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# Effects of pressure on glucose transport in human erythrocytes

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The operation of the human red cell glucose transporter has been studied at normal and high hydrostatic pressure to identify the step(s) which involve a volume change. Pressure inhibited zero-*trans* and equilibrium exchange influx to similar extents, by decreasing the $V_{max}$ but not significantly changing the $K_m$. The $B_{max}$ and $K_d$ of specific [$^3$H]cytochalasin B binding were unaffected by pressure indicating no change to the number or affinity of functional transporters at pressure. Passive glucose transport was inhibited by pressure in a manner consistent with permeation across the lipid bilayer. These data indicate that there is a major change in volume during the translocation step of the glucose transporter which is rate-limiting for transport.

Glucose transport; Hydrostatic pressure; Red cell; Facilitated diffusion; Activation volume; Membrane transport

## 1. INTRODUCTION

The facilitated diffusion of glucose across the human red cell membrane can be described by the classical 4-state model [1,2]. In this model, the transporter presents glucose binding sites alternately to the exterior and interior of the cell. The binding/dissociation steps are very rapid [3], and hence the translocation step is rate-limiting for the overall process [1,2].

At low temperatures glucose transport is asymmetrical with the maximum rate of zero-*trans* (ZT) efflux exceeding that for influx by about 10-fold. Furthermore, the maximum rate for EE influx exceeds that of ZT influx by about 100-fold [2]. This latter phenomenon is termed *trans*-stimulation and is a characteristic of many carrier-mediated transporters [1]. These properties of the transporter indicate that the rates of re-orientation between inward- and outward-facing conformations are markedly higher for the loaded than the unloaded transporter, and also that the rate constants for inward-reorientation exceed those for outward-reorientation with the result that most of the glucose transporters face inwards at 0°C [2].

It is well known that many isolated enzymes require a volume change for activity [4,5]. Increasing hydrostatic pressure can markedly inhibit enzyme function indicating that there is a volume increase (i.e. positive $\Delta V^*$) associated with the rate-limiting step. There are also examples where pressure increases the rate of a process, indicating that a volume decrease (i.e. negative $\Delta V^*$) occurs. These volume changes are thought to result from physical changes to the enzyme (e.g. conformational state, binding/dissociation of substrate, hydration) [5] and are therefore fundamental for enzyme operation. The only technique available for measuring the volume changes associated with transport is hydrostatic pressure, however there have been relatively few studies in this field. One pertinent study on the human red cell anion exchanger, has suggested that the large volume change associated with its operation is due primarily to the conformational change of the transporter rather than anion binding/dissociation [6]. In the present paper we have investigated the effects of pressure on both glucose permeation and specific inhibitor binding in an attempt to identify the step(s) which involve a change in volume for the well-characterized glucose transporter.

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*Abbreviations:* ZT, zero-*trans*; EE, equilibrium exchange; $\Delta V^*$, apparent activation volume; FDNB, 1-fluoro-2,4-dinitrobenzene.

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

[$^{14}$C]Glucose and [$^3$H]cytochalasin B were obtained from Amersham International and biochemicals from either Sigma Chemical Co., or B.D.H., Poole.

### 2.2. *Blood*

Fresh human blood was collected into heparin, centrifuged (3,000×*g*, 5 min) and the plasma/buffy coat removed by aspiration. The red cells were washed (5×) by centrifugation, aspiration and resuspension in a saline (A) comprising 85 parts 165 mM NaCl and 15 parts phosphate-Na (pH 7.4). The phosphate-Na solution was made by mixing 165 mM $NaH_2PO_4$ with 110 mM $Na_2HPO_4$ to give pH 7.4. Haematocrits were measured by haemoglobin release at 540 nm [7]. In order to reduce mediated glucose transport so that fluxes could be measured using our pressure apparatus, a fraction of the transporters was inactivated by FDNB (60% haematocrit, 4 mM, 45 min at 30°C)

