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KINETICS OF GLUCOSE TRANSPORT IN HUMAN ERYTHROCYTES

BY JESPER BRAHM

From the Department of Biophysics, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

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SUMMARY

1. The rate of unidirectional D-[^{14}C]glucose efflux from human red blood cells was determined at self-exchange and net-efflux conditions by means of the Millipore–Swinnex filtering technique and the rapid continuous flow tube technique with which initial rates can be measured within fractions of a second.

2. Determinations at 38, 25 and 10 °C of the concentration dependence of glucose self-exchange flux and net efflux showed that both self exchange and net efflux followed simple Michaelis–Menten kinetics at all temperatures. At 38 °C the maximal self-exchange flux and the maximal net efflux were identical (6×10^{-10} mol/cm^2 . sec). The cellular glucose concentration for half-maximal flux ($K_{\frac{1}{2}}$) was 6·7 mM for self exchange and 8·2 mM for net efflux. By lowering temperature the maximal glucose self-exchange flux progressively exceeded the maximal net efflux, and was about three times larger at 10 °C. $K_{\frac{1}{2}}$ for self exchange increased to 12·6 mM at 10 °C, while $K_{\frac{1}{2}}$ for net efflux decreased to 4·4 mM.

3. At 38 °C the glucose permeability at self exchange at a constant extracellular glucose concentration of 40 mM showed a bell-shaped pH dependence between pH 6 and pH 9. A maximum was found at pH 7·2, whereas the apparent permeability coefficient was halved both at pH 6 and pH 9.

4. The temperature dependence of glucose transport was determined between 47 and 0 °C at a cellular glucose concentration of 100 mM which ensured $> 85\%$ saturation of the glucose transport system within the temperature range. The Arrhenius activation energy of glucose transport was not constant. By lowering the temperature, the activation energy increased gradually for net efflux from 55 kJ/mole between 38 and 47 °C to 151 kJ/mole between 0 and 10 °C. The temperature dependence of self-exchange flux showed a more pronounced change around 10 °C. The Arrhenius activation energy was found to be 61 kJ/mole above and 120 kJ/mole below 10 °C.

INTRODUCTION

D-glucose transport in human red blood cells proceeds by facilitated diffusion. The concentration-dependent permeability is more than three orders of magnitude larger than permeability coefficients between $2{\cdot}4 \times 10^{-10}$ cm/sec (Jung, 1971) and $2{\cdot}6 \times 10^{-8}$ cm/sec (Lidgard & Jones, 1975) of bimolecular lipid membranes through which glucose is transported by simple diffusion. The kinetics of the facilitated

glucose transport have been studied extensively for decades. LeFevre (1948) showed that glucose efflux was a function of the intracellular glucose concentration, and a few years later Wilbrandt (1954) first demonstrated asymmetry of the glucose transport system. Numerous later studies which have confirmed and extended these observations have been analysed and discussed in recent reviews (Jung, 1975; LeFevre, 1975; Naftalin & Holman, 1977; Widdas, 1980). It is currently accepted that glucose transport differs in self-exchange and net-flux modes, because previous results have suggested that the maximum flux (J_{max}) and the half-saturation constant ($K^{\frac{1}{2}}$), which are used to describe the kinetics of facilitated transport, are both larger in the glucose self-exchange mode than in the net-flux mode. There is, however, a considerable spread in the values for $K^{\frac{1}{2}}$ and J_{max} of the two types of transport, published from different laboratories. This may, in part, reflect that glucose transport proceeds so rapidly that the experimental determination of glucose-transport parameters is difficult.

Since most of the previous studies were performed at temperatures below 25 °C it has been an open question whether data at low temperatures can be extrapolated to the physiological temperature range. In the present study, glucose-transport kinetics were therefore studied at 38 °C and re-investigated at lower temperatures using methods particularly well suited for measuring rapid transport processes: the Millipore–Swinnex filtering technique (Dalmark & Wieth, 1972) and the continuous flow tube method (Brahm, 1977, 1982, 1983). Unidirectional glucose fluxes were determined in the self-exchange mode and in the net-efflux mode by means of tracer measurements. At 38 °C glucose self-exchange fluxes and net effluxes were equal. By lowering the temperature to 0 °C, glucose self exchange decreased by a factor of 125, and net efflux by a factor of 560, giving rise to an increasing difference between net efflux and the larger self-exchange flux. The difference was however less pronounced than suggested by previous studies, for example, Mawe & Hempling (1965) determined the ratio of self-exchange flux/net efflux to be 2·3 at room temperature, whereas this study gives a factor of 1·4. Though the temperature dependence of glucose self exchange and net efflux differs, both types of transport followed simple Michaelis–Menten kinetics at 38 °C, as well as at lower temperatures.

Several transport models have been published to describe glucose transport in human red blood cells (see LeFevre, 1975; Naftalin & Holman, 1977; Widdas, 1980). Complicated kinetic models, for example, the tetramer model of Lieb & Stein (1970) and the allosteric-pore model of Holman (1980) were suggested to account primarily for the large difference between values of the half-saturation constant ($K^{\frac{1}{2}}$ mM) determined in self-exchange and net-flux modes. In this study, the $K^{\frac{1}{2}}$ of self exchange and net efflux was 8·1 and 5·8 mM, respectively, at room temperature. The $K^{\frac{1}{2}}$ of self exchange is much lower than the published values of 32–38 mM (Miller, 1968*b*; Eilam & Stein, 1972) used for model considerations. The present results suggest a reconsideration of the existing models.

METHODS

Glucose self-exchange and net-efflux experiments were performed by measuring the rate of the unidirectional efflux of radioactive D-glucose from packed labelled red blood cells suspended in a large extracellular compartment. In glucose net-efflux experiments glucose-loaded cells were

suspended in a glucose-free medium, and the exit of labelled glucose was followed in a short time interval to give 'initial rates' (see *Experimental procedures*).

Electrolyte medium. The electrolyte medium had the following composition (mM): 150, KCl; 0·5, KH_2PO_4, and 1–120, D-glucose (Merck, Darmstadt). The medium was titrated to the desired pH of the subsequent experiment with 1 M of either HCl or KOH.

