Biochimica et Biophysica Acta, 649 (1981) 503–514
Elsevier/North-Holland Biomedical Press

BBA 79510

EVIDENCE FOR NEGATIVE COOPERATIVITY IN HUMAN ERYTHROCYTE SUGAR TRANSPORT

G.D. HOLMAN, A.L. BUSZA, E.J. PIERCE and W.D. REES

Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY (U.K.)

(Received June 9th, 1981)

Key words: Sugar transport; D-Glucose; Negative cooperativity; Allosteric pore model; (Human erythrocyte)

1. When D-glucose exchange influx is measure over a wide range of concentrations then two affinity constants (2.27 and 26.0 mM) are evident. This is consistent with a transport model (the allosteric pore model) in which there is negative cooperativity between subunits of the transport protein. 2. The equations for the allosteric pore model interacting with two substrates (or a substrate and an inhibitor) have been derived and have been used to analyse data from exchange inhibition and for mixed infinite-*trans* uptake experiments. 3. The exchange inhibition of tracer 3-*O*-methyl-D-glucose, D-xylose and D-fructose uptake by D-glucose also shows evidence for negative cooperativity and for two inhibition constants which are approximately equal to the D-glucose equilibrium exchange affinity constants. 4. The uptake of D-glucose into infinite-*trans* D-glucose or 3-*O*-methyl-D-glucose gives K_m values of 2.6 and 2.33 mM, respectively. The uptake of 3-*O*-methyl-D-glucose into infinite-*trans* D-glucose or 3-*O*-methyl-D-glucose gives K_m values of 6.0 and 4.6 mM, respectively. V values are slightly higher when the internal sugar is 3-*O*-methyl-D-glucose. 5. In cells that are treated with fluorodinitrobenzene the apparent K_i value for D-glucose inhibition of tracer D-fructose uptake is lowered. It is proposed that this is due to a partially selective effect of FDNB on the internal subunit interface stability constant (the internal pore gate).

Introduction

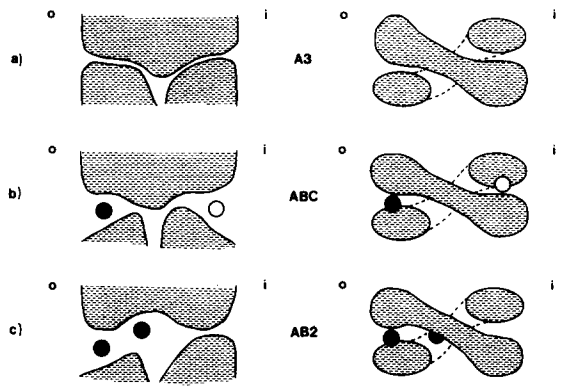
Sugar transport into human erythrocytes has been extensively studied over many years and it has gradually become more difficult to account for the accumulating experimental parameters in terms of a simple carrier model with a site alternatively available at inner and outer sides of the membrane. Instead, multiple site models have been suggested [1–3]. We have recently described an allosteric pore model [4] which can account for the experimental parameters for D-glucose and D-galactose transport. Because of the multiple site interactions predicted by this model it is appropriate to test the model by measuring the transport parameters under conditions in which the transporter combines with two sugars. It is shown that the equations for the allosteric pore model when it combines with two sugars can be reduced to simple equations in the special case of exchange inhibition of one sugar on the tracer flux of another and in the case of infinite-*trans* fluxes.

Abbreviation: FDNB, fluorodinitrobenzene.

Theoretical

The allosteric pore model

The allosteric pore can be simultaneously occupied from the inside and outside sites. Substrate binding induces a conformational change which opens gates to the pore. The gates to the pore are regarded as interfaces between protein subunits. A linear arrangement of three subunits was assumed which gave two linear subunit interfaces. A cross-section of this arrangement of units could be envisaged as providing discrete upper and lower channels (Scheme I). Klingenberg [5] has recently reviewed evidence that dimers are involved in the transport of many substances, including sugars. An irregular arrangement of two protein units with two domains per subunit and two contact



Scheme I. The scheme shows features of the allosteric pore. (a) in the absence of substrate the interfaces between subunits are stable and the pore is closed. (b) The pore is simultaneously occupied from inside and outside by transported substrate. The A subunits are converted to B subunits by substrate outside (•) and to C subunits by substrate inside (○). (c) When neighbouring subunits are both occupied from outside then the interfaces between subunits (pore gates) are greatly destabilised and negative cooperativity results. The right panel of this scheme shows cross sections through these pores. The dotted line indicates that two subunits with separate domains at inner and outer surfaces would be equivalent to a linear arrangement of three subunits since both arrangements have inner and outer subunit interfaces.

points per neighbouring subunit would be equivalent to three subunits with only one point of contact per neighbouring subunit. In the case of sugar transport the exact structure of the transporter has not been clearly resolved, so that at present we shall continue to consider the transporter as consisting of three subunits.

