)}(0)]}{C^{(i)}(0) \cdot \{Q^{(i)}(0) + C^{(i)}(0) + K_{1/2ue}\}} \cdot \frac{t}{\ln(f)} = \frac{Q^{(i)}(0) \cdot K_{1/2ue}}{C^{(i)}(0) \cdot \{Q^{(i)}(0) + C^{(i)}(0) + K_{1/2ue}\}} \quad (6)$$

f is the ratio of cpm at time t, to cpm at $t = 0$ in the N cells (for further details, see Ref. 17).

Eqn. 5 is the basic equation used in this study to obtain the Michaelis-Menten parameters, $K_{1/2ue}$ and V_{ue}^{max} by nonlinear regression analysis.

All fitting procedures were done by use of the computer program Statgraphics (STSC, Inc., Rockville, MD) that calculates a 'standard error' of the estimated parameters. This standard error is abbreviated S.E. in this study.

Results

The present study was carried out at 20°C (pH 7.2) to make the results directly comparable to those commonly used in model considerations. Our preferred terminology of transport modes, in accordance with, e.g., that used in anion transport literature, is summarized in Table IA, and Table IB shows the conversions factors used by us to make the fluxes in different studies comparable to our flux units (molcm^{-2}s^{-1}).

TABLE IA

Terminology of experimental conditions used to measure glucose transport across the red cell membrane

The synonyms are listed in columns (1) and (2). Column (3) lists the experimental conditions.

(1)	(2)	(3)
Infinite-cis exit	Net efflux	$C^{(i)} > C^{(o)}$
Infinite-cis entry	Net influx	$C^{(i)} < C^{(o)}$
Zero-trans exit	Unidirectional efflux	$C^{(i)} > C^{(o)} = 0$
Zero-trans entry	Unidirectional influx	$C^{(o)} > C^{(i)} = 0$
Equilibrium exchange	Self exchange	$C^{(i)} = C^{(o)}$
Infinite-trans entry	Unidirectional tracer influx	$^*C^{(o)} > {}^*C^{(i)} = 0$
	Net efflux of solute	$C^{(i)} > C^{(o)}$
Infinite-trans exit	Unidirectional tracer efflux	$^*C^{(i)} > {}^*C^{(o)} = 0$
	Net influx of solute	$C^{(o)} > C^{(i)}$

Further, we have performed experiments, self exchange as well as unidirectional efflux of glucose, both in KCl and NaCl media to ensure that the substitution of sodium for potassium, commonly used in anion transport studies and routinely used in our laboratory, had no effect on glucose transport (results not shown).

Self-exchange experiments

As the chemical concentrations remains constant under self-exchange conditions the rate coefficient for glucose tracer efflux is constant as illustrated by the linearity of the semilogarithmic plot shown in Fig. 1 (filled circles). The unidirectional efflux of glucose was calculated by use of Eqn. 2, and the resulting flux values at different concentrations of internal glucose are depicted in Fig. 2. Each point (±S.D.) represents an average of two or more flux values. The flux values at $C^{(i)} = C^{(o)} = 1$ mM were obtained by means of the continuous flow tube technique while at higher concentrations the Millipore-Swinnex Filtering method was used. As discussed below (cf. Discussion) the cell glucose concentration was slightly lower than the glucose concentration in the flux media. The flux is, therefore, depicted as a function of the intracellular concentration in Fig. 2. The Michaelis-Menten parameters obtained by nonlinear fit to the experimental data are $K_{1/2ex} = 10$ (S.E. ± 1) mM and $J_{ex}^{max} = 1.8$ (S.E. ± 0.1) · 10^{-10} mol cm^{-2} s^{-1}.

Unidirectional efflux

Under conditions of unidirectional efflux the chemical concentration of intracellular glucose is gradually lowered and the efflux rate coefficient is, therefore, not constant, but increases with time. The concentration dependence of the rate coefficient is illustrated in Fig. 1.

