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# GLUCOSE TRANSPORT CARRIER ACTIVITIES IN EXTENSIVELY WASHED HUMAN RED CELL GHOSTS

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SUMMARY

Human red cell ghosts were prepared and extensively washed in the presence of divalent cations ($Ca^{2+}$ and $Mg^{2+}$) and their residual glucose transport carrier activities were studied. 99.18 % of the original cellular contents of hemoglobin were removed by the isolation procedures. The glucose carrier activities of such ghosts exhibited the following characteristics. (1) An extremely high permeability to D-glucose ($2.0 \cdot 10^{-5}$ cm/sec at 24°) and to 2-deoxy-D-glucose ($2.9 \cdot 10^{-5}$ cm/sec at 24°), compared with the permeability to D-mannitol ($5.2 \cdot 10^{-8}$ cm/sec at 24°). (2) Stereospecificity towards various sugars; relative rates of permeation by 2-deoxy-D-glucose, D-glucose, D-mannose and D-ribose were 1.5, 1.0, 0.35 and 0.020, respectively. (3) Saturability (for D-glucose at 24°, a $K_m$ of 19.5 mM and a $\Phi_{max}$ of 6.4 $\mu$moles$\cdot$cm$^{-3}\cdot$sec$^{-2}$). (4) Sensitivity to stilbestrol (competitive inhibition with a $K_i$ of 4.69 $\mu$M at 24°) and to dinitrofluorobenzene (irreversible inhibition). (5) Transient uphill movements of D-glucose by the addition of a second sugar, 2-deoxy-D-glucose, a counter-transport phenomenon. (6) Relative insensitivity to change in pH, between 5.2 and 9.0. (7) High degree of temperature dependency with a negative $Q_{10}$ for $K_m$ at low temperatures. (8) A relative resistivity to heat inactivation up to 65°.

In conclusion, this work provides (1) the first evidence that such purified ghosts maintain fully functional glucose carrier activity and (2) a new detailed description of the nature of the temperature dependence and heat denaturation of this carrier system in ghosts.

## INTRODUCTION

Considerable efforts have been directed to isolate "carrier" systems of biological membrane transport in recent years[1,2]. One obvious approach to this goal would be to isolate and purify the minimal membrane structure in which the carrier system resides and operates. The carrier-mediated glucose transport system of human red cells[3-5], because of its relative simplicity, appears to offer an excellent opportunity for such an approach[6].

Previously LEFEVRE[7] has shown that isolated human red cell ghosts retain most of the glucose carrier activity of the intact cells. His ghost preparation, however,

retained almost a quarter of the original cell hemoglobin content. In this report we have applied more rigorous isolation procedures in an attempt to define the condition of maximum purification under which the carrier function still remains active. In actual fact, present methods permit measurement of the specific carrier function only in those ghost preparations which retain the integrity of the nonspecific membrane diffusion barrier. Therefore, the ghost preparation studied here was chosen which retained only a minimal trace of the intact cell hemoglobin contents yet did not damage this barrier[6].

## EXPERIMENTAL PROCEDURE

200 ml of fresh human blood were drawn by venipuncture from selected healthy volunteers into an ACD Blood-Pack (Fenwall, Morton Grove, Ill.) and used within 4 days. The whole blood was spun down at 4000 × *g* for 20 min (Type RC2-B Automatic Superspeed Refrigerated Centrifuge, Sorvall) and the buffy coats of the white cells and platelets as well as plasma were removed by aspiration. The red cells were further washed with usually 10–20 vol. of isotonic NaCl, phosphate buffered at pH 7.4, for 3 times by resuspension and centrifugation with removal of any remaining contaminating cells or debris. The washed cells were resuspended into isotonic NaCl–Tris buffer (pH 7.4) at a cell concentration of $5 \cdot 10^9$ cells/ml.

The red cell ghosts were isolated by hemolyzing and washing 1 vol. of the packed cells with 20 vol. of 0.1 isotonic balanced salt solution ($Na^+$, 12.5 mM; $K^+$, 0.5 mM; $Ca^{2+}$, 0.38 mM; $Mg^{2+}$, 0.25 mM; all as chlorides, buffered with Tris–HCl buffer, 0.5 mM, at pH 9.0). The hemolyzed cell membranes were recovered by centrifugation, and the procedure was repeated three to five more times. Isolated ghosts were finally resuspended in 10–20 vol. of 0.1 isotonic or isotonic balanced salt solution at pH 7.4.

For each of the ghost preparations, hemoglobin content was assayed by reading the absorbance of the pyridine hemochromogen as described by DODGE *et al.*[8] and compared against that of the intact whole cell at a proper dilution. The protein was measured by the method of LOWRY *et al.*[9] and the non-hemoglobin protein content was calculated as the difference of the total protein and its hemoglobin content.