The kinetic considerations of the allosteric pore demand only two subunit interfaces and a sufficent size or number of internal channels for substrate entering the pore from opposite sides of the membrane to pass one another.

When the pore is occupied from outside by substrate (S) a single subunit undergoes a conformational change. An A (unoccupied) subunit is converted to a B (occupied from outside) unit. When the pore is monovalently occupied from inside an A subunit is converted to a C (occupied from inside) unit. Monovalent occupancy of the pore by S results in low $K_m(1/K_S)$ and low $V(V_L)$ transport parameters because the *trans* gates are not fully open. However, monovalent occupancy exposes a high K_m site (negative cooperativity). If the concentration of substrate is sufficiently high, then binding to this site can destabilize the *trans* gate and result in a high $V(V_H)$ for transport. The degree of negative cooperativity is dependent on the stability of the interface between two B subunits compared with the stability of two A subunits (K_{BB}). This has been defined more fully elsewhere [4].

The net influx equation for S_o (in the absence of other forms of the pore) combines the fractional concentrations of the monovalent (A2B) and divalent (AB2) pores and their respective low and high translocation rates

$$[A2B] = [A3]\, K_S S_o$$

$$[AB2] = [A3]\, K_S^2 K_{BB} S_o^2$$

$$U_{oi}^S = \frac{\mathrm{Tot}(V_L K_S S_o + V_H K_S^2 K_{BB} S_o^2)}{1 + K_S S_o + K_S^2 K_{BB} S_o^2} \qquad (1)$$

where Tot is the total concentration of all forms of the transporter. S_o represents substrate (S) concentration. [A3] is the concentration of the three unoccupied A subunits (empty pores). K_{BB} is the stability constant for two subunits occupied from outside.

In the case of efflux monovalent occupancy also occurs with an association constant, K_S, but divalent occupancy from inside gives a greater destabilization of subunit interfaces. This destabilization is given by the interface stability between two C subunits compared with the stability of two A subunits (K_{CC}). The stability constant K_{CC} is less than K_{BB}.

In applying similar considerations to the flux of S_o in the presence of another substrate, T_o, and to the flux of S_o in the presence of internal sugar (S_i and T_i) we give a general equation in the Appendix and return to a more detailed consideration of subunit interactions only for particular experimental situations.

Equilibrium exchange

We have previously shown by computer simulations that equilibrium exchange in the allosteric pore predominantly involves low K_m, low V_{max} (V_L) exchange and high K_m, and high V_{max} (V_{HE}) exchange components. This is confirmed in the Appendix where the full exchange equation (Eqn. A3) is reduced to an equation containing only these

components (Eqn. A5). The low K_m, low V_{max} exchange occurs in pores that are monovalently occupied from both outside and inside. An A subunit outside is converted to a B subunit and an A subunit inside is converted into a C subunit. Thus the (ABC) pore has stable subunit interfaces. The high K_m, high V_{max} exchange occurs predominantly in pores that are divalently occupied from outside but monovalently occupied from inside (B2C) and in pores that are divalently occupied from inside but monovalently occupied from outside (C2B). Since these pores (B2C and C2B) are divalently occupied from a single side of the membrane (either outside or inside), then the interfaces between subunits are unstable and the interface stability constant $(K_{BB}+K_{CC})\,K_{BC}$ for the combined B2C and C2B pores gives rise to high K_m and high V_{max}.

The inhibition of the tracer flux of T by S at equilibrium gives a similar equation (Eqn. A7). Tot/ denominator of both these equations is equal to the concentration of available pores. This concentration is unaltered by the tracer T and the denominators of Eqns. A5 and A7 are identical. The numerators in these equations are equal to the tracer permeabilities through the available pores and the apparent affinity constants will be dependent upon the ratio of the permeabilities through the high and low affinity components. The exchange inhibition equations are (in coefficient form) equivalent to the net flux equation (Eqn. 1). The parameter values are, of course, different. Eqn. 2 can thus be used for curve fitting to net flux and exchange flux data:

$$\frac{U_{oi}^{T}}{T}=\frac{\mathrm{Tot}(\upsilon\_K_L+\upsilon\_K_H S)}{1+K_L S+K_H S^2} \tag{2}$$

We have previously shown [7] that the carrier equation for the effect of S on the tracer flux of T is

$$\frac{U_{oi}^{T}}{T}=\frac{1/(R_{ZZ}K_T)}{1+\dfrac{R_{ee}^{S}}{K_S R_{ZZ}}\cdot S}$$

where $R_{ee}^{S}/K_S R_{ZZ}$ is the reciprocal K_i value and should equal the equilibrium exchange K_m for S *.