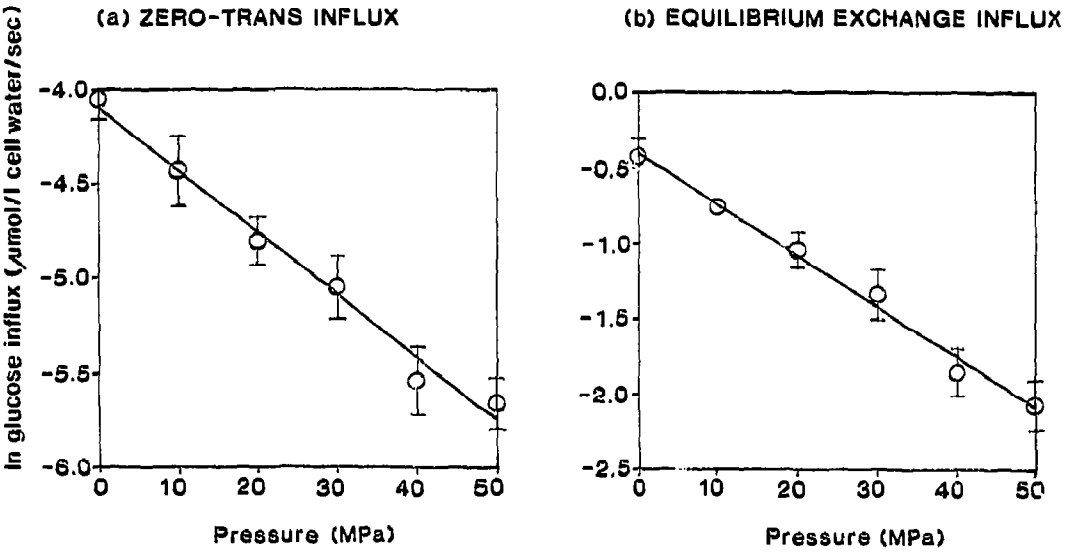


Fig. 1. Inhibition of (a) zero-*trans* and (b) equilibrium exchange glucose influx in FDNB-treated human red cells by pressure. Influx was measured at saturating glucose concentrations (1.2 mM and 100 mM, respectively) at 1°C. Data are means ±S.E.M. ($n$=3) and lines drawn are linear regressions with slopes of 0.0033±0.0003 and 0.0035±0.0003 $MPa^{-1}$ for zero-*trans* and equilibrium exchange influx, respectively; values for the $\Delta V^*$ are 74.2±6.7 and 78.4±7.1 ml/mol, respectively.

followed by washing 3× in saline A. This reduced the flux by about 90% allowing first-order influx measurements to be performed over 30 min.

### 2.3. *Flux measurements*

Initial rates of ZT and EE influx of [$^{14}$C]-D-glucose were measured as described [2] with the following modifications since the fluxes were started in the pressure vessel. Briefly, an aliquot (0.1 ml) of red cell suspension (~10% haematocrit) was pipetted into a syringe (including a mixing bar) and a layer (0.2 ml) of dibutylphthalate (density 1.04 g/ml) placed on top. The radioactive buffer solution containing [$^{14}$C]glucose at the required concentration (in 1 ml), was then pipetted onto the oil, all air excluded and the syringe sealed. The syringes were then placed in a water-filled, temperature-controlled (1°±0.2°C) pressure vessel [8]; atmospheric pressure controls were treated identically. A parallel set of tubes were prepared with 100 μM phloretin to determine the passive glucose flux [2]. Pressure was then applied and the samples equilibrated for 20 min. The flux was started by inverting the vessel allowing the mixing bar to fall through the oil and completely mixing the solutions. After a selected incubation time, the syringes were removed and their contents rapidly ejected into a large volume of an ice-cold stopping solution comprising saline A with phloretin (100 μM). The cells were then quickly washed (2×) by centrifugation, aspiration and resuspension with 50 vols of stopping solution. The final cell pellet was deproteinized (5% w/v TCA) before counting for radioactivity.