Both net and unidirectional fluxes were measured by an isotopic tracer technique using $^{14}C$-labeled sugars and mannitol (Tracer Lab., Waltham, Mass.). All flux measurements were done with a ghost suspension density of $10^9$ ghosts per ml in 0.1 isotonic, or isotonic, balanced salt solution at a specified pH and temperature. pH in the range of 5.2–9.0 was obtained with Tris–maleate buffer. Temperatures above the ambient were maintained in water baths (Metabolic Water Bath Shaker, New Brunswick Science Co., New Brunswick, N.J.). Temperatures lower than ambient were maintained in a water bath with an immersion bath cooler (Model TEV-45, with Bath Cooler Model PCB-2, Neslab Instruments, Portsmouth). A water bath in a cold room (Hotpack, Philadelphia, Pa.) was also used. Each temperature was maintained constant within $\pm$ 0.1° during the experiments. For net flux experiments, the ghosts originally free from the permeating species were rapidly suspended into the medium containing the permeant at a low chemical concentration (0.1–0.25 mM) with its radioactive tracer at the specific activity of 0.25–0.5 $\mu$C/ml. For the unidirectional flux measurements, the ghosts were loaded with the permeating species at a specified concentration with (in the case of efflux measurement) or without (in the case

of influx measurement) the corresponding radioactive tracer. The preloaded packed ghosts were rapidly suspended at $t = 0$ into the medium of the identical concentration of the permeating species without (efflux experiments) or with (influx experiments) the tracer present. To ensure an instantaneous mixing of the ghosts in the suspension at $t = 0$, and to keep the ghost suspension well mixed during the flux measurements, the suspension was continuously stirred with a magnetic stirrer (MS-7, Tri-R Instruments, Rockville Center, N.Y.). In short-term experiments, usually 6–8 aliquots (1–2 ml each) were sampled with the aid of an automatic sampler (B-D Conwall Continuous Pipetting Outfit) within 12–20 sec. Each sampled aliquot was immediately mixed into 9 ml of prechilled $HgCl_2$ (2 mM) solution[10] adjusted isotonic to ghost with NaCl, and centrifuged immediately at 4° at 30000 × $g$ for 20 min. It was confirmed that the $HgCl_2$ treatment under the stated condition arrests the rapid (carrier mediated) sugar flux within a second. The supernatants of the centrifuged samples were separated and the tube walls were gently swabbed off in order to separate the membrane pellets with minimum contamination by medium. It was found that the $HgCl_2$ treatment drastically enhanced compact packing during centrifugation, a condition which was prerequisite to quantitative separation of the pellet from the supernatant.

The radioactivities of the pellets and the supernatants, after a proper dilution, were counted in a liquid scintillation counter (Nuclear Chicago) using BRAY'S[11] solution. Each counting was corrected for quenching by an internal standard method[12]. Long-term experiments were done in the same manner as the short term experiments, as described above, except that usually 4–6 samples were taken during a 1–3-h period. Each flux experiment was accompanied by estimation of tritiated water space and [$^{14}$C]inulin space of the packed ghosts in order to estimate the intra and extra-ghost spaces of the pellet. The flux data were converted to intra-ghost $^{14}$C-labelled permeant space at each sampling time point, and finally expressed as fractional equilibration to the intraghost water space.

The data of the time-course of isotopic equilibration at complete chemical equilibration were analyzed according to equation

$$\log \frac{S^*_{i,\infty} - S^*_i}{S^*_{i,\infty} - S^*_{i,0}} = -0.434 \frac{(V_i + V_e)\Phi}{V_i V_e [S]} t \qquad (1)$$

where $S^*$, $[S]$, $V$, $\Phi$ and $t$ denote specific radioactivity (counts/min per mmole), chemical concentration, volume, flux and time (sec), respectively, and subscript i, e, $\infty$, and o denote intraghost, extraghost, at equilibrium, and at initial time, respectively. This equation is essentially identical to the one previously derived by BRITTON[10]. The flux was estimated from an half-equilibration time, $t_{1/2}$, obtained from the time-course, using the relationship,

$$\Phi = 0.693 \frac{V_i V_e [S]}{(V_i + V_e) t_{1/2}} \qquad (2)$$

The flux was related to permeability, $P$, at a sufficiently low permeant concentration (see RESULTS AND DISCUSSION), by equation,

$$P = \Phi / A \cdot [S] \qquad (3)$$

where $A$ is the total ghost membrane area.

Each batch of ghost preparations was examined for their gross morphology by phase contrast microscopy. The three-dimensional image of isolated membranes was constructed by observing the membrane suspension on a slide at various levels of depth. The packed volume of the membrane preparations was estimated by counting the ghosts on a hemacytometer (Hausser Hy-Lite) under a phase microscope (Phase-star, American Optics).

## RESULTS AND DISCUSSION

### *General description of ghosts*

The human red cell ghosts studied here were prepared by washing 4–6 times, each time with 20 vol. of 0.1 isotonic balanced salt solution at pH 9.0. The ghosts retained 0.82 % (an average of twelve preparations with a standard error of the mean of 0.42 %) of their cellular hemoglobin contents. This particular pH was used since it was shown to give ghosts with less retention of hemoglobin without showing any reduction in the glucose flux[6]. This preparation also showed the semipermeability to be least affected compared with other preparations[6]. Phase microscopy of this preparation revealed that they are mostly swollen spheres or cups in 0.1 isotonic balanced salt solution, and responded osmotically upon resuspension into higher osmolarity.