* K_T and K_S in this equation are defined in Refs. 6 and 7 and bear no relation to the allosteric pore K_T and K_S.

This K_i value should be independent of which tracer is used. This can be contrasted with the predictions of the allosteric pore where the K_i values will be dependent upon the interaction of S and T within the available pores. The ratio of tracer permeability (V/K) through the low- and high-affinity components may be different for different tracers and the K_i values need not equal the equilibrium exchange K_m values for S. For the allosteric pore only one K_m (or K_i) will be apparent if the ratio $\upsilon\_K_L K_L/\upsilon\_K_H$ is approximately equal to 1.

Infinite-trans uptake

The initial rates of infinite-*trans* uptake (counterflow) of a sugar S can be measured either with an infinite internal concentration of S or using an infinite concentration of another sugar, T [7,8].

We have previously derived [7] the equation for the interaction of the carrier model with two substrates. For infinite-*trans* uptake of S_o into infinite T_i

$$\frac{U_{oi}^{S}}{S_o}=\frac{1/R_{ee}^{ST}}{\dfrac{R_{io}^{T}}{R_{ee}^{ST}}K_S+S_o} \tag{3}$$

For infinite-*trans* uptake of S_o into infinite S_i

$$\frac{U_{oi}^{S}}{S_o}=\frac{1/R_{ee}^{S}}{\dfrac{R_{io}^{S}}{R_{ee}^{S}}\cdot K_S+S_o} \tag{4}$$

where R_{ee}^{ST} is the reciprocal of the V_{max} for the mixed exchange of S_o into T_i or T_i into S_o. R_{io}^{T} and R_{io}^{S} are the reciprocals of the V_{max} values for zero-*trans* exit of T_i and S_i, respectively, and R_{ee}^{S} is the reciprocal of the exchange V_{max} for S.

We have previoulsy shown that

$$R_{ee}^{S}+R_{ee}^{T}=R_{ee}^{ST}+R_{ee}^{TS} \tag{5}$$

where R_{ee}^{T} and R_{ee}^{TS} are the reciprocals of the V_{max} values for exchange of T and for the mixed exchange of T_o into S_i or S_i into T_o. This equation means that the sum of the reciprocals of the V_{max} values for the self-exchange of S and T should equal the sum of the reciprocals for the heteroexchange V_{max} values.

For the carrier model it is clear that the infinite-*trans* exchange will be fast because flux will not be

limited by the return of the empty carrier. The K_m will be low only if the zero-*trans* rates are approximately equal to the exchange rates. Hence the carrier model is inconsistent with the data of Baker and Naftalin [9], who showed a low K_m for infinite-*trans* efflux of D-glucose into infinite D-galactose. The carrier model predicts a high K_m because the ratio exchange/net influx is high.

These predictions of the carrier can be contrasted with those of the allosteric pore model where a fast infinite-*trans* exchange rate is due to *trans* destabilization of the pore gates. In this model the infinite-*trans* K_m values are low because of the destabilization of both the inner and outer pore gates that occurs at high (infinite-*trans*) sugar concentrations. For the allosteric pore model simple Michaelis-Menten equations for the infinite-*trans* experiment are also obtained (see Eqns A8 and A9). For the allosteric pore model the sum of the reciprocals of the V_{max} values for the self-exchange of S and T are not necessarily equal to the sum of reciprocals of the heteroexchange V_{max} values. Thus an observation of inequality in the relationship (Eqn. 5) would be inconsistent with the carrier model but not the allosteric pore model. However, an equality would be consistent with either model.

Materials and Methods

D-Glucose, D-xylose, 3-*O*-methyl-D-glucose and D-fructose were from Sigma. D-[U-^{14}C]-Glucose, D-[U-^{14}C]xylose, and 3-*O*-[^{14}C]methyl-D-glucose and D-[U-^{14}C]fructose were from the Radiochemical Centre, Amersham. Phloretin was from K and K laboratories and other reagents were from B.D.H.