TABLE IB

Conversion of different flux units to mol cm^{-2} s^{-1}

It is assumed that a 'standard' human red blood cell has a volume of 87 fl of which 58.3 fl (67%) is cell water and 28.7 fl is cell solids, mainly hemoglobin (29–32 pg/cell). The area of the cell membrane is assumed to be $1.42 \cdot 10^{-6}$ cm^2, and 1 kg of cell solids under standard conditions equals $3.1 \cdot 10^{13}$ cells that have a total membrane area of $4.4 \cdot 10^7$ cm^2 (For further discussion, see Ref. 36). The table lists the factors to multiply the flux values in the units listed below to obtain the values in mol cm^{-2} s^{-1}.

mmol/(liter isotonic cell water) per min	$7.15 \cdot 10^{-13}$
μmol/(ml cell water) per min	$7.15 \cdot 10^{-13}$
μmol/(10^{10} cells) per min	$1.17 \cdot 10^{-12}$
mmol/(liter cells) per s	$6.13 \cdot 10^{-11}$
Isotone/min = 310 mmol/(liter cell water) per min	$2.22 \cdot 10^{-10}$
mmol/(kg cell solids) per min	$3.79 \cdot 10^{-13}$
mmol/(kg cell solids) per s	$2.27 \cdot 10^{-11}$

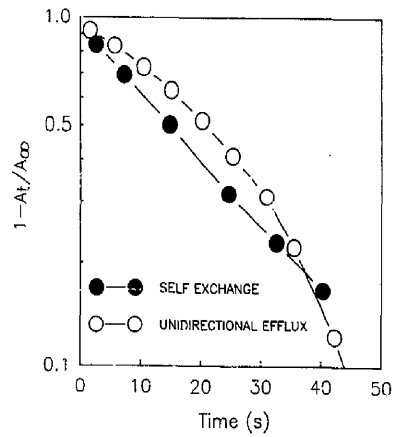

Fig. 1. Semilogarithmic plot of D-[^{14}C]glucose efflux from labeled red blood cells loaded with 72.5 mM glucose at 20°C and pH 7.2 under conditions of self exchange and unidirectional efflux. The ordinate expresses the fraction of tracer that remains in the cells at the sampling time. Under self-exchange conditions the chemical concentrations are constant, and the linearity of the plot indicates that one rate coefficient, equal to the numerical value of the slope, determines the tracer efflux rate. In the unidirectional efflux mode the cells lose glucose, both labeled and non-labeled, and concomitantly water. The progressive desaturation of the transport system and the cell shrinkage enhance the efflux rate coefficient as indicated by the increasingly steep curve.

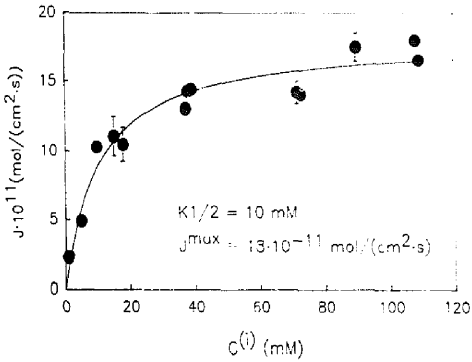

Fig. 2. Glucose self exchange flux as a function of the intracellular glucose concentration in human red blood cells at 20°C (pH 7.2). The flux at each concentration was calculated from $J_{ex} = k \cdot (V_c^{(i)}/A_c) \cdot C^{(i)}$, where k is the rate coefficient (cf. Fig. 1). $(V_c^{(i)}/A_c)$ is the ratio of cell water volume to membrane area, and $C^{(i)}$ is the internal glucose concentration. The curve was fitted to the data points, assuming transport kinetics of simple Michaelis-Menten type, by means of nonlinear regression analysis.

As time passes, and the internal concentrations decreases, the slope of the efflux curve (open circles) becomes more steep. From the initial slopes of the plots of Fig. 1, we see that the rate of efflux under self-exchange conditions is higher than under conditions of unidirectional efflux at 20°C.

Initial rates. The flux results obtained as described in Materials and Methods by the initial rate method in the concentration range 1–120 mM glucose are depicted in Fig. 3 as a function of internal glucose concentration. Each point (±S.D.) represents an average of at least two determinations of the unidirectional efflux. The saturation kinetics reveal that $K_{1/2ue}$ is 6.6 (S.E. ± 0.5) mM, and J_{ue}^{max} is 8.3 (S.E. ± 0.1) · 10^{-11} mol cm^{-2} s^{-1}.