### *Determination of the flux of various sugars and a polyol in ghosts*

Fig. 1 is a typical result of the experiments where the time-courses of net uptake of various sugars and a related polyol by ghosts were followed for 3 h. Both D-glucose and 2-deoxy-D-glucose equilibrated almost completely within 1 min, the

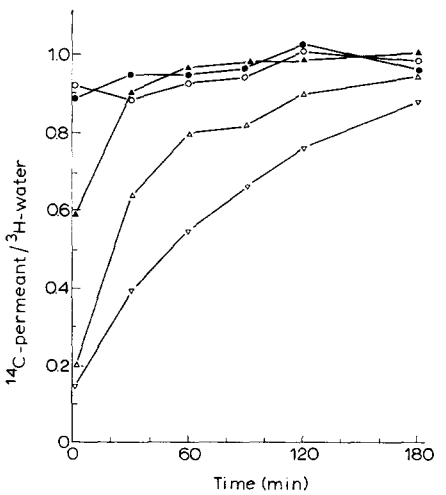


Fig. 1. 3-h time-courses of net uptake of D-glucose (●), 2-deoxy-D-glucose (○), D-mannose (▲) D-ribose (△), and D-mannitol (▽) by ghosts. The ghosts, free of permeating species, were suspended into the solutions containing permeating species at a concentration of 5 mM with corresponding tracer at a specific activity of 0.25 $\mu$C/ml at $t = 0$. The volume ratio of the ghosts to the medium measured as tritiated water distribution was 0.11. 2-ml aliquots were taken at each time point, which were immediately mixed with chilled $HgCl_2$ solutions and centrifuged in the cold to separate the membrane pellet from the medium. Uptakes were expressed as fractional equilibration of each permeant to the water space of the ghosts.

earliest sampling point in this experiment, whereas D-mannose, D-ribose and D-mannitol reached an half-equilibrium apparently only after 1 min, 20 min and 1 h, respectively. The result demonstrates that such a ghost preparation possesses a marked stereospecificity toward different but closely related permeants.

Fig. 2 represents a result of the first 20-sec time-courses of the net flux of D-mannitol, and of the exchange flux of D-glucose in the absence or in the presence (2 mM) of $HgCl_2$, by the same ghost preparation as in Fig. 1. The following points are to be noted. First, in the absence of $HgCl_2$, glucose reached virtually a complete equilibration during this 20-sec period, whereas hardly any movement of mannitol was detected. Second, this fast glucose equilibration was virtually completely inhibited in the presence of $HgCl_2$ down to the level of the speed of the mannitol equilibration. The glucose movement seen in the absence of $HgCl_2$ was found to be kinetically fairly precisely described as a single first order process which takes place between two well-mixed compartments. This is evident in Fig. 3, where the D-glucose data of Fig. 2 were replotted according to Eqn. 1. A good fitting of the experimental points to the theoretical straight line is evident. Such a fitting was consistently observed in the flux experiments throughout the present studies. Knowing the concentration, $[S]$, and the water spaces $V_i$ and $V_e$, the flux, $\Phi$, was calculated from the observed half-equilibration time, $t_{1/2}$, according to Eqn. 2. From the value of the flux $\Phi$ and using an estimated surface area $A$ of the ghost, the permeability constant, $P$, was obtained according to Eqn. 3. Such a measure of permeability has a unique value in a saturable system when done at sufficiently low concentrations. This value of permeability is proportional to permeant specificity ($1/K_m$) if the maximum carrier-mediated flux ($\Phi_{max}$) is of fixed value for each permeant[4].

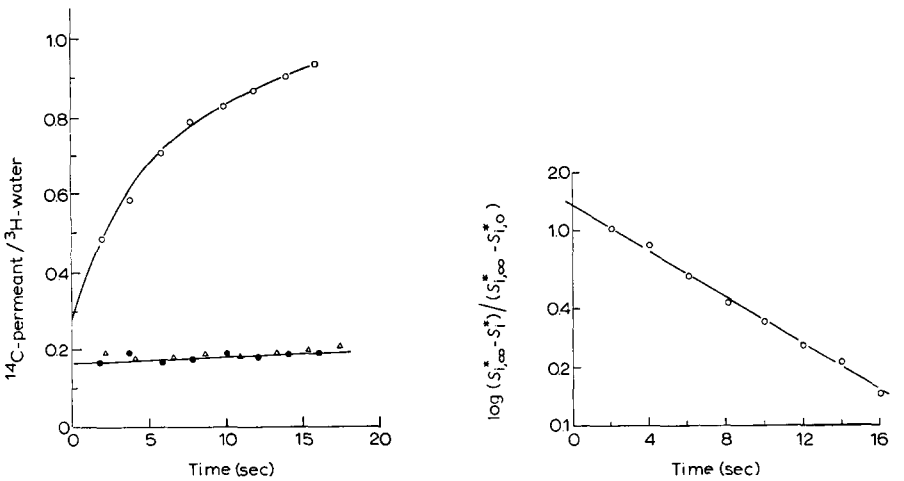


Fig. 2. 18-sec time-courses of the uptake of D-glucose in the absence (○) and in the presence (●) of $HgCl_2$ (2 mM), and of the uptake of D-mannitol (△). The glucose uptakes were measured at complete chemical equilibrium across the ghost membranes, whereas the mannitol uptake was measured without chemical pre-equilibration. The concentration of permeants was 5 mM in each case. The uptakes were expressed as fractional equilibrations to the water space of the ghost pellets.

Fig. 3. Time-courses of unidirectional flux of D-[$^{14}$C]glucose as plotted according to Eqn. 1. The solid line represents the least squares solution of the data taking the first sample point, $t = 2$ sec, as the initial condition, $S^*_{i,0}$. See the text for further details.