Transport experiments

Outdated human erythrocytes were washed five times in phosphate-saline buffer, pH 7.4 (154 mM NaCl/12.5 mM sodium phosphate). For the infinite-*trans* experiments cells were loaded to give 80 mM internal sugar. 50 μl cells (80% cytocrit) were mixed with 1.6 ml of external sugar. To terminate the reaction 10 ml of stopping solution (0.3 mM phloretin and 0.16 μM mercuric chloride in phosphate-saline buffer) were added at 0, 1 and 1.5 s and after equilibration. For exchange inhibition experiments cells were equilibrated with a substrate concentration of 10 μM (except in the case of D-fructose which was 100 μM) and with an appropriate concentration of D-glucose for 5–15 min. Transport was initiated by adding radiolabelled substrate with or without D-glucose. After appropriate time intervals (which ranged from 1 s for D-glucose to 15 min for D-fructose) tansport was terminated by the addition of stopping solution. In both types of experiment, after centrifugation of cell pellets in stopping solution, the supernatants were removed and the cells were washed in a further 5 ml of stopping solution, respun, and then treated with 10% trichloroacetic acid. Aliquots, after removal of precipitated protein, were used to estimate radioactivity by liquid scintillation counting. Exchange uptake rates (v/s) were calculated from the equation

$$v/s = \ln(1/(1-f))/t$$

where f is the fractional filling and t is the time.

The infinite-*trans* equations (Eqns. A8, A9) are linear and data were fitted by weighted regression to give the apparent K_m and V.

The exchange inhibition equation (Eqn. 2) is nonlinear and the data were fitted by a weighted least-squares procedure. This procedure uses the Marquardt method to give values for v_K_L, v_K_H, K_L and K_H. A computer programme was written using the subroutine NAG E04 GAF.

In experiments using FDNB, washed cells (25% cytocrit) were treated with the reagent at the indicated concentrations of FDNB for 1 h at 25°C. The cells were then washed three of four times before use in sugar uptake estimations.

Results

Exchange inhibition experiments

The allosteric pore model, using previous estimates [4] for parameter values predicts that over the normal range of sugar concentrations used to study exchange no nonlinearity in reciprocal plots should be detectable. However, as can be seen from the predicted plot Fig. 1 some departure from linearity should occur at very low D-glucose concentrations (tracer concentrations). In view of this we decided to look for a low K_m component using low D-glucose concentrations. Fig. 1 shows the exchange uptake of D-glucose measured at 20°C. On the same plot are

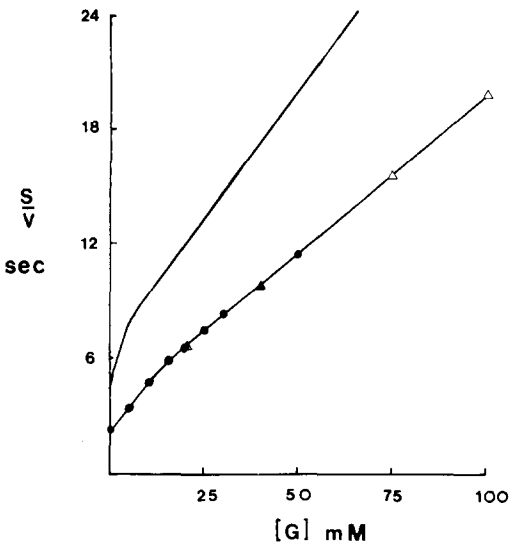


Fig. 1. D-Glucose exchange at 20°C: •, exchange influx; △, exchange efflux. The lowest D-glucose concentration is 10 μM. The line through the data is derived by least-squares analysis. The influx data are from three experiments with triplicate observations. The efflux data are from Ref. 10. Also shown is the simulated D-glucose exchange plot derived from previously assigned (Ref. 4) parameters. These parameter values were assigned to illustrate features of the model and were not based on least-squares fitting.