For EE influx, aliquots of FDNB-treated erythrocytes (~20% haematocrit) were pre-incubated overnight at 21° then 2 h at 37°C in saline A, including non-radioactive glucose (concentration range 5–100 mM) to allow equilibration with the intracellular pool. At the end of the loading period, the cells were washed (5×, 10,000×g, 10 s) in ice-cold unlabelled medium with glucose at the required concentration. The cells were then resuspended in ice-cold saline A containing the required concentration of glucose and placed in the syringes as described above for the ZT experiments.

### 2.4. *Binding studies*

The binding of [$^3$H]cytochalasin B at normal and high pressure was determined by equilibrium dialysis as follows. Human red cell ghosts [9] were used in preference to erythrocytes to increase the sensitivity of the measurements. A concentrated suspension of ghosts (protein concentration of 1–2 mg/ml) was added to saline A and aliquotted into dialysis bags (Medicell Intl. London), which were then and placed within large (50 ml) syringes with 3–4 dialysis bags/syringe. The saline (containing [$^3$H]cytochalasin B, concentrations (0.035–2 μM) was then drawn into each syringe, the syringes were sealed and placed in a water bath (at 0.1 MPa) or pressure vessel, both kept at 16°C for up to 24 h and mixed regularly. Non-specific binding was measured by preparing additional syringes at a range of cytochalasin B concentrations containing 200 nM of unlabelled glucose. Control experiments established that equilibration of the radioactive inhibitors across the dialysis membrane was complete within 12 h. At the end of the experiment, the syringes were quickly removed from the pressure vessel and water bath. The dialysis bags were then removed from the syringes and blotted free of external saline. Samples from inside the bags (i.e. bound + unbound inhibitor) and the suspending medium (unbound inhibitor) were taken for counting. Correction was made for loss of membrane protein during ghost preparation [9]; data were analysed by Scatchard plots [10].

Table I

Effects of hydrostatic pressure on (a) kinetic properties of the glucose transporter and (b) specific inhibitor binding to the transporter

| (a) Glucose transport | ($n$) | Pressure (MPa) | Kinetic properties $K_m$ (mM) | $\Delta V^*$ (for $V_{max}$) (ml/mol) |
|---|---|---|---|---|
| Zero-*trans* | 3 | 0.1 | 0.23 ± 0.07 | |
| | 3 | 30 | 0.19 ± 0.04 | 72.5 ± 3.1 |
| Equilibrium | 3 | 0.1 | 9.47 ± 2.14 | |
| Exchange | 3 | 30 | 11.40 ± 1.40 | 74.0 ± 2.0 |
| (b) [$^3$]cytochalasin B binding | | | $K_d$ (μM) | $B_{max}$ (nmol/mg) |
| Inhibitor binding | 7 | 0.1 | 0.17 ± 0.06 | 0.74 ± 0.10 |
| | 6 | 50 | 0.21 ± 0.04 | 0.71 ± 0.12 |