Table I summarizes the results of the permeabilities of the ghosts, estimated in the manner described above, to various sugars and a related polyol, D-mannitol. A low concentration (0.25 mM) of the permeants was adopted so that the measured permeabilities to sugars represent those unhindered by the saturation of the carrier

TABLE I

PERMEABILITIES OF GHOSTS TO VARIOUS SUGARS AND A RELATED POLYOL

Each figure represents the mean ± S.E. An isotopic exchange flux was measured at complete chemical equilibrium, except in the case of D-mannitol, for which the net flux was measured. Chemical concentration of the permeants in each case was 0.25 mM. The temperature was 24°. The surface area of the ghosts was assumed to be identical to that of intact cells, and a reported value of $1.55 \cdot 10^{-6}$ cm² was used[13]. The volume of water space of ghosts, $V_i$, was obtained from the difference of tritiated water and [$^{14}$C]inulin space of the ghost pellets.

| *Permeants* | *Permeabilities (cm/sec)* | *Number of experiments* |
|---|---|---|
| D-Glucose | $2.0 \cdot 10^{-5} \pm 0.77 \cdot 10^{-5}$ | 8 |
| 2-Deoxy-D-glucose | $2.9 \cdot 10^{-5} \pm 0.91 \cdot 10^{-5}$ | 3 |
| D-Mannose | $6.9 \cdot 10^{-6} \pm 2.1 \cdot 10^{-6}$ | 4 |
| D-Ribose | $3.9 \cdot 10^{-7} \pm 1.8 \cdot 10^{-7}$ | 3 |
| D-Mannitol | $5.2 \cdot 10^{-8} \pm 3.6 \cdot 10^{-8}$ | 5 |

function. The following points should be noted. First, the permeability to D-glucose observed with the ghosts at 24°, $2.0 \cdot 10^{-5}$ cm/sec, if due correction is made for the temperature influence ($Q_{10}$ of about 2 at this temperature range, as shown in a later section of the text), is closely comparable to the value reported for that of intact cells at 37°, $4 \cdot 10^{-5}$ cm/sec (ref. 1). Second, the permeation specificity sequence of the ghost carrier system to those permeants tested (2-deoxy-D-glucose, D-glucose, D-mannose, D-ribose and D-mannitol, in that order) is identical to that of the intact cell system[3]. The ghost permeation rates for these permeants, relative to the value for D-glucose, have the following values, listed in the same sequence as above; 1.5, 1.0, 0.035, 0.020 and 0.0026. These permeation values cover the same range as do those for the intact whole cell system[3], with the exception of D-mannitol. This exception is due to the relatively higher permeability of ghost to this polyol, since the corresponding permeability of intact red cells to D-mannitol was measured to be $1 \cdot 10^{-9}$–$2 \cdot 10^{-9}$ cm/sec (ref. 6), 25–50-fold less than the mannitol permeability measured for ghosts. D-Mannitol is usually considered to have negligible affinity for the carrier, and is rather considered to be transferred by simple diffusion.

*Kinetics of D-glucose flux in ghosts*

In a series of experiments each of which was similar to that of Fig. 3, rates of D-glucose equilibration across ghost membranes at 24° were studied as a function of glucose concentration in the medium. A typical result of such experiments is presented in Fig. 4, where the observed fluxes were correlated with the concentration of the sugar used, according to the double reciprocal form of LINEWEAVER AND BURK[14]. An excellent fit of the data to the kinetics is evident in the figure. Five independent series of such experiments using a ghost preparation derived from different donor individuals gave sets of values for the maximum flux ($\Phi_{max}$) and the half-saturation

concentration ($K_m$) shown in Table II. The sizable spread in the observed values of $K_m$ may be due to differences among the blood donors used. Some reported values for these parameters for intact human red cells along with the conditions and type of measurement were also included in the table for comparison. The following points are to be noted. First, the available values for the intact cell system of the kinetic parameters, particularly those for $K_m$, vary so vastly that it is not possible to compare

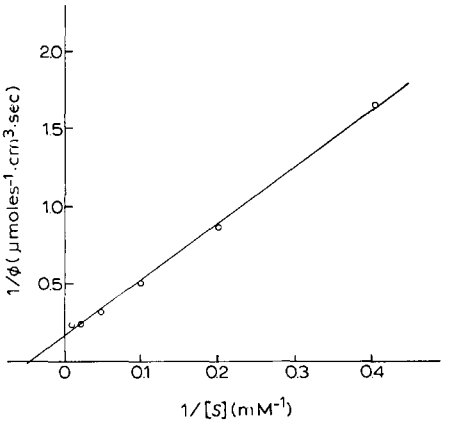


Fig. 4. Flux of D-glucose as a function of its concentration. The fluxes measured were isotopic influx at complete chemical equilibration at each specified concentration. The reciprocals of the rates, $1/\Phi$, were plotted against the reciprocals of the concentrations, $1/[S]$, according to the equation[14]: $1/\Phi = 1/\Phi_{max} + K_m/\Phi_{max} \cdot [S]$. Each point in the figure represents a single determination. The solid line, $1/\Phi = 0.16 + 3.61/[S]$ is the least squares solution of the data. With this particular set of data, $\Phi_{max}$ of 6.6 $\mu$moles·cm$^{-3}$·sec$^{-1}$ and $K_m$ of 22.5 mM were calculated.