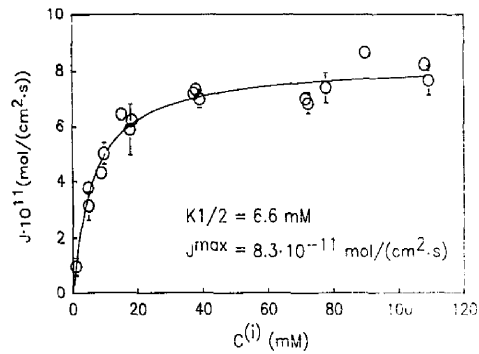

Fig. 3. Unidirectional glucose efflux as a function of the intracellular glucose concentration from human red blood cells at 20°C (pH 7.2). The points of the plot represent the initial efflux at each concentration as described in Materials and Metnods. The curve fitting to the data points was carried out by nonlinear regression analysis, assuming kinetics of simple Michaelis-Menten type.

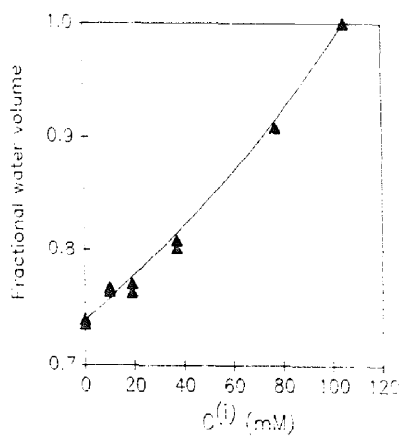

Fig. 4. The relative cell water volume depicted as a function of the intracellular glucose concentration ($C^{(i)}$, mM) in human erythrocytes at 20°C (pH 7.2). Samples of red cells washed in a 120 mM glucose + 150 mM KCl medium were resuspended in media with decreasing glucose concentration and increasing KCl concentrations to keep osmolarity constant. The cell water volumes were determined by drying cells samples, and correcting for trapped extracellular volume and the contribution of glucose to the cell solids. The curve was drawn according to: $V^{(i)}/V^{(i)}(0) = Q^{(i)}(0)/(Q^{(i)}(0) + C^{(i)}(0) - C^{(i)})$, where $V^{(i)}(0)$ and $V^{(i)}$ is the cell water volume before and after, respectively, washes in the low glucose medium. $Q^{(i)}(0)$ is the concentration of impermeants at time zero (osmol/liter cell water), and $C^{(i)}(0)$ and $C^{(i)}$, respectively, is the internal glucose concentration at time zero and after equilibration (washes) in the medium with the lower glucose concentration. The equation describes the relation for red cells performing as perfect osmometers.

Integrated rates. One assumption included in the integrated rate method is that the cells perform as perfect osmometers, that is the cells adjust the cell water volume completely and 'instantaneously' during the loss of the osmotic active glucose molecules. The high osmotic water permeability of human red blood cells [18] implies a sufficiently rapid adjustment of the cell volume. We here tested the completeness of the adjustment. Cells were washed repeatedly in medium A-120, divided in six portions of which the five were washed several times in five media (pH 7.2, 20°C) containing 80, 40, 20, 10 and 0 mM glucose, respectively. KCl was added such that the osmolarities of the six wash media varied < 2%. Determinations of the intracellular glucose concentrations and the water content of the cells (cf. Materials and Methods) are shown in Fig. 4, where the relative cell volume ($V(C^{(i)})/V(\text{initial})$) is depicted vs. $C^{(i)}$. We see that the red cells adjust their water volume as is expected for perfect osmometers.

The integrated rate method was also applied to our results of glucose efflux. Basically, the experiments are identical to the initial rate experiments with the exception that they proceed longer. We could, therefore determine several values of the Michaelis-Menten parame-