Preparation of cells. Red blood cells from a heparinized blood sample were washed once in the medium with the appropriate glucose concentration and titrated to the desired pH with either CO_2 or 150 mM-$KHCO_3$. At extreme pH values small amounts of 150 mM-HCl or -KOH were added. After titration the red blood cells were washed three times in the glucose medium before labelling with [^{14}C]glucose (0·5–1 μc/ml. cell suspension, The Radiochemical Centre, Amersham). The preparation procedures were carried out at 38 °C, and the efflux experiments were performed immediately afterwards to reduce maximally the fraction of metabolized glucose.

Preliminary results at 38 °C show that glucose permeability equals the permeability of the non-metabolized 3-*O*-methylglucose, even at a low sugar concentration of 1 mM, indicating that metabolism of glucose cannot affect the flux results during the experiments (J. Brahm & J. Vinten, unpublished experiments). Cell samples for efflux experiments, for determinations of distribution of tracer between cells and medium, and of cell water content were obtained by centrifugation as described previously (Brahm, 1977). Independent of glucose concentration and temperature, the distribution of radioactively labelled glucose between the intracellular and the extracellular water phases ($r^{glucose} = C_i/C_0$) was less than one where C_i and C_0 are the intracellular and extracellular concentrations of glucose respectively. For example, in the series of experiments determining the concentration dependence of glucose transport at 38 °C $r^{glucose}$ was 0·87 (S.D. 0·04, $n = 15$). Since the ratio was independent of concentration in the extracellular concentration range 1–120 mM-glucose, one can exclude significant intracellular metabolism of glucose at low concentrations and insufficient glucose equilibration, during the preparation procedure, at high glucose concentrations as causes of the deviation of the ratio from unity. Further, in red blood cell ghosts the ratio is one (J. Brahm, unpublished results). It is therefore possible that about 10–15 % of the intracellular water in red blood cells is not accessible to glucose.

Determination of radioactivity. The radioactivity of cells and medium was determined by liquid scintillation spectrometry after precipitation with perchloric acid as described by Dalmark & Wieth (1972). The radioactivity of the cell-free filtrates and equilibrium sample of the experiments was measured without preceding precipitation because traces of haemoglobin do not affect the spectrometrical measurements.

Determination of cell water content. Cell water was determined by drying cell samples to constant weight. As about 2 % of extracellular medium was trapped between the cells during the packing procedure (Brahm, 1977), a correction for this contribution was carried out. The ratio between intracellular solvent volume (V, cm^3) and cell membrane area (A, cm^2) was calculated assuming that 1 g of cell solids equals $3·1 \times 10^{10}$ normal erythrocytes with a total membrane area of $4·4 \times 10^4$ cm^2 ($1·42 \times 10^{-6}$ cm^2/cell, see Wieth, Funder, Gunn & Brahm, 1974; Brahm, 1982). In cell samples with high glucose concentrations, the cell solids content was corrected for the contribution by glucose.

Experimental procedures. Two different methods were used depending on the rate of tracer efflux. In self-exchange experiments, with rate coefficients of tracer efflux less than $\sim 0·6$ sec^{-1}, corresponding to half-times above $\sim$ 1 sec, efflux rates were determined with the Millipore–Swinnex filtering technique described by Dalmark & Wieth (1972). Faster rate coefficients are above the upper limit of determinations by this method. Efflux rates with half-times in the millisecond range were, therefore, determined with the continuous flow tube method previously described (Brahm, 1977, 1982, 1983). The overlap of the performance of the two methods was tested by measuring glucose self exchange at 20 mM-glucose (25 °C, $T_{\frac{1}{2}} \sim 3·7$ sec). In three experiments the Millipore–Swinnex filtering technique gave a mean rate coefficient of 0·191 sec^{-1} (range 0·180–0·203), and the continuous flow tube method 0·185 sec^{-1} (range 0·180–0·190). Besides, in determinations of rapid self-exchange rates, the flow tube method was used in net-efflux experiments to obtain so-called 'initial rates' by following the efflux of tracer in less than 10 % of the half-time. A sufficiently short observation period, compared to the half-time of the net efflux, implies that neither the intracellular glucose concentration nor the cell volume change significantly within the experimental period, reducing greatly errors in determinations of initial rates. With both methods the rate of tracer efflux was determined by suspending labelled red blood cells in a large extracellular volume which ensures a low haematocrit (< 1 %). The rate of tracer efflux was well described by a two-compartment model

with compartments of constant volume, and the kinetics were found to follow the equation:

$$1-(a_t-a_0)/a_\infty = e^{-kt}, \quad (1)$$

where, a_0, a_t and a_∞ denote the extracellular radioactivity at zero time, at time t, and at equilibrium, respectively. The radioactivity in the extracellular phase at the start of the experiment (a_0) was due to isotope in the extracellular medium trapped between the cells, during the packing of the cell sample for the efflux experiment. In the samples used for determinations with the Millipore–Swinnex filtering method, about 2 % of the total amount of radioactivity was found extracellularly, whereas it amounts to ~ 10 % in the samples for the continuous-flow experiments. The left-hand side of equation (1), represents the fraction of tracer that remains intracellular at time t. The rate coefficient, k (sec^{-1}), of tracer efflux was determined by linear regression analysis of the logarithmic expression obtained from equation (1).

The rate coefficient is related to the permeability coefficient, P (cm/sec) by:

$$P = k\,V/A \quad (cm/sec), \quad (2)$$

where V/A (cm) is the ratio between the intracellular solvent volume and the membrane area.

Glucose transport showed saturation kinetics of the Michaelis–Menten type (cf. Fig. 4). The apparent glucose permeability (P_{app}) is thus a function of glucose concentration and decreases with increasing glucose concentration. The unidirectional flux, J_{uni} (mole/cm^2 . sec), is defined by:

$$J_{uni} = P_{app} C_i \quad (mole/cm^2 . sec), \quad (3)$$

where C_i is the intracellular glucose concentration (mole/cm^3).

The temperature dependence of glucose transport was calculated by linear regression analysis of the relation:

$$\ln J_{uni} = -E_A/RT + constant, \quad (4)$$

where, E_A (J/mole) is the Arrhenius activation energy for the transport process, R is the gas constant (8·32 J/mole °K), and T is the absolute temperature (°K).