TABLE II

KINETIC PARAMETERS OF D-GLUCOSE TRANSPORT IN GHOSTS AND IN INTACT CELLS

Each set of parameters for ghosts represents a single determination in experiments similar to those represented in Fig. 4.

| $\Phi_{max}$ ($\mu moles \cdot cm^{-3} \cdot sec^{-1}$) | $K_m$ ($mM$) | *Experimental conditions* | *Experimental methods* | *Ref.* |
|---|---|---|---|---|
| *Ghosts* | | | | |
| 5.5 | 10.8 | 24°, pH 7.4 | Tracer equilibration | |
| 6.4 | 18.8 | 24°, pH 7.4 | Tracer equilibration | |
| 6.6 | 22.5 | 24°, pH 7.4 | Tracer equilibration | |
| 7.1 | 20.2 | 24°, pH 7.4 | Tracer equilibration | |
| 6.6 | 25.1 | 24°, pH 7.4 | Tracer equilibration | |
| *Intact cells* | | | | |
| 5 | 1 | 20°, pH 7.4 | Tracer and chemical equilibration | 15 |
| 2.17 | 2.75 | 25°, pH 7.4 | Densitometry* | 16 |
| 1.7 | 1.01 | 20°, pH 7.4 | Densitometry* | 16 |
| 3.3 | — | 22.5°, pH 7.4 | Tracer equilibration | 10 |
| 2.75 | 3–5 | 25°, pH 7.4 | Densitometry* | 17 |
| 1.73 | 1.8 | 20°, pH 7.4 | Densitometry* | 18 |
| 4.33 | 38 | 20°, pH 7.4 | Tracer equilibration | 19 |

* Densitometry measures net flux in the presence of a chemical concentration gradient.

these values with the values obtained here for the ghost system. Second, it would appear, however, that the values for the ghost system are fairly comparable to those (shown as ref. 18 in the table) for the intact cells obtained by isotopic exchange experiments, the method essentially identical to that adopted in the present report for the ghost system. It has been noted[19] already that the net uptake measured by densitometry[16] gives lower values for the $K_m$ in intact red cells, suggesting that the values obtained by different methods may not be comparable with each other. For $\Phi_{max}$, if the observed temperature effects on this parameter were properly corrected (see later section of the text), the comparability would still be improved. These considerations would indicate that the isolated system behaves, kinetically, identically to the intact system.

*Inhibition of* D-*glucose flux in ghosts by 2,4-dinitrofluorobenzene (FDNB)*

Since FDNB is a known irreversible inhibitor of the glucose carrier activity in intact cells[20], its effects on the glucose transport in ghosts were studied. The ghosts were treated with FDNB (1.4 mM) by incubating at 37° for varying time intervals. The treated ghosts, after being freed from unbound FDNB by washing twice with 20 vol. of balanced salt solution, were subjected to D-glucose flux measurement. It was found that the FDNB treatments inhibited the glucose flux irreversibly, and the inhibition progressed gradually as the time interval of the treatment was increased. Fig. 5 shows a typical result of such experiments. The 20-min treatment with FDNB resulted in an inhibition of 80 % of the uninhibited rate, with an observed maximum inhibition of 95 % due to the 60-min treatment. These results were in close agreement with the FDNB effects on the glucose flux observed with intact human red cells by FORSLING *et al.*[20].

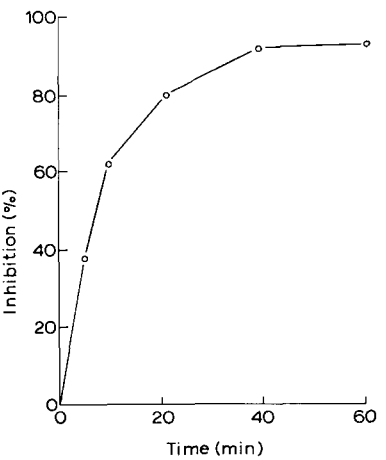


Fig. 5. Inhibition of flux of D-glucose as a function of the incubation time with FDNB. Each point represents a single determination. See text for more detail.

*Inhibition of* D-*glucose flux in ghosts by stilbestrol*

The glucose carrier function of intact red cells is known to be competitively inhibited by stilbestrol[21]. Fig. 6 represents the results of the experiments where the inhibition of D-glucose flux in the ghosts by stilbestrol was studied as a function of

inhibitor concentration. Two different glucose concentrations were used. It is obvious in this result that the data for each of the two glucose concentrations gave a straight line with a different slope but with the same intercept on the $y$ axis, indicating that the inhibition is competitive[22]. The carrier-permeant-dissociation constant ($K_s$) and the inhibition constant ($K_i$) of 10.2 mM and 4.69 $\mu$M, respectively, were obtained from analysis of the data. The value of the inhibition constant is reasonably comparable to the value of 6.6 $\mu$M for intact red cells reported by LEFEVRE AND MARSHALL[23].