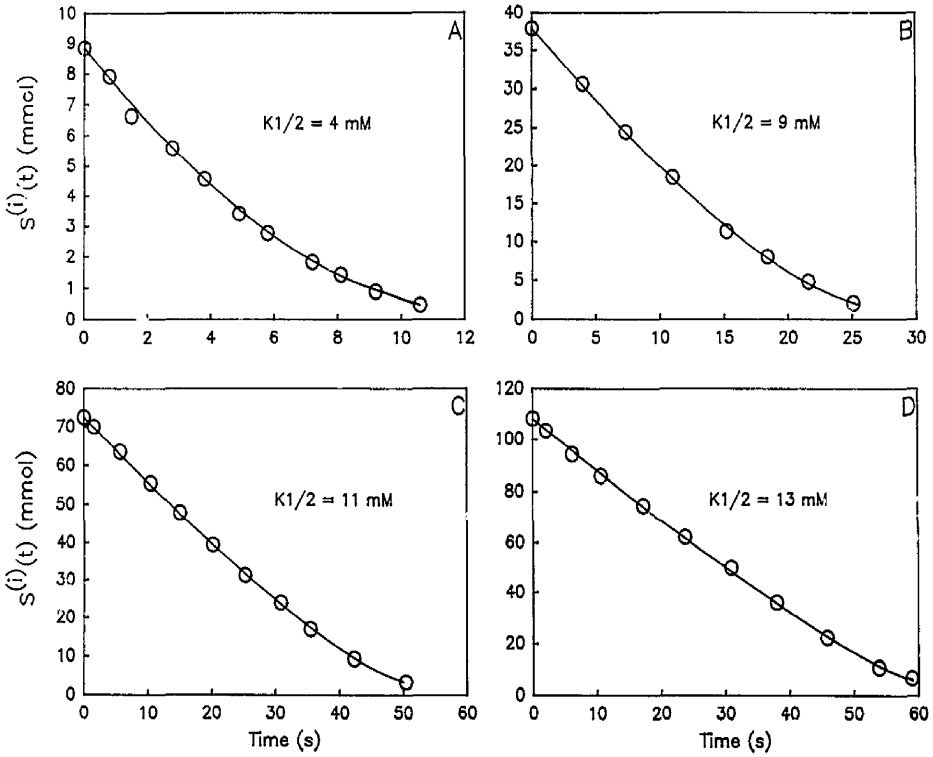

Fig. 5. Unidirectional efflux of glucose from human red blood cells at 20°C (pH 7.2). The intracellular amount of glucose is depicted as a function of time, and the curves are drawn according to the integrated rate equation. Note the difference in the ordinates and the time scales. The glucose concentrations of the wash media used in the four experiments (A, B, C and D) are 10, 40, 80 and 120 mM, respectively. The initial intracellular glucose concentrations in the four experiments are 5–10% lower than in the wash media. For further explanation see Discussion. The experiment in (C) is identical to the unidirectional efflux experiment of Fig. 1.

TABLE II

Values of J_{ue}^{max} and $K_{1/2ue}$ at 20°C (pH 7.2) obtained by means of the integrated rates method and initial rates method

$C^{(i)}$ (mM)	J_{ue}^{max} (10^{-11} mol cm^{-2} s^{-1})		$K_{1/2ue}$ (mM)		$J_{ue}^{max}/K_{1/2ue}$ (10^{-5} cm/s)
	mean	range	mean	range	
Integrated rates method					
4.8	6.2	4.1–10.0	3.7	2.2– 6.9	1.68
8.8	6.3	5.1– 7.7	2.7	1.1– 3.8	2.33
9.7	7.4	6.9– 8.2	3.3	3.0– 3.8	2.24
15.0	9.3	8.6–10.4	4.9	3.9– 6.3	1.90
17.8	10.0	7.8–12.0	9.5	3.5–14.6	1.05
18.2	9.5	8.6–11.3	7.8	5.8–11.4	1.22
37.2	9.0	8.6– 9.5	6.3	5.0– 7.4	1.43
37.9	9.6	8.0–10.4	8.0	3.6–12.3	1.20
72.5	7.9	7.2– 8.4	8.8	6.4–10.9	0.90
80.0	8.9	8.3– 9.5	11.0	6.1–15.0	0.81
89.8	9.6	7.4–10.4	11.0	3.3–14.8	0.87
108.1	9.6	9.3–10.2	12.1	5.1–19.0	0.79
109.2	8.5	7.2– 9.1	10.6	0.6–19.6	0.80
Range	6.2–10.0		2.7–12.1		
Initial rates method					
Mean ± S.E.	8.3 ± 0.1		6.6 ± 0.5		1.26

ters, one set for each initial concentration. Fig. 5 shows four efflux curves with four different initial concentrations. The four cell samples used were washed in media containing 10, 40, 80 and 120 mM glucose, respectively. The initial cellular concentrations were a little lower (90–97% of the glucose concentration in the wash medium) (cf. Discussion). The figure shows that the higher is the initial concentration the longer is the time where the slope is constant, that is the flux is constant. At lower concentrations the slope gradually decreases because the transport system desaturates. Fitting Eqn. 5 to the experimental results yields the Michaelis-Menten parameters that are summarized in Table II.