RESULTS

The pH dependence of glucose permeability at 38 °C

Glucose permeability was determined at a constant glucose concentration of 40 mM in the pH range from 6 to 9. Fig. 1 shows that the glucose permeability coefficient (P cm/sec, upper graph) varied in the pH range under study. A maximum permeability was found at pH 7·2 at which the permeability coefficient was $1{\cdot}4 \times 10^{-5}$ cm/sec. The permeability decreased at both more acid and more alkaline pH values and was almost halved at pH 6 and 9. The permeability coefficient is the product of the rate coefficient (k sec^{-1}, second graph) and the ratio between the intracellular solvent volume and the membrane area (V/A cm, lower graph). Both the rate coefficient and the intracellular solvent volume (V cm^3) were varied by raising pH. Cell water content was decreased smoothly by increasing pH, whereas the rate coefficient showed a bell-shaped dependence of pH, like that of the calculated apparent permeability coefficient. The subsequent examinations of concentration dependence and temperature dependence of glucose transport were performed at pH 7·2.

[^{14}C]glucose efflux during self exchange and net efflux

The glucose permeability was concentration dependent. Fig. 2 shows the rate of glucose self exchange at 38 °C at three different glucose concentrations. The logarithmic ordinate expresses the fraction of tracer remaining in the cells at a given time. The slope of the curves equals the rate coefficient of the exchange process under the

experimental conditions of a very low haematocrit (cf. Methods). The rate of self exchange at 1 mM was obtained with the continuous flow tube method and the curve of the Figure demonstrates that the method was well suited to determine rate coefficients of exchange processes with half-times ($T^{\frac{1}{2}} = \ln 2/k$, sec) below 1 sec. The efflux curves at 40 and 80 mM-glucose were obtained with the Millipore–Swinnex filtering technique. The Figure illustrates that the rate of self exchange decreased, and the half-time thereby increased, as the glucose concentration was raised.

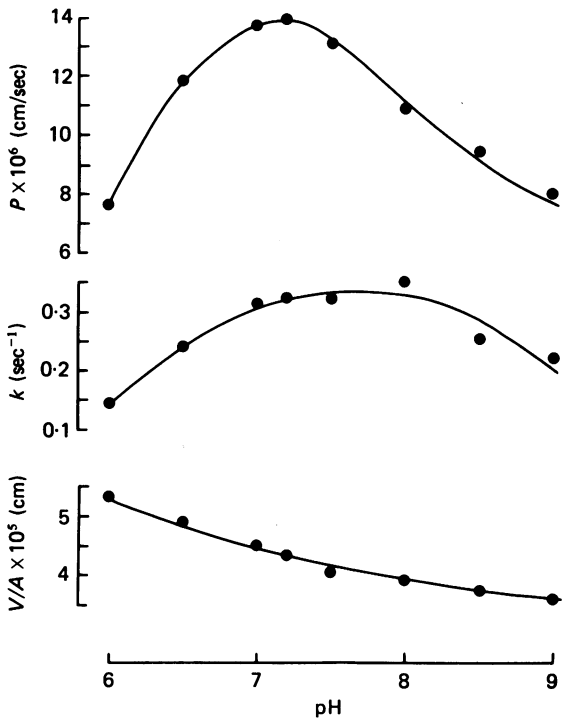

Fig. 1. The pH dependence of glucose permeability in human red blood cells at 38 °C. The apparent permeability coefficient (P) was determined at a glucose concentration of 40 mM. The permeability coefficient is the product of the rate coefficient, k (sec^{-1}) (second graph), and the ratio between the cellular solvent volume, V (cm^3), and the membrane area, A (cm^2) shown at the lower graph. The bell-shaped pH dependence of permeability showed a maximum at pH 7·2 at which the apparent permeability was $1{\cdot}4 \times 10^{-5}$ cm/sec.

In self-exchange experiments the chemical concentrations remained constant with time, and the efflux of labelled glucose could be followed through two or more half-times. In contrast, net-efflux experiments were performed by suspending glucose-loaded cells in a glucose-free electrolyte medium, and the intracellular glucose concentration decreased with time. Consequently, the rate of glucose efflux increased with time, as the intracellular glucose concentration decreased. If, however, one restricts the observation period to the initial small fraction of the first half-time of labelled glucose exit (i.e. $< 10\%$), 'initial' rates could be obtained. Fig. 3 compares the rate coefficients of glucose self exchange (Fig. 3B) and of net efflux (Fig. 3A) at

38 °C in red blood cells loaded with 40 mM-glucose. Note that both the ordinate and the abscissa in Fig. 3*A* have been extended by a factor of ten compared to the co-ordinates of Fig. 3*B*. The rate of labelled glucose efflux in the net-efflux experiment was determined with the continuous flow tube method by following the exit of ~ 8% of the intracellular radioactivity at time zero. Identical rate coefficients were found in both experimental conditions, and the linearity of the efflux curve of Fig. 3*A* indicates its applicability to calculate the initial rate.

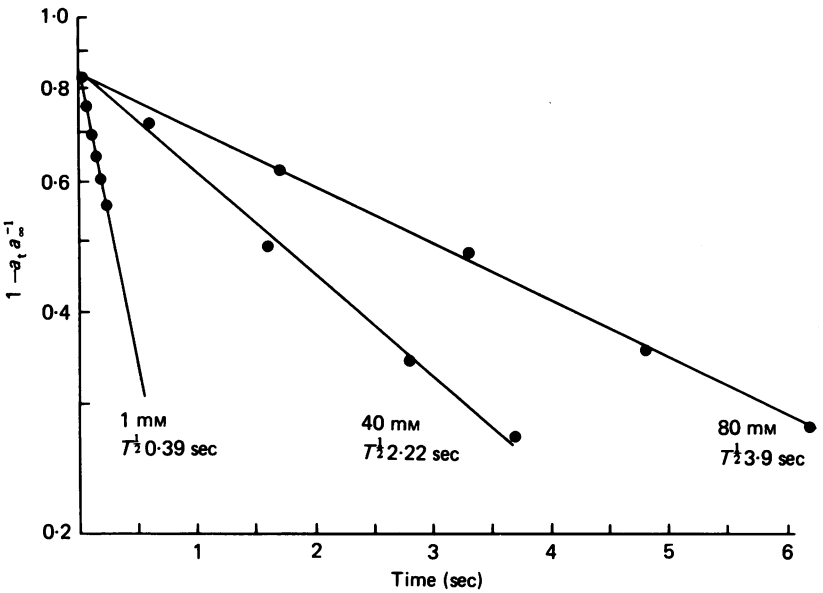

Fig. 2. The rate of glucose self exchange in human red blood cells (38 °C, pH 7·2) at three different glucose concentrations, determined as the unidirectional efflux of [^{14}C]glucose from labelled cells into an isotope-free extracellular medium. The rate of self exchange at 1 mM was obtained by means of the continuous flow tube method. At 40 and 80 mM-glucose, the rates were determined with the Millipore–Swinnex filtering technique. The ordinate shows the fraction of tracer remaining in the cells at time t. The exchange is well described by a closed two-compartment system with an extracellular compartment constituting more than 99% of the total volume. The rate coefficients, k (sec^{-1}), equal the negative slope of the wash-out curves and were determined by linear regression analysis. The half-time of the exchange processes is related to the rate coefficient by: $T^{\frac{1}{2}} = \ln 2\ k^{-1}$ (sec).