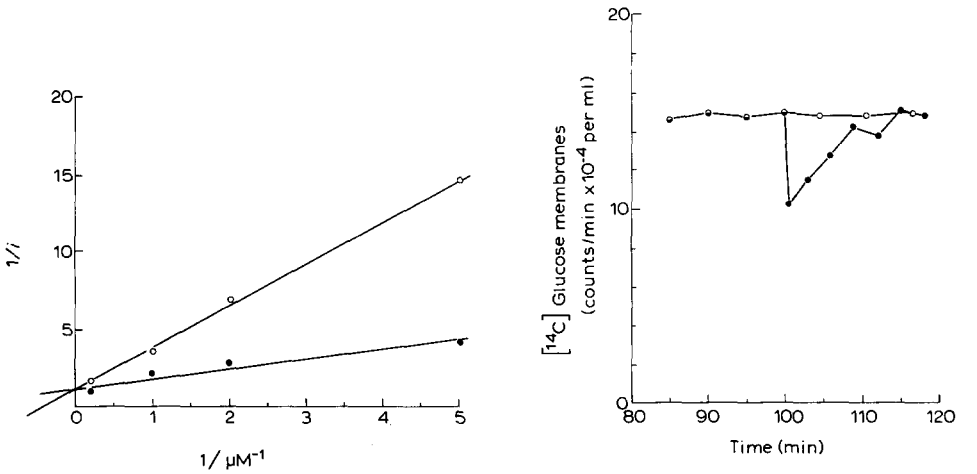


Fig. 6. Inhibition of flux of D-glucose by stilbestrol as a function of inhibitor concentration. The glucose flux, $\Phi^1$, was measured in the presence of stilbestrol at varying inhibitor concentrations, $[I]$, then compared to the flux without the inhibitor, $\Phi^\circ$, in order to compute the fractional inhibition, $i$, which is defined[22] as $i = 1 - (\Phi^1/\Phi^\circ)$. Reciprocals of the fractional inhibitions were plotted against reciprocals of the inhibitor concentrations, according to the equation: $1/i = 1 + K_i(1 + [S]/K_s)/[I]$, where $K_i$ and $K_s$ are constants. Two different glucose concentrations, 5 (●) and 50 mM (○) were used. Solid lines are the least squares solutions of the data, $1/i = 1 + 7.0 \cdot 10^{-3}/[I]$ and $1/i = 1 + 27.7 \cdot 10^{-3}/[I]$, respectively.

Fig. 7. Transient uphill movements of D-glucose induced by an addition of 2-deoxy-D-glucose. The symbols represent the glucose contents in ghost pellets before the addition (◒), and after the addition (●) of 2-deoxy-D-glucose, and after the addition of blank (○). Both 2-deoxy-D-glucose and the blank were added at the 100-min point. See text for more detail.

*Demonstration of a counter-flow*

In Fig. 7, evidence is presented to indicate that the ghosts show a counter-flow phenomenon, a property of the glucose transport carrier operation in intact red cells[24]. A suspension of the membrane preparation was first fully equilibrated with D-[$^{14}$C]-glucose at 50 mM for 95 min and divided into two equal portions of 30 ml each. 0.4 ml of a concentrated 2-deoxy-D-glucose solution was added into the first portion to give a final concentration of 33.3 mM of 2-deoxy-D-glucose, and the same volume of the plain suspension medium (containing no 2-deoxy-D-glucose) was added into the second portion. Isotopic glucose distributions were followed with both the portions in parallel. The addition of the plain suspension medium without 2-deoxy-D-glucose did not disturb the glucose distribution, whereas the addition of the 2-deoxy-D-glucose solution induced a rapid depletion of the intra-ghost glucose contents to such an extent that the concentration became only 70 % of the original equilibration within

a 0.5 min. This indicated a movement of glucose out of the ghosts against a concentration gradient. This decrease was fully recovered during the following 20-min period.

*The pH effects on* D-*glucose flux in ghosts*

The effects of pH on D-glucose flux in ghosts were also studied. At eight different pH values between 5.2 and 9.0 pH units, the glucose carrier activities were assayed in terms of the rate of D-[$^{14}$C]glucose exchange flux measured at complete chemical equilibrium across the ghost membranes. Two different concentrations, 5 and 130.4 mM of the sugar were employed. The temperature was fixed at 24°. The flux at each pH tested was found to be fitted as a single first-order process. The results of the experiments are summarized in Fig. 8. The flux at both of the concentrations

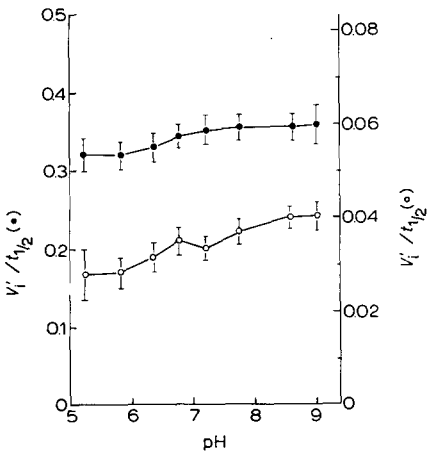


Fig. 8. Relative flux of D-glucose in ghosts as a function of pH. All ghosts were prepared in an identical manner, as described in the experimental procedures, then suspended in the medium of each specified pH for 1 h at room temperature prior to the flux measurements. The fluxes in terms of $t_{1/2}$ were obtained from the initial 12–16-sec time-course. The relative fluxes on the ordinate were estimated in terms of $V_i'/t_{1/2}$, where $V_i'$ is the normalized intra-ghost water space at a given pH, the experimentally measured space relative to that obtained at pH 7.4. The surface area of ghosts appears to be invariant with change in pH. This estimation gives a fairly good approximation of a relative permeation rate (with an error less than 5%) under the conditions employed here. The glucose concentrations of 5 mM (●) and 130.4 mM (○) were used. Each point represents the mean of three independent measurements with its standard error shown by a bar.

showed only a small degree of pH dependency. The flux measured at the lower concentration of the permeant only increased less than 15% as the pH was increased from 5.2 to 9.0. The flux measured at the higher concentration of the permeant, however, increased about 30% under the same conditions. The pattern of the change in both cases was roughly linear and no optimum pH could be detected. Sets of values for $\Phi_{max}$ and $K_m$ were estimated at three different pH values and are shown in Table III. It is apparent here that both of the kinetic parameters increased to a small but equal extent (about 30%) as the pH was increased in the range tested. This observation roughly corresponds with the observed pH effects on glucose flux with intact human red cells reported by SEN AND WIDDAS[16]. They have observed about 75 and 15% linear increases in $\Phi_{max}$ and $K_m$, respectively, as the pH was raised from 5.4 to 8.4. Our results, on the other hand, are at variance with the observation of

FAUST[25] on intact red cells, who has reported a marked pH optimum at pH 7, at sugar concentrations higher than 0.1 M.