Discussion

LeFevre [19] demonstrated that glucose transport depends on the internal glucose concentration, and later Wilbrandt [20] showed that the saturable glucose transport in human red cells is asymmetric. Since then several models have been proposed to account for the high values of $K_{1/2}$ obtained under conditions of self exchange and unidirectional efflux, and the low value of $K_{1/2}$ of glucose net influx. For example, a tetramer model [21], an allosteric model [22], and a model with multiple binding sites and allosteric effects [23,24] have been suggested in a continuous discussion of glucose transport kinetics in human red blood cells.

We have been disturbed by the continuous attempts to describe glucose transport kinetics in terms of rather complicated models that essentially should take into account the great difference between the half-saturation constants obtained under conditions of self exchange, unidirectional efflux, and unidirectional influx, because our previous studies [13,25] suggest that the functional asymmetry of the glucose transport system is less than assumed (the structural asymmetry appears well-established by Mueckler et al. [1]).

The perception of the asymmetry of glucose transport basically refers to studies at 20°C under conditions of (1) self exchange showing $K_{1/2}$ values of 32–38 mM [26,27], (2) unidirectional efflux revealing $K_{1/2}$ values of 22–25 mM [17,28], and (3) unidirectional influx where $K_{1/2}$ is 1–2 mM [28–30]. While the $K_{1/2}$ of influx was obtained by determining the initial influx rates, $K_{1/2}$ for unidirectional efflux was estimated by use of the integrated rate equation method, and $K_{1/2}$ for self exchange was found by means of a stopper solution method and measuring the tracer efflux rates. In the present study we have determined $K_{1/2}$ by means of tracer efflux, both under conditions of self exchange and unidirectional efflux. Though the experimental approach basically is similar, the interpretation of the tracer efflux data is different and will be discussed separately.

Glucose self exchange

Under self exchange conditions no net flow of glucose takes place, and any information about the exchange rate requires the use of radioactive labeled glucose to determine either the influx or efflux rate of the tracer. Eilam and Stein [27] used a stopper solution method in which samples after start of the experiment were taken serially at standard times of 10, 20, and 30 s, transferred to an icecold stopper solution containing 171 mM NaCl, 1 μM $HgCl_2$, 1.25 mM KI, and 0.1 mM phloretin. The rationale of the low temperature is obvious as is the presence of the inhibitor. The control experiments to test the efficacy of the stopper solution showed that incubation of the radioactive labeled red cells in the stopper solution at 0°C led to no increase of radioactivity in the external solution within 20 min. However, it is not clear whether their zero-time value represents a control value or a value after the cells were exposed shortly, < 1 min, to the stopper solution. Hence, it is not clear from their Table I whether the inhibition indeed was instantaneous. If the time of the process of transferring a cell sample from the reaction chamber to the stopper solution is not taken into consideration, as in the efflux experiments, the onset of inhibition is delayed, and the intracellular radioactivity has decreased. This delay time was probably rather fixed, and may therefore have played an increasingly critical role as an experimental error when the glucose concentration was lowered (from 140 to 26 mM) and the exchange rate increased. Unfortunately, the appropriateness of the experimental procedure was not considered in light of Mawe and Hempling's earlier work [32] showing that half times of glucose self exchange decreased from 24 s at 165 mM glucose to 5.6 s at 41 mM glucose. Hence, Eilam and Stein [27] used fixed sampling times beginning approximately after two half times had elapsed at the lowest glucose concentration, that decreases the precision of the method.

Miller [26] determined a $K_{1/2ex}$ of 38 mM. Miller [31] realized the necessity of taking samples at intervals of approx. 2 s, and combined several one point determinations to obtain the efflux rate. The tracer efflux was stopped by either filtration or inhibition of a suspension sample and may have built in the same kind of problems of the time resolution as in Eilam and Stein's study.

Recently Lowe and Walmsley [30] determined a $K_{1/2ex}$ of 17 mM by measuring D-[^{14}C]glucose influx that was stopped at the desired times by addition of a stopper solution to the cell suspension. The procedure did not include a transfer step of the cell suspension that diminishes the time lag.