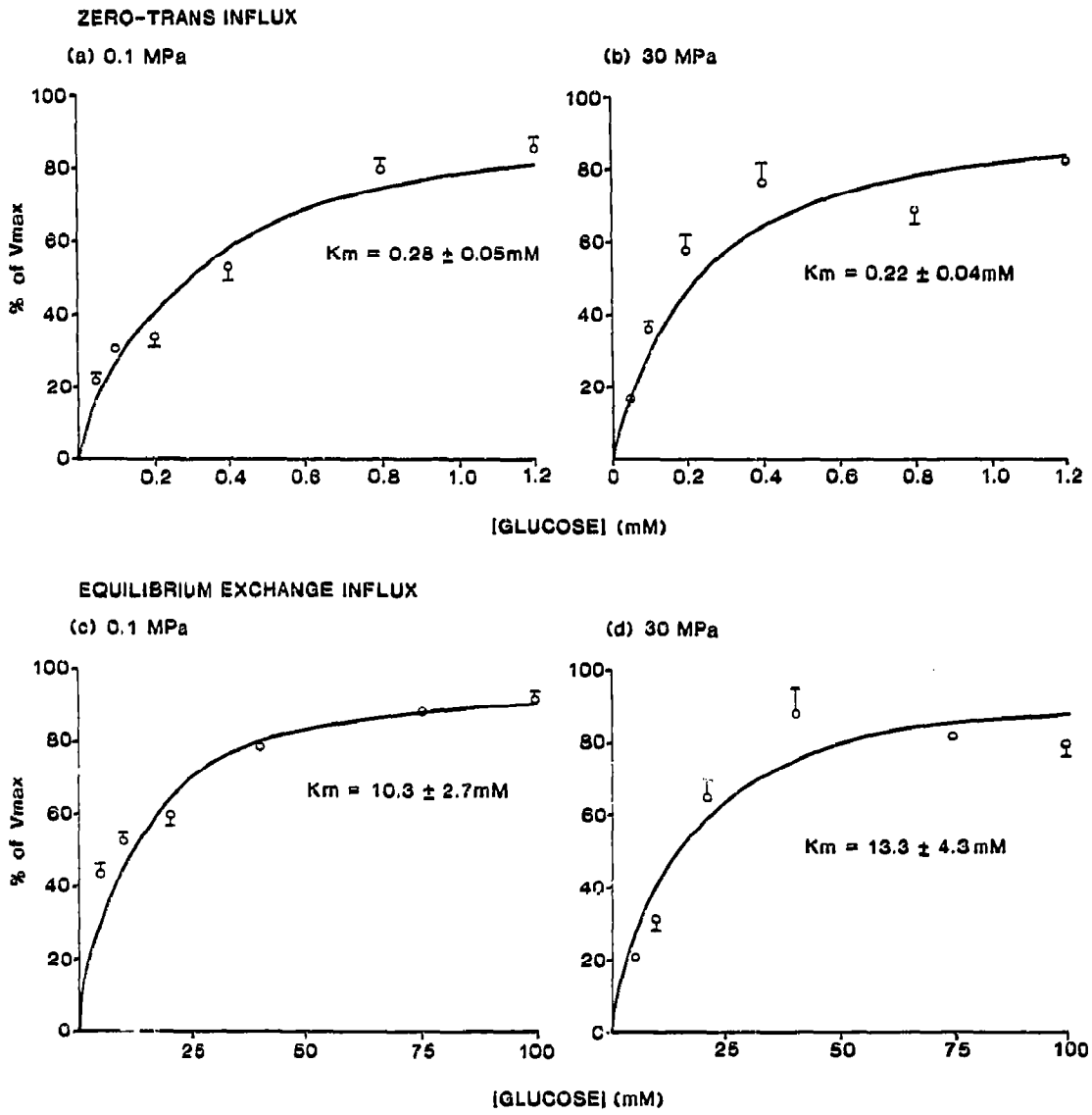


Fig. 2. The influence of pressure on the kinetic properties of the glucose transporter operating either in the zero-*trans* mode at (a) 0.1 MPa or (b) 30 MPa, or in the equilibrium exchange mode at (c) 0.1 MPa or (d) 30 MPa. For clarity, data from one representative experiment (means ±S.D. of triplicate determinations) are expressed as a percentage of the $V_{max}$ obtained at the relevant pressure; pooled data for 3 sets of experiments are given in Table I.

2.5. *Calculation of $\Delta V^*$*

Values for the apparent activation volume were calculated using the following standard equation [4,8];

$$\Delta V^* = RT \frac{(\ln k_1 - \ln k_2)}{(P_2 - P_1)}$$

The symbols are defined as follows; $k_1$ and $k_2$ represent the flux rate at pressures $P_1$ and $P_2$, respectively; $R$ is the gas constant (82.07 ml-atmosphere/°C/mol) and $T$ the absolute temperature (274°K) yielding the activation volume in units of ml/mol.