TABLE III

KINETIC PARAMETERS OF D-GLUCOSE TRANSPORT IN GHOSTS AT DIFFERENT pH

Each set of parameters was measured in experiments similar to those of Fig. 4 and Table II, at the specified pH values.

| *pH* | $\Phi_{max}$ ($\mu moles \cdot cm^{-3} \cdot sec^{-1}$) | $K_m$ (*mM*) |
|---|---|---|
| 5.2 | 5.7 | 16.1 |
| 5.2 | 5.8 | 18.8 |
| 7.4 | 6.4 | 18.8 |
| 7.4 | 7.1 | 20.2 |
| 9.0 | 7.4 | 19.1 |
| 9.0 | 7.6 | 23.3 |

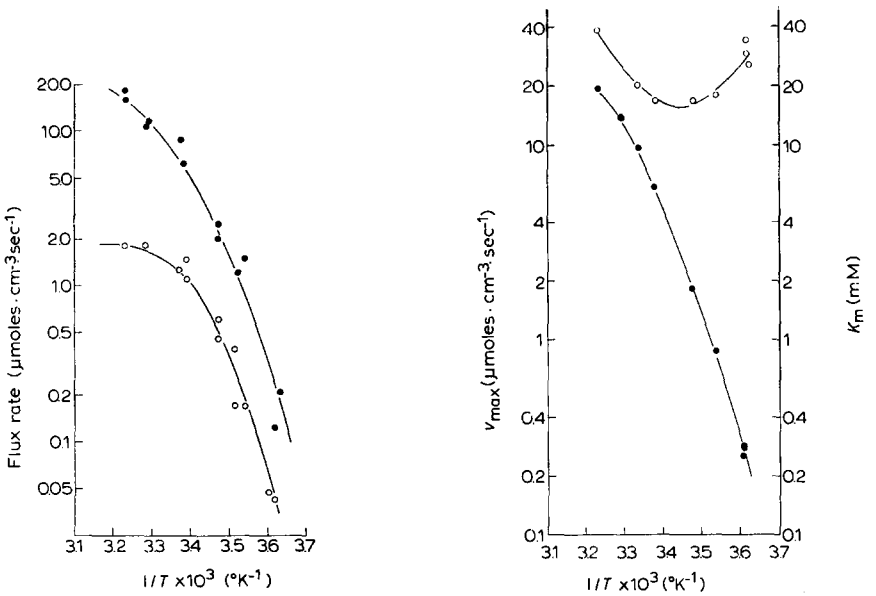


Fig. 9. Flux of D-glucose in ghosts as a function of temperature. Two different sugar concentrations, 5 mM (○) and 130.4 mM (●), were used. All the ghosts were prepared in an identical manner, as described in the experimental procedures, then pre-equilibrated at each specified temperature for 1 h prior to the experiments. The fluxes were plotted against reciprocals of absolute temperature on a semilog scale. Each point represents a single determination. The solid lines are predicted variations to fit data based on the model of DAWSON AND WIDDAS[28].

Fig. 10. The maximum flux ($\Phi_{max}$) and the half-saturation concentration ($K_m$) of D-glucose flux in ghosts as a function of temperature. The kinetic parameters at each temperature were obtained from a set of experiments similar to those of Fig. 4 and Table II. The parameters, $\Phi_{max}$ (●) and $K_m$ (○), were then plotted against the reciprocal of the absolute temperature on a semilog scale. Each point represents a single set of experiments. The solid line for each parameter was drawn by the least squares solution of the data according to the model of DAWSON AND WIDDAS[28].

*Temperature effects on* D-*glucose flux in ghosts*

At ten different temperatures ranging from 4 to 37°, the glucose carrier activity in ghosts was assayed in terms of the D-[$^{14}$C]glucose exchange flux. The fluxes were measured during complete chemical equilibration of the sugar across the membranes at two different concentrations, 5 and 130.4 mM. The pH of the flux system was fixed at 7.4. The results are presented in Fig. 9. That the flux is highly sensitive to change in temperature is evident. The degree of the sensitivity, however, was different at each of the two concentrations. As temperature was increased from 4 to 37°, the rate increased about 40-fold at the lower concentration whereas it increased more than 150-fold at the higher concentration. In both cases, the effects were much greater at lower temperatures than at higher temperatures. The 10° increase in temperatures below 20° increased the flux more than 10-fold at both concentrations, whereas the same increase in temperatures above 20° increased the flux only less than a factor of 1.5 (with 5 mM) and 2 (with 130.4 mM).

The effects of temperature on the kinetic parameters $\Phi_{max}$ and $K_m$ were studied next. As evident in Fig. 10, both of these parameters showed a pronounced temperature effect, although the pattern of the effect was quite different in the two cases. Neither of these parameters showed a linear dependency in these plots as the Arrhenius equation would predict. (The presentation of the data for $K_m$ in this particular plot is mainly for a convenience without theoretical consideration.) The curve for $\Phi_{max}$ was concave downwards, having greater effects ($Q_{10}$ of 7–8) in the lower range of temperature than in the higher range ($Q_{10}$ of 2–3). The behavior of $K_m$ was much more peculiar, the value being first decreased as temperature was lowered from 37 to around 20°, but then increased as temperature was further lowered; thus the observed value for $K_m$ at 4° was almost approaching that at 37°.