Concentration dependence of glucose transport

Experiments at 38 °C. In Fig. 4*A* glucose self-exchange flux and net efflux at 38 °C (pH 7·2) are depicted against the intracellular glucose concentration. Both types of glucose transport saturate. The curves were drawn assuming simple Michaelis–Menten kinetics described by: $J_{uni} = J_{uni}\,(max) \times C_i/(K^{\frac{1}{2}} + C_i)$. The maximal glucose transport (J_{uni} (max), mole/cm^2 . sec) and the half-saturation constant ($K^{\frac{1}{2}}$, mM), defined as the intracellular glucose concentration at which the glucose transport is half maximal, were obtained from the Hanes plot of Fig. 4*B* depicting the reciprocal of permeability *vs.* the cellular glucose concentration. In this plot the reciprocal of the slope of the

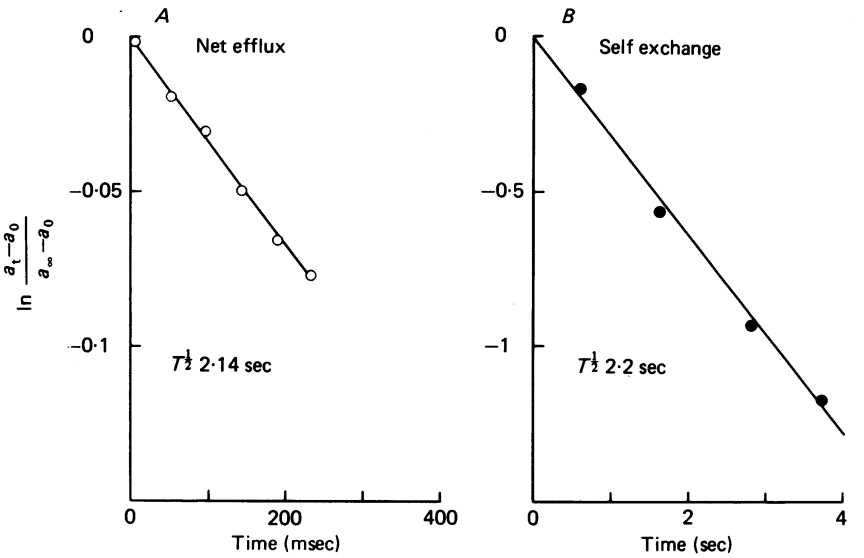

Fig. 3. The rate of [^{14}C]glucose efflux at glucose self exchange and net efflux at 38 °C (pH 7·2) from red blood cells loaded with 40 mM-glucose. The net-efflux experiment was carried out with the flow tube method by following the exit of less than 10% of the intracellular radioactivity to obtain initial rates. The axes of the diagram depicting the wash-out curve for net efflux have been extended by a factor of ten. The slope of the curve is, therefore, directly comparable to the slope of the self-exchange curve obtained with the Millipore–Swinnex filtering technique through two half-times of exchange. The equal slope of the two curves indicates that glucose permeability equals at self exchange and net efflux.

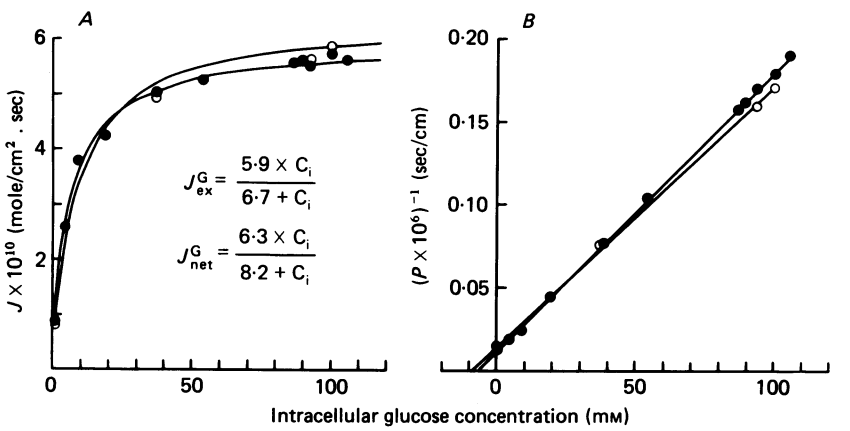

Fig. 4. Glucose self-exchange flux (●) and net-efflux (○) as a function of intracellular glucose concentration at 38 °C (pH 7·2). The curves in *A* were drawn according to the inserted equations for self-exchange flux (J^G_{ex}) and net efflux (J^G_{net}) describing glucose transport by simple Michaelis–Menten kinetics. The maximal glucose flux at self exchange and net efflux of $5{\cdot}9 \times 10^{-10}$ and $6{\cdot}3 \times 10^{-10}$ mole/cm^2 . sec, respectively, was determined as the reciprocal of the slope of the curve in the Hanes plot in *B*. The half-saturation constants ($K\frac{1}{2}$) of 6·7 and 8·2 mM were found by the intersection of the curves with the abscissa.