In the present study the rate of self exchange was determined by means of the Millipore-Swinnex filtering technique, as originally described by Mawe and Hempling [32] and later modified by Dalmark and Wieth

[14], and the continuous flow tube method. In studies of chloride self exchange it was shown that the Millipore-Swinnex filtering method could be used to determine transport rates with half times down to 1–2 s [33], and the method is therefore suitable to determine glucose self exchange at 20°C. At low glucose concentrations the efflux rate is too rapid to be determined precisely enough with the Millipore-Swinnex filtering technique, and the continuous flow tube method was therefore used. This method has a time resolution of a few milliseconds and has shown its ability to measure fast transport processes [13,15]. At $C^{(i)} = 5$ mM and under conditions of unidirectional efflux both methods were applied. The flux values obtained by the two methods were almost identical.

Our studies show that $K_{1/2ex}$ is 7.5–10 mM (Refs. 13 and 25, and this study), and make us suggest that the earlier values of $K_{1/2ex}$ of 32–38 mM [26,27] are far too high, and that the high values were obtained because of the use of methods with inappropriate time resolutions. The much lower $K_{1/2ex}$ of 17 mM in Lowe and Walmsley's study [30] would suggest that the transfer time in the studies of Miller and Eilam and Stein [26,27] may be a source of error in determining $K_{1/2ex}$. It may be argued that the great ranges of $K_{1/2}$ values under the two experimental conditions are related to the problem of using either outdated or fresh blood cells. A comprehensive list of $K_{1/2}$ values [35] may suggest that outdated blood cells have a lower affinity ($K_{1/2}$ is higher) than fresh cells to glucose. However, our previous glucose transport studies at 25°C in both fresh and outdated red blood cells (Refs. 13 and 25, see Ref. 35), performed with the same methods, do not support the high $K_{1/2}$ values of outdated blood cells reported previously [17,27,28].

TABLE III

Comparison of $K_{1/2}$ and J^{max} at 20°C from different studies

Outdated blood was used in all studies but [30] and the present study

	$K_{1/2}$ (mM)	J^{max} (10^{-10} mol cm^{-2} s^{-1})
Self exchange		
Miller [26]	38	1.9
Eilam and Stein [27]	32	2.6
Challiss et al. [28]	21.5	2.6
Lowe and Walmsley [30]	17	3.6
This study	10	1.8
Unidirectional efflux		
Karlish et al. [17]	25	0.9
Challiss et al. [28]	22.9	1.4
Lowe and Walmsley [30]	4.6	1.6
This study		
Integrated rate method	7.7 (2.7–12)	0.9 (0.6–1.0)
Initial rate method	6.6	0.8

In contrast to the four fold variation of $K_{1/2ex}$ in the cited studies, J_{ex}^{max} varies less as shown in Table III. Because the combination of the two methods in our study has demonstrated the ability to determine such rapid transport processes [13,33], we feel confident with the value of J_{ex}^{max} in our study though it is in the lower range of the cited values.

Unidirectional efflux

By the unidirectional efflux of glucose the intracellular amount of osmotically active solutes decreases, and the cells shrink. This shrinkage tends to keep up the intracellular glucose concentration. However, the concentration further decreases (towards zero) by the continuous equilibration of glucose between the two compartments. Hence, the unidirectional efflux is controlled by a complex interplay between (the changes of) the cell water volume ($V_c^{(i)}$, cm^3), and the intracellular amounts of glucose ($S^{(i)}$, mol) and impermeants, according to Eqn. 5.

If the initial internal glucose concentration is high, that is $\gg K_{1/2ue}$, the transport system is saturated, and an efflux curve, as depicted in Fig. 1, reveals that the efflux rate becomes increasingly higher with time (the slope $= -k$, s^{-1}) towards a limit value for $C^{(i)} \approx 0$. If, on the other hand the internal amount of glucose is depicted against time (cf. Fig. 5), we see that the linearity of the initial part of the plots becomes less pronounced when the initial glucose concentration decreases from approx. 110 mM to approx. 9 mM (D through A).