The effects of temperature on the carrier-mediated glucose flux in human red cells have been extensively studied in several different laboratories. FAUST[25] has observed that the net glucose flux shows a calculated $Q_{10}$ of 2–2.8 between 29 and 37° at pH values of 6 and 7. SEN AND WIDDAS[16], in the temperature range of 7–47° (at pH 7.4) have observed that the $\Phi_{max}$ of the glucose flux was increased by a factor of 39 as the temperature was raised in this range, whereas the $K_m$ was increased only by a factor of 10. They have further shown in this work that the Arrhenius plot of these data shows a concave downward curve for the $\Phi_{max}$, but a straight line for the $K_m$. BOLIS *et al.*[27], in a more critical work, recently have confirmed these results of SEN AND WIDDAS, but with one exception. In their hands, the Arrhenius plot of the data for the $K_m$ resulted in a concave upwards curve, instead of a straight line. (Both of these studies adopted essentially the same method of measuring the net glucose flux by means of a photoelectric densitometer.) BOLIS *et al.*[27] pointed out that the straight line relationship for the $K_m$ reported by SEN AND WIDDAS may be an artifact induced by the defective graphic analysis adopted to the data which otherwise would have given a concave upward curve similar to their own work. It is interesting to note in this connection that DAWSON AND WIDDAS[28] have reported temperature effects on the glucose carrier system of the foetal guinea-pig which plot as a curve concave upward for the $K_m$ and a curve concave downward for the $\Phi_{max}$ in an Arrhenius plot.

These peculiar temperature effects on the kinetic parameters observed with intact cells closely correspond with the findings made here with ghosts. Detailed

analysis of these temperature effects inevitably involves an adoption of a kinetic model. Since no proper model is available at present which accounts for all the data accumulated on this transport system, it would be appropriate not to attempt any further inference at this moment.

*Effect of heat pretreatment on inactivation of* D-*glucose flux in ghosts*

It is a well known fact that enzyme activities or biological phenomena in general show a well defined heat inactivation[26]. In order to study this aspect of the temperature effect on this system, the ghosts were pretreated for 60 min at a high temperature, up to 80°, and the fluxes of glucose and mannitol were measured at these same temperatures (Table IV). The following points are notable. The observed glucose flux at the higher sugar concentration (130.4 mM) was increased considerably as the temperature was raised from 37 to 65°. The flux at the lower sugar concentration (5 mM), on the other hand, showed a broad optimum at around 45°, with the flux at 65° being less than the maximum by only 30% and still greater than the flux at 23°.

TABLE IV

FLUXES OF D-GLUCOSE AND D-MANNITOL IN GHOSTS PRETREATED AND MEASURED AT HIGHER TEMPERATURES

All ghosts were prepared in an identical manner as described in the experimental procedures. The fluxes were estimated from the initial 12–16-sec time-course of the equilibration at pH 7.4. Two different glucose concentrations were applied. The fluxes for mannitol could not be measured precisely with such a short equilibration time as adopted here, and represent only an indication as to whether the ghosts are leaky or not. Each value represents a single estimation.

| *Temp.* | *Flux of* D-*glucose* ($\mu moles \cdot cm^{-3} \cdot sec^{-1}$) | | *Flux of* D-*mannitol* ($\mu moles \cdot cm^{-3} \cdot sec^{-1}$) |
|---|---|---|---|
| | *5 mM glucose* | *130.4 mM glucose* | *5 mM mannitol* |
| 23° | 1.28, 1.31 | | |
| 37° | | 16.1, 15.9 | <0.01 |
| 45° | 1.81, 1.76 | 22.3, 21.7 | <0.01 |
| 50° | | 25.4, 23.3 | <0.01 |
| 55° | 1.45, 1.57 | 26.7, 24.7 | <0.01 |
| 60° | | 27.1, 26.4 | <0.01 |
| 65° | 1.30, 1.37 | | <0.01 |
| 70° | >4, >4 | | >5 |
| 75° | >4, >5 | | >5 |
| 80° | | | >4 |

In no case was a drastic decrease observed in the flux following such a heat treatment, implying that little heat inactivation had occurred in this range of temperatures. Further increase in temperature beyond 65° merely resulted in a general damage to the diffusion barrier of the membranes, as monitored by the mannitol flux. It is not known whether the glucose carrier activity is still operative in these higher temperatures, where the diffusion barrier is lost. The estimated values of $\Phi_{max}$ and $K_m$ of the D-glucose flux in ghosts at 50°, after 60 min incubation at the same temperature, were 37 moles$\cdot$cm$^{-3}\cdot$sec$^{-1}$ and 80 mM, respectively (a single set of measurements).

Phase-microscopic examinations carried out parallel to these flux measurements after the incubations at these elevated temperatures revealed that some spherical

transformation of the ghosts from cup form occurred already at 45–50°. At 65°, all the ghosts showed a perfect sphere of darker outline, occasionally with a small surface dot, interpreted as a very minute vesicle indicating membrane degeneration. At 70°, small, clearly visible vesicles, 3 or 4 in number, surrounding the spherical thick outline of each ghost were observable. At 80°, these small vesicles were increased in number with a visible decrease in the size of the residual ghosts. Some changes in gross appearance to the naked eye were also noted at these elevated temperatures. At 60°, the membrane suspension was slightly cloudy, and this cloudiness progressively increased as the temperature was further raised. At 60–65°, there was a tendency for the membranes to settle down to the bottom of the container. The degree of this tendency also increased as the temperature was elevated. It is thus apparent that some definite changes in the ghosts are already initiated at temperatures below 65°, where the glucose carrier functions are not yet significantly inactivated.

Much other evidence is now available suggesting that many transport carriers are macromolecular proteins[1,2,29]. The relative resistivity to heat denaturation observed here appears to be rather unusual if the glucose carrier should be a macromolecular protein.

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