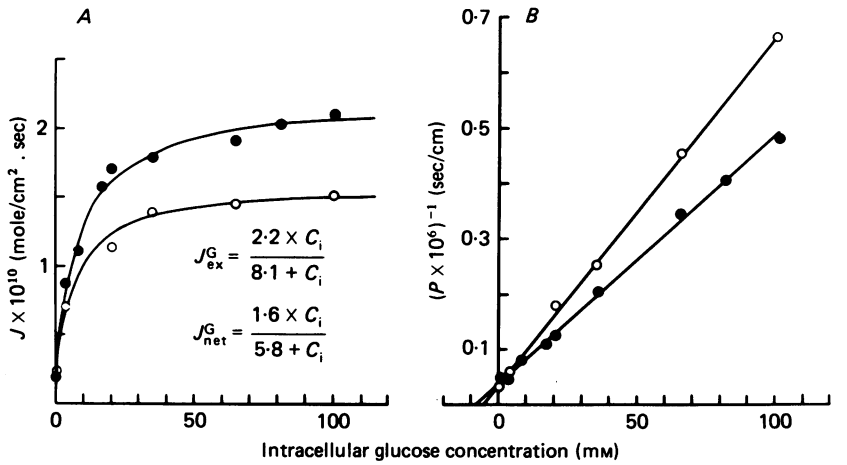

Fig. 5. The dependence of glucose transport and glucose permeability at self exchange and net efflux on intracellular glucose concentration at 25 °C (pH 7·2) depicted in plots similar to Fig. 4. Both self exchange and net efflux followed simple Michaelis–Menten kinetics. The maximal fluxes and the half-saturation constants used in the inserted equations were found as described in the legend to Fig. 4.

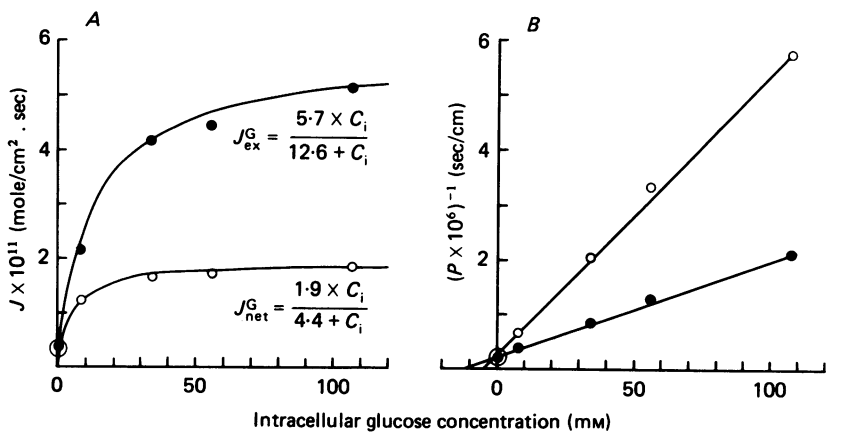

Fig. 6. Glucose self exchange (J^G_{ex}) and net efflux (J^G_{net}) dependence on intracellular glucose concentration at 10 °C (pH 7·2). Again the curves shown in *A* were drawn by assuming simple Michaelis–Menten kinetics. The constants of the inserted equations were found from the plot shown in *B*.

straight curves equals the maximal glucose fluxes and the intersection of the curves with the abscissa gives the half-saturation constants. As indicated by the same location and slope of the curves for self exchange and net efflux the half-saturation constants and the maximal fluxes were almost equal. $K^{\frac{1}{2}}$ for self exchange was 6·7 mM and for net efflux 8·2 mM. The respective maximal fluxes were $5{\cdot}9 \times 10^{-10}$ and $6{\cdot}3 \times 10^{-10}$ mole/cm^2 . sec.

Experiments at 25 °C. Fig. 5 shows the results of similar experiments carried out at room temperature. Both self-exchange flux and net efflux again followed simple Michaelis–Menten kinetics. However, the maximal net efflux of

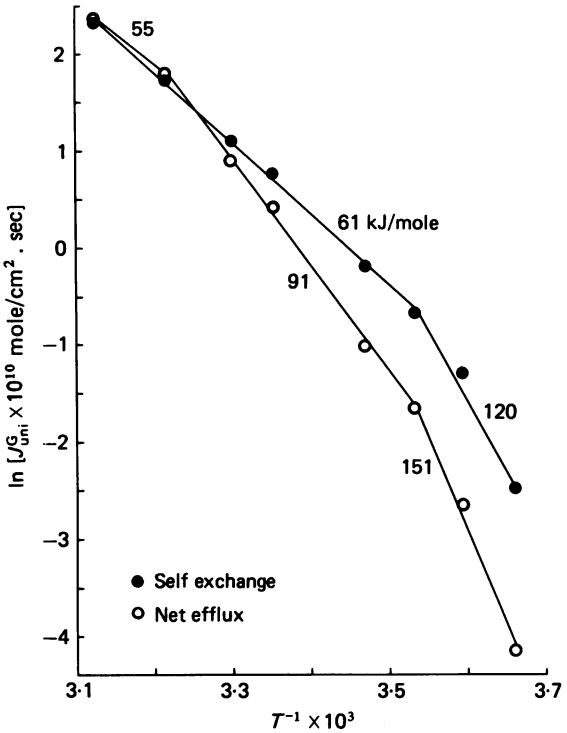

Fig. 7. The temperature dependence of glucose self exchange (●) and net efflux (○) between 0 and 47 °C. The experiments were performed with red blood cells containing 100 mM-glucose which ensured a high saturation of the transport system. The temperature dependence of net efflux decreases more gradually by raising temperature while the apparent activation energy of self-exchange flux shows a more abrupt change about 10 °C. The activation energy in the various temperature ranges was determined by linear regression analysis, except for net efflux at 38–47 °C, where the activation energy was calculated from: $E_A = (\ln J_1 - \ln J_2) \times RT_1 T_2/(T_1 - T_2)$ according to Exner (1964).

$1{\cdot}6 \times 10^{-10}$ mole/cm^2 . sec was only two thirds of the maximal self-exchange flux of $2{\cdot}2 \times 10^{-10}$ mole/cm^2 . sec in the Hanes plot of Fig. 5*B*. The half-saturation constant for self exchange increased to 8·1 mM, whereas $K^{\frac{1}{2}}$ for net efflux decreased to 5·8 mM.

Experiments at 10 °C. By a further decrease in the temperature to 10 °C, the difference between maximal net efflux and self-exchange flux became even more pronounced as demonstrated in Fig. 6. The Hanes plot of Fig. 6*B* gave respective maximal fluxes for self exchange and net efflux of $5{\cdot}7 \times 10^{-11}$ and $1{\cdot}9 \times 10^{-11}$ mole/cm^2 . sec. $K^{\frac{1}{2}}$ for self exchange increased further to 12·6 mM, and $K^{\frac{1}{2}}$ for net efflux decreased to 4·4 mM.