Determinations of $K_{1/2ue}$ and J_{ue}^{max} by means of the initial rate method are subject to two major problems that are mutually connected, the constancy of the flow and the duration of the observation time. We exploited that the flux $(dS^{(i)}(t)/dt)$ initially was constant at the high concentrations (cf. Fig. 5) while at low initial concentrations the rate coefficient $(d \ln[S^{(i)}(t)]/dt)$ showed less variation than the flux. Arbitrarily, we set 5 mM as the concentration above which the flux was determined as $dS^{(i)}(t)/dt$. The second problem of restricting the observation time to a minimum where the internal glucose concentration changes but little was settled by applying our methods with high time resolutions. By means of the Millipore-Swinnex filtering technique six cell-free filtrates can be serially collected within appropriate time intervals, down to 5–6 s. This time resolution appears high enough except at low concentrations ($\leqq 5$ mM), where we used the continuous flow tube technique.

An alternative way to determine $K_{1/2ue}$ and J_{ue}^{max} is to incorporate the changes of cell volume and glucose concentration during the glucose efflux as is done in the integrated rate equation (cf. Eqn. 5). Ideally both methods, initial rates and integrated rates, should of course reveal identical results with regard to $K_{1/2ue}$ and J_{ue}^{max}.

The conflicting values of $K_{1/2uc}$ determined by the integrated rates method (22–25 mM at 20°C [17,28]) and the initial rates method (5.8 mM at 25°C [13]) cannot, as emphasized by Brahm [13] be due to a temperature effect. Consequently, the difference predominantly must be due to a disagreement about theory or an experimental problem. Our reevaluation of the integrated rate equation [2] as shown above in its final form (Eqn. 5) revealed no serious disagreement with previous presentations of the equation (in different forms) [17,28] that can account for the difference. We next combined the integrated rate method with our rapid filtering methods to expose the possible technical source of error. The advantage of the Millipore-Swinnex technique, and in particular the continuous flow tube method, is that it takes only a short time to separate the cells from the suspending medium, stopping the efflux process. Further, because the cell-free filtrates are examined for the increasing radioactivity with time, the rate of efflux is determined with a high degree of precision though it decreases by the process approaching tracer equilibrium. We generally do not use data points obtained after approx. 90% of the equilibrium value has been attained. Hence, the problem of the method may be to determine the very late data points. In contrast, the stopper solution method was used to determine the radioactivity that remains in the cells at a given time [17,28] that should provide an increasing precision in determination of the changes of intracellular tracer with time. It should be noted that all the data points in our efflux experiments (cf. Fig. 5) represent samples, including the very first one shortly after time zero, collected during the experimental time.

In the studies by Karlish et al. [17] the first 'zero time' intracellular radioactivity was obtained by a separate determination, in parallel to the efflux experiments, and the intracellular glucose concentration was set equal to the concentration in the incubation medium. Challiss et al. [28] constructed an efflux curve from several one point determinations, and assumed the intracellular and extracellular glucose concentrations to be equal at time zero (cf. Fig. 2 in Ref. 28).

The important point to note in connection to the use of the integrated rate equation, in particular of the linear transformations (cf. Eqn. 6) that were convenient to use until more sophisticated computer programs were available, is that the analysis is very susceptible to the zero time point, giving rise to great variations of $K_{1/2uc}$.

The assumption that $C^{(i)} = C^{(o)}$ at time zero is questionable as our control experiments show that the distribution ratio, $r = C^{(i)}/C^{(o)}$, is 0.90–0.97 in human red cells. The reason for a distribution ratio < 1 is not clear, but we exclude incomplete equilibration of glucose across the red cell membrane because (1) $r = C^{(i)}/C^{(o)}$ remains < 1 during long term incubation and (2) r becomes < 1 if cells with an initially high glucose concentration are washed in a medium with a lower glucose concentration (cf. Fig. 4). A likely explanation is that part of the red cell water phase is not accessible to glucose. In that case the intracellular glucose concentrations are underestimated by a few percent.

Because we can obtain several samples within the 10 seconds time interval where no samples were taken in Karlish et al.'s study (Ref. 17, Fig. 1), and one or two samples in Challiss et al.'s study (Ref. 28, Fig. 2), we may also determine initial rates of glucose efflux. While the two previous studies used only one starting glucose concentration (approx. 40 mM [28], and approx. 80 mM [17]), we did experiments at several concentrations including the two ones used by Karlish et al. and Challiss et al. [17,28]. Our results obtained at approx. 5–110 mM (cf. Fig. 5 and Table II) show that the average values of $K_{1/2uc}$ were in the range 2.7–12.1 mM if the data were analyzed by means of the integrated rate equation, and 6.6 mM when analyzed by means of the initial rates method. Hence, the experiments analyzed in one or the other way reveal values of $K_{1/2uc}$ much lower than the previously published values of 22–25 mM.