## 3. RESULTS AND DISCUSSION

Hydrostatic pressure inhibited the $V_{max}$ for ZT and EE glucose influx to very similar extents (Fig. 1a and b; Table I). The values for the $\Delta V^*$ of ZT or EE glucose influx measured over a range of pressures (Fig. 1, Table I) were not significantly different ($P>0.05$). In kinetic experiments carried out at varying concentrations of glucose, pressure had no effect ($P>0.05$) on the $K_m$ for ZT or EE glucose influx (Fig. 2, Table 1). The $V_{max}$ of other membrane transporters shows varying degrees of sensitivity to the application of hydrostatic pressure. For example, ion (Na, K, Ca) channels are relatively insensitive to pressure ($\Delta V^*$ up to about +30 ml/mol; [11,12]) whereas the red cell anion exchanger exhibits a large $\Delta V^*$ of about 150 ml/mol [6]. For the Na/K pump and nucleoside transporter pressure decreases the $V_{max}$ and the $K_m$ when transport is measured in the zero-*trans*

mode [13,14] (unpublished observations). However for the amino acid transporter System C of sheep red cells, the $V_{max}$ is reduced by pressure, whereas the $K_m$ is unaltered (unpublished observations).

The transport data taken with the lack of effect of pressure on specific [$^3$H]cytochalasin B binding (Table I) indicate that the rate-limiting step for glucose transport is accompanied by a significant increase in the volume of the system. Previous studies of the temperature dependence of glucose transport [2] are consistent with the existence of transition states of both the glucose-loaded and unloaded forms of the transporter and hence the present results indicate that the attainment of these transition states involves a relatively large increase in volume. The volume change could, in principle, arise from (1) dissociation of water from the transport protein, (2) a change in conformation of the transporter (possibly also involving (1)), or (3) a re-arrangement of the membrane lipids in the environment of the transporter in such a way as to constrain the transporter. It is not possible with our data to distinguish between these options unequivocally, however (3) seems rather unlikely. This is because at the temperature used for these studies (close to 0°C) the membrane lipids are probably highly ordered and therefore little further influenced by pressure. Furthermore, when plotted semilogarithmically, the linear pressure-dependence of the transporter (Fig. 1a and b) suggests no change in lipid phase state. This leaves a more direct effect of pressure on the transporter conformational change, possibly involving hydration, as the most likely source of the change in volume.

A role for water dissociation in the rate-limiting step of the glucose transport mechanism would be consistent with the large changes in enthalpy and entropy previously shown to be associated with the formation of the transition states of the glucose transporter [2]. Furthermore, since the $\Delta V^*$s are similar for zero-*trans* and equilibrium exchange influx (Fig. 1, Table I) yet the rate-limiting steps which involve changes in reorientation of the unloaded transporter (ZT mode) or glucose-loaded transporter (EE mode) are different, it follows that any volume change associated with glucose binding is relatively small. This is in keeping with the lack of effect of pressure on the $K_m$ for glucose transport and $K_d$ for cytochalasin B binding.

Passive glucose permeation, when plotted semi-logarithmically, was also linearly reduced by raised pressure (up to 50 MPa; $r$>0.985) giving a value for the $\Delta V^*$ of 33.6±0.3 ml/mol (mean±S.E.M, $n$=3). This value is similar to that obtained for the effects of pressure on D-glucose diffusion in liposomes (~37 ml/mol; [15]), and for lysine, alanine, glycine permeation in red cells (~30 ml/mol) [14] supporting the notion that in the absence of carrier-mediated transport, diffusion is primarily across the lipid bilayer.

In summary, these data are the first demonstration that a volume change associated with translocation but not binding is fundamental to the operation of the red cell glucose transporter.

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