The temperature dependence of glucose self exchange and net efflux

The temperature dependence of glucose self exchange and net efflux was determined between 0 and 47 °C (pH 7·2) at an intracellular glucose concentration of 100 mM which ensured that glucose transport was studied at high saturation (> 85%) at all temperatures. The Arrhenius diagram of Fig. 7, depicting the logarithm of the

unidirectional glucose flux (J^{G}_{uni}) as a function of the reciprocal absolute temperature, shows that the over-all temperature dependence of net efflux was larger than that of self-exchange. Both types of transport showed a non-linear dependence of temperature indicating that the Arrhenius activation energy differs at low and high temperatures. The temperature dependence of self exchange can be described as compounded of two activation energies of 120·3 kJ/mole (S.D., ±19·0; regression coefficient, 0·987) below and 61·3 kJ/mole (S.D., ±1·1; regression coefficient, 0·999) above an apparent breaking point at 10 °C. The same simplified description of the temperature dependence was not applicable for net efflux, because the activation energy decreased more gradually from 151 kJ/mole between 0 and 10 °C to 55 kJ/mole between 38 and 47 °C.

The temperature dependence of glucose self exchange was also studied at an intracellular glucose concentration of 36 mM (results not shown) at which the glucose transport system was considerably more unsaturated. Linear regression analysis indicated a kink of activation energy at 15 °C. In the 38–15 °C range, the activation energy was 65·3 kJ/mole (S.D. ±2·0; regression coefficient, 0·998) and in the 15–0 °C range it was 92·4 kJ/mole (S.D. ±4·3; regression coefficient 0·998).

DISCUSSION

The pH dependence of glucose permeability at 38 °C

The pH dependence of glucose permeability was studied in the self-exchange mode where the net flux is zero. The cells were titrated to the appropriate pH value so that pH equilibrium across the erythrocyte membrane was maintained during the experiments. Sources of error, due to cell volume changes and to titration of intracellular buffers during the experiments, were thereby excluded. The results of Fig. 1 show that glucose permeability at a constant extracellular concentration of 40 mM-glucose was strongly dependent on pH with a maximum at pH 7·2. Glucose permeability decreased at both more alkaline and more acidic pH values; the apparent permeability coefficient for glucose being reduced by almost 50% both at pH 6 and at pH 9. Glucose transport can be described by simple Michaelis–Menten kinetics (see below). The decrease in permeability as pH is changed from 7·2 may be caused either by a decrease in the maximal glucose flux or by a decrease in the affinity of the transport system for glucose as indicated by an increase in the half-saturation constant $K^{1}_{\frac{1}{2}}$. Experiments performed at an intracellular glucose concentration of 100 mM showed a similar decrease in permeability at pH 6 and 9 (J. Brahm, unpublished results) and therefore exclude a pH-dependent change of $K^{1}_{\frac{1}{2}}$ as a predominant factor. It appears that the bell-shaped pH-dependent glucose permeability was due to a decrease in maximal glucose transport at acidic and alkaline pH values caused by titration of groups in the transport system involved in glucose transport and with apparent pK values somewhere near pK 6 and pK 9.

Using a volumetric method to measure glucose exit, Sen & Widdas (1962) found a gradual increase of 66% in maximal glucose transfer in pH-equilibrated erythrocytes at pH 5·4–8·4 at 37 °C. With a similar technique, Bloch (1974) showed that the initial efflux rate at 25 °C was constant in the same pH range. By using labelled glucose, Bloch (1974) further showed that glucose influx and exchange at 7 °C in the pH range

5·5–8·5, were not pH-dependent. In Bloch's experiments extracellular pH was varied, while cell pH remained constant. Lacko, Wittke & Geck (1972*a*) studied the initial uptake of labelled glucose in the exchange mode at 25 °C between pH 2–11. Glucose flux showed a bell-shaped pH dependence with a broad maximum between pH 6·5 and 8·5. It is not clear from their paper whether the cells were pH-equilibrated prior to the influx experiments, but according to Widdas (1980) their cell samples were exposed to a changed extracellular pH only during the [^{14}C]glucose-influx period of a few seconds. The pH dependence of glucose exchange thus determined should reflect an effect of hydrogen ions on the external side of the transport system. Lacko *et al.* (1972*a*) concluded that the pK of the acidic group was 5·2 and that of the alkaline group 9·5. The present results (Fig. 1) show a more peaked pH dependence of glucose permeability at 38 °C with a pH optimum at pH 7·2. The pK of the titratable groups of 6 and 9 differs only a little from the values of Lacko *et al.* It is not known whether the difference in the respective pK values is due to a temperature effect and/or to the different experimental conditions of a constant intracellular pH (Lacko *et al.* 1972*a*) or pH equilibration across the erythrocyte membrane in the present study.

The concentration dependence of glucose transport

Glucose transport in human red blood cells has been studied very little at body temperature. Using photometric methods, which relate the change of light scattering to cell volume changes, Sen & Widdas (1962) determined glucose net efflux to be $2{\cdot}5 \times 10^{-10}$ mole/cm^2.sec at an intracellular glucose concentration of 76 mM and Miller (1965) found $4{\cdot}4 \times 10^{-10}$ mole/cm^2.sec in red blood cells with 128 mM-intracellular glucose. Half-saturation constants of 4 mM (Sen & Widdas, 1962) and 5·6 mM (Miller, 1965) were obtained by varying the extracellular glucose concentration between 1 and 18 mM. Britton (1964) investigated glucose self exchange by measuring isotope efflux with a stopper-solution technique and found a glucose flux of $6{\cdot}3 \times 10^{-10}$ mole/cm^2.sec which was independent of glucose concentrations between 67 and 267 mM. At lower glucose concentrations the more rapid equilibration rate of labelled glucose was above the upper limit of determination by the method (Britton, 1964). Hankin & Stein (1972) studied the temperature dependence of both glucose self exchange and net efflux in red blood cells loaded with 300 mM-glucose up to 45 °C. Their high temperature data gave a self-exchange flux of 8×10^{-10} and a net efflux of 5×10^{-10} mole/cm^2.sec at body temperature. As stressed by the authors, the determination of glucose transport at high temperatures was complicated by the experimental conditions with samples taken 5 sec after the start of the experiments, at which time the cells had already equilibrated almost 50% of the initially intracellular radioactive glucose.