Inspection of Table II shows that $K_{1/2uc}$ increased with an increasing initial glucose concentration. At all glucose concentrations intracellular metabolism with formation of e.g. glucose 6-phosphate, CO_2, HCO_3^-, and lactate that are all candidates for ^{14}C labeling, takes place. The contribution of each component to the total tracer efflux is not easy to sort out, even if e.g. ^3H-labeled glucose is used. Our routine determinations of external glucose by means of the GOD-Perid method, however, do not show that the products of the metabolism play any significant role, except at low (< 1–2 mM) glucose concentrations where the internal concentrations may be significantly reduced if the cells were not kept on ice and used as promptly as possible.

The other possible error of significance is that the cell may not perform as perfect osmometers. Our results as depicted in Fig. 4 support the conclusion that cells, once washed in a solution with a higher glucose concentration, and next suspended in a solution with a lower glucose concentration and KCl added to maintain osmolarity indeed regulate completely as the water content declined in accordance with the concept of red cells behaving as perfect osmometers. This test does not rule out that the other part of the function as a perfect osmometer, an instantaneous regulation takes place. However, the human red blood cells have a high osmotic water permeability [18] that excludes a delay in volume regulation as the osmotic active glucose molecules leave the cell water phase considerably slower. We therefore conclude that the variation of $K_{1/2uc}$ with the initial glucose concentration is not due to the red cells functioning as imperfect osmometers.

Naftalin et al. [34] reported that $K_{1/2uc}$, determined by means of the integrated rate method, at 2°C in-

creased 3-fold from 40 mM by a rise of the initial glucose concentration from 7.5 to 120 mM, and their results at 20°C with $K_{1/2ue}$ of 17.7 mM ($C^{(i)} \approx 75$ mM) and 32.9 mM ($C^{(i)} \approx 120$ mM) suggest the same trend (Table 1 in Ref. 34). Naftalin et al. [34] suggested that the concentration dependence of $K_{1/2ue}$ is due to an internal compartementalization of the red cells, glucose being either bound or free. Their suggestion was further supported by the observation that the ratio $J_{ue}^{max}/K_{1/2ue}$ decreased with increasing concentration, in accordance with their proposed model. In our study at 20°C the same qualitative and quantitative changes were found (cf. Table II) in favor of the model of Naftalin et al. [34], but it should be noted that the absolute values of $K_{1/2ue}$ at 20°C in their study are considerably larger than the values in the present study. If the hypothesis of internal compartmentalization of glucose holds true the course of tracer efflux under conditions of self exchange should also be affected. However, control experiments under conditions of self exchange (data not shown) where > 90% of the tracer equilibration was monitored followed a monoexponential course, indicating that only one rate coefficient determined the transport process. However, if a small fraction of internal glucose is confined to a relatively slow releasing compartment only the very last part of the efflux curve will be affected. As mentioned above our filtering methods are not suitable for determining the very late data points, so small deviations from the monoexponential course due to internal compartementalization of glucose tracer may not be detected.

Concluding remarks

Our study at 20°C shows that $K_{1/2}$ under conditions of self exchange is approx. 10 mM, and in the unidirectional efflux mode is approx. 7 mM. We have not determined the external affinity of the transport system to glucose, but we note that a high affinity, represented by a low $K_{1/2}$ of 1.6 mM for unidirectional influx has been determined [28–30]. We agree with Wheeler and Whelan [35] who recently concluded that the so-called 'carrier model', that also accounts for conformational changes in the band 4.5 integral membrane protein mediating glucose transport in human red cells, suffices to describe the kinetics. Our values of $K_{1/2ex}$, $K_{1/2ue}$, J_{ex}^{max} and J_{ue}^{max} show good agreement with the values predicted in Wheeler and Whelan's model of the simple carrier [35]. Whether or not the kinetics of glucose transport show asymmetry that appears insignificant at 20°C [34] (for a discussion of the criteria, see, for example, Stein [10]) it seems that complicated models [21–24] can be abandoned. Our results also underline the need to reexamine and repeat experiments so thoughtfully planned to characterize glucose transport kinetics, but carried out by means of methods that may not be sufficiently accurate.

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