In the present study the combined use of the Millipore–Swinnex filtering technique and the very rapid continuous flow tube method ensured that glucose transport measurements were done with sufficiently high time resolution. Thus determinations both of the rapid glucose self exchange at low glucose concentrations (cf. Fig. 2) and the 'initial' rates in net-efflux experiments (cf. Fig. 3) were carried out with a technique developed for measuring rapid transport processes (Brahm, 1977, 1982, 1983). The results of Fig. 4 show that both self-exchange flux and net efflux saturate

and that the concentration dependence of glucose transport can be described by simple Michaelis–Menten kinetics. The half-saturation constant was similarly low, both for net efflux and for self exchange indicating that the magnitude of the maximal glucose fluxes of self exchange and net efflux are almost equal. The $K_{\frac{1}{2}}$ of 6·7 mM for self exchange explains why Britton (1964) reached the conclusion that self-exchange flux was independent of concentration between 67 and 167 mM-glucose, because the transport system was 90–98 % saturated.

The $K_{\frac{1}{2}}$ of self exchange at 38 °C had not been previously determined. On the basis of self-exchange studies at lower temperature, it had been assumed that the $K_{\frac{1}{2}}$ of self exchange exceeded the $K_{\frac{1}{2}}$ of net efflux considerably at body temperature. At 20 °C, the $K_{\frac{1}{2}}$ of self exchange between 14 and 38 mM has been published (Edwards, 1974; Lacko, Wittke & Kromphardt, 1972*a*; Eilam & Stein, 1972; Miller, 1968*b*). The present study shows, however, that the half-saturation constant of self exchange at 25 °C was only 8·1 mM (Fig. 5). The large difference between this value at 25 °C and the much larger values of 32 mM (Eilam & Stein, 1972) and 38 mM (Miller, 1968*b*) at 20 °C cannot be ascribed to the difference in temperature of the experiments, but presumably reflects the insufficient time resolution of the methods used. Eilam & Stein (1972) determined the rate of labelled glucose efflux by taking three samples at standardized time intervals of 10, 20 and 30 sec after the start of the experiments, independent of the applied glucose concentration between 26 and 140 mM. However, even at 20 °C, the rate of glucose self exchange is too rapid ($T_{\frac{1}{2}}$ 5–6 sec at 25 mM-glucose and 8–9 sec at 50 mM-glucose as extrapolated from the present results at 25 °C) to be determined at the lower glucose concentrations with sufficient accuracy 10 sec after the start of the experiments, where more than 50 % of the intracellular radioactivity had already equilibrated with the extracellular phase. Eilam & Steins' value for $K_{\frac{1}{2}}$ of 32 mM was determined from a Hanes-like plot of the reciprocal rate coefficient against the glucose concentration (Fig. 4 of Eilam & Stein, 1972) in which the slope of the curve depends crucially on the critically low rate coefficients obtained at the lower glucose concentrations.

The $K_{\frac{1}{2}}$ for net efflux has been determined by Miller (1971) to be 7·4 mM, and by Karlish, Lieb, Ram & Stein (1972) to be 25·4 mM at 20 °C. In both studies the efflux of labelled glucose from glucose-loaded cells into a glucose-free extracellular medium was measured. In the study of Karlish *et al.* (1972) NaCl was added to the glucose-free suspension medium, to maintain isotonicity with the glucose-loaded cells at the instant of mixing. However, samples were taken 20 sec later, preventing determinations of initial rates under conditions which reduced cell volume changes to a minimum. Karlish *et al.* (1972) reported that the $T_{\frac{1}{2}}$ for efflux from 80 mM-glucose-loaded cells was 25 sec (20 °C) as calculated from the integrated rate equation for glucose efflux. The poor agreement between their findings and the present results is illustrated by the fact that the initial $T_{\frac{1}{2}}$ at a similar intracellular glucose concentration was only 12 sec (25 °C). In Miller's study (1971), the larger intracellular osmolarity was not compensated for by the addition of an extracellular impermeant. Neither Miller's work (1971) nor the paper describing the method employed (Miller, 1968*a*) contain any information about the time resolution of the tracer method used, but it might have been the same or higher than that used by Karlish *et al.* (1972).

The present $K_{\frac{1}{2}}$ of 5·6 mM for net efflux (Fig. 5) is about three times larger than

that determined by Sen & Widdas (1962). As indicated by Widdas (1980) the Sen & Widdas experiments reveal the K^1_2 for glucose influx, while the experimental procedures of Miller (1971) and Karlish *et al.* (1972), as well as those of present study give the K^1_2 for efflux. Thus the present and Miller's K^1_2 values indicate that the asymmetry of affinities of the glucose transport system is less marked than had been anticipated from the work of Karlish *et al.* (1972).

The present study shows that the maximal glucose net efflux while being almost equal to the maximal self-exchange flux at 38 °C (Fig. 4) became progressively smaller than the self-exchange flux as the temperature was lowered to 25 °C (Fig. 5) and 10 °C (Fig. 6). It must be noted, however, that although the maximal flux, and thereby the K^1_2 for self-exchange flux and net efflux respectively, show a different temperature dependence, both types of transport can be described in terms of simple Michaelis–Menten kinetics at all temperatures.

The temperature dependence of glucose transport

The Arrhenius diagram of Fig. 7 illustrates that the logarithm of the glucose flux in self-exchange and net-efflux modes was a non-linear function of the reciprocal absolute temperature. It must be considered whether the non-linearity is due to a temperature-dependent degree of saturation of the transport system, or to a phase transition of membrane components, or may reflect that different rate-limiting steps, with different apparent activation energies, regulate glucose transport at high and low temperatures, respectively.

Saturation of the transport system. The intracellular glucose concentration was 100 mM in all experiments. With a K^1_2 of self exchange varying from 6·7 mM at 38 °C to 12·6 mM at 10 °C, one can calculate that the degree of saturation decreases from 94 % at body temperature to 89 % at 10 °C, and to 86 % at 0 °C by extrapolating the data obtained at the higher temperatures. This small decline of saturation of the transport system by lowering temperature from 38 to 0 °C cannot, however, explain the very pronounced change of the Arrhenius activation energy from 61 kJ/mole in the physiological temperature range to 120 kJ/mole in the low-temperature range, for self-exchange flux.

For net efflux the temperature dependence also increased by lowering the temperature and was more pronounced than for self exchange, despite the fact that a decrease of temperature increased the saturation of the transport system from 92 % at 38 to 96 % at 10 °C. The small temperature-dependent changes in the saturation of the glucose transport system, which for net efflux even goes in the opposite direction of that for self-exchange, make it highly unlikely that the breaks in the temperature curves of glucose transport are due to the degree of saturation of the transport system.

Phase transition of the red blood cell membrane. Sen & Widdas (1962) showed that the glucose exit was less temperature-dependent at 37 °C (36 kJ/mole) than at 20 °C (84 kJ/mole). Lacko, Wittke & Geck (1973) demonstrated that the activation energy for initial uptake of labelled glucose into glucose-loaded cells changed from 42 kJ/mole above to 92 kJ/mole below 20 °C. In contrast, Hankin & Stein (1972) found a constant temperature dependence between 5 and 45 °C both for self exchange (68 kJ/mole) and net efflux (75 kJ/mole) in red blood cells loaded with 300 mM-glucose.

They argued that the constant activation energy was due to saturation of the transport system. Their argument, however, is no longer tenable in the light of the present determination of K_t^1 values for net efflux and self exchange, and the above considerations. The constant activation energy found by Hankin & Stein (1972) may possibly be explained if their method over-estimated the glucose flux progressively more, as the temperature was increased. Lacko *et al.* (1973) suggested that the change of temperature dependence was due to a phase transition of the erythrocyte membrane. Though the present temperature study of self exchange might support their suggestion, it is more difficult to apply the same explanation to the gradual increase of the Arrhenius activation energy for net efflux, as the temperature was decreased. One would expect *a priori* that an over-all phase transition of the membrane affects the temperature dependence of self exchange and net efflux equally which was not the case (cf. Fig. 7). Further, phase transitions of the cholesterol-rich erythrocyte membrane in the considered temperature range, appear to be highly questionable (Oldfield & Chapman, 1972) though local crystallizations of the lipids changing the local permeability properties can not be excluded (Chapman, 1975). However, if a local change of the membrane viscosity causes the temperature-dependent changes of glucose transport, one must assume that the transport system is affected differentially at self exchange and net efflux by the same physical property of the lipid annulus surrounding the glucose transport system.

Different rate-limiting steps. The third possibility, is that glucose transport becomes rate limited by different steps in the transfer of glucose across the membrane, as temperature is changed. The simplest kinetic model of glucose self exchange involves binding of glucose to the transport site at the one side of the membrane; translocation of the glucose-loaded transport site, and liberation of glucose on the opposite side of the membrane. The change of activation energy of glucose self exchange may be more gradual than indicated by the two regression lines intersecting at 10 °C in Fig. 7. However, the interpretation of the temperature-dependent change of activation energy is simplified by the assumption of only one low activation energy (61 kJ/mole) above and one high (120 kJ/mole) below 10 °C. Hence, according to the simple kinetic model binding (and liberation) of glucose is rate limited in either of the two temperature ranges, and translocation is rate limiting in the other temperature range. Net efflux showed a more gradual increase of activation energy with decreasing temperature from 47 to 0 °C (Fig. 7). It appeared that the temperature dependence of net efflux was better described by assuming that transport is rate limited by three different steps in the transport process. The simplest kinetic model for self exchange which operates with one rate coefficient for the translocation of the glucose-loaded transport site (and one rate coefficient for binding/liberation of glucose), may also describe net flux if the translocation rate of the unloaded and the loaded glucose transport site have the same temperature dependence. A picture like that of Fig. 7 would appear if the temperature dependence of the rate coefficients was different. It is suggested that the rate limiting step between 10 and 38 °C with an activation energy of 91 kJ/mole represents the translocation of the empty glucose transport site. It must be emphasized that the present findings do not allow any conclusion whether binding (liberation) of glucose or translocation of the glucose-loaded transport site is the rate-limiting factor for self exchange above 10 °C and for net efflux above 38 °C.

Critical rate. The statistical treatment of self-exchange flux data at an intracellular glucose concentration of 100 mM indicates a critical transport rate at 10 °C, where the self-exchange flux was 51×10^{-12} mole/cm^2 . sec. In agreement with the interpretation of a critical rate, Ginsburg & Yeroushalmy (1978) have shown that the activation energy of galactose self exchange, following a rise of temperature, changed from 277 kJ/mole to 97 kJ/mole at 5 °C at which the maximal galactose self-exchange flux was 52×10^{-12} mole/cm^2 . sec. The activation energy of 3-*O*-methylglucose self exchange at 40 mM also changes from 139 kJ/mole to 71 kJ/mole at a self-exchange flux of 51×10^{-12} mole/cm^2 . sec at 10 °C (J. O. Wieth, personal communication). In the present study the temperature dependence of glucose self exchange at 40 mM showed a kink at 15 °C, at which the flux was of the same magnitude (69×10^{-12} mole/cm^2 . sec). The self exchange of the polyol, erythritol, mediated by the hexose-transport system, on the other hand, showed a constant activation energy of 125 kJ/mole between 0 and 38 °C (Wieth, 1971). The erythritol flux was, however, considerably lower than the hexose self-exchange fluxes, and did not exceed the critical rate within the temperature range studied.

It thus appears that the temperature dependence of hexose self-exchange flux changes at a critical rate at which the flux is $\sim 5 \times 10^{-11}$ mole/cm^2 . sec, which equals $4{\cdot}4 \times 10^7$ molecules/cell . sec. Cytochalasin B binding studies (for a review, see Jones & Nickson, 1981) show $0{\cdot}4$–$3{\cdot}5 \times 10^5$ binding sites per cell membrane. If the number of Cytochalasin B binding sites equals the number of glucose transport sites, the 'one way' turnover number for glucose molecules being translocated from one side of the membrane to the other side, is $0{\cdot}1$–$1{\cdot}1 \times 10^3$ molecules/site . sec. At 38 °C the turnover number increases to $1{\cdot}2$–13×10^3 molecules/site . sec.

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