some previous observations [10] on D-glucose exchange exit at 20°C. As must be the case for a non-accumulating facilitative transfer system the influx and efflux exchange rates are compatible. However, our exchange influx rates at low D-glucose concentrations are faster than those which have been previously reported by Eilam [11] for exchange efflux of low D-glucose concentrations. Eilam [11] noted that the literature results on D-glucose exchange flux showed a different apparent K_m depending upon the concentration range of sugar used. However, on re-examination of D-glucose exchange, Eilam found no evidence for nonlinearity in exchange efflux reciprocal plots. Edwards [12] reported exchange efflux rates that are compatible with our demonstration of nonlinearity. We chose to study exchange influx over short incubation times because of the possibility of some metabolism of the D-glucose label at low D-glucose concentrations. The problem associated with measuring exchange efflux is that a significant metabolism of the D-glucose label occurs, at low concentrations of sugar, during the cell loading period. The metabolized label is trapped so that efflux of nonmetabolized label (when measured as a fraction of the total label) may appear to be slow. Slight metabolism of the non-labelled internal D-glucose will be less important as this will be rapidly replaced from the loading solution. This metabolism can be partially avoided by using a short loading time but in our view the 5 min loading time used by Eilam is too long and we find that a significant proportion of the cellular label is metabolized in 2–3 min at low D-glucose concentrations. An alternative explanation for the discrepancy may be related to structural changes due to ageing of the cells but we have no detailed information on this.

The difference between our results on D-glucose exchange and those reported by Eilam is not of crucial importance, as we now have supportive evidence for two exchange affinity constants for D-glucose using other tracers. In Fig. 2 the inhibition of tracer uptakes of 3-*O*-methyl-D-glucose and D-xylose by equilibrated D-glucose are shown. Neither of these sugars shows evidence for breakdown of cellular label but each shows a similar pattern of D-glucose inhibition to that observed in D-glucose equilibrium exchange. In all three cases the plots show evidence for two affinity constants. Visual examination of the plots appears to indicate that two intersecting straight lines could be drawn through the data points. One line

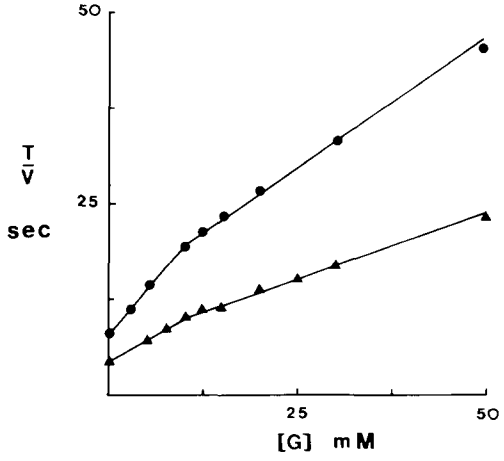


Fig. 2. D-Glucose inhibition of tracer (10 μM) 3-*O*-methyl-D-glucose (▲) and D-xylose (•) exchange uptake at 20°C. From two experiments with triplicate observations in each case.

would appear to have an intercept on the abscissa close to 7 mM. The other line would appear to have an intercept on the abscissa close to 26 mM. The ratio of the two intercepts on the ordinate for these lines would be about 1.5. Least-squares fitting of the data (Table I) to the allosteric pore (Eqn. 2) also indicates that there are two affinity constants. The high K_m is about 26 mM, while the low K_m is about 2 mM. From Eqn. 2 it can be seen that at high sugar concentrations a linear relationship between T/V and S will exist (Eqn. 6) with an intercept on the ordinate equal to the K/V ratio for the high K_m component.

$$T/V = (K_L/v\_K_H) + (K_H/v\_K_H)\ S \qquad (6)$$

The tracer V/K ratio for the low K_m component is equal to the permeability in the absence of S. Hence the component ratio $v\_K_L \cdot K_L/v\_K_H$ should equal the ratio of tracer permeabilities through the two components and approximately correspond to the ratio 1.5 which was obtained from the ordinate intercepts from lines drawn by visual examination of the data. Computer fitting indicates that this ratio is closer to 2 than 1.5. Visual examination of the plot thus underestimates the component ratio and overestimates the low K_m but gives a good estimate of the high K_m. This is because part of the flux at low but not zero D-glucose concentrations occurs through the high K_m component.

Our analysis of the carrier model [7] showed that the K_i for D-glucose inhibition of the flux of any tracer should equal the equilibrium exchange K_m for D-glucose. We were aware that the literature values for the exchange K_m for D-glucose (20–40 mM) were much higher than the K_i for D-glucose inhibition of the transport of the low affinity sugar L-sorbose (7 mM) [13]. Clearly the evidence is inconsistent with the carrier model but we wished to determine whether the inhibition by D-glucose of the flux of a low affinity sugar could be resolved into two components. As L-sorbose was not available we chose the structurally related ketose, D-fructose. The results showed that inspite of a tracer permeability (V/K) almost 600-times lower than for D-glucose, two K_i values were clearly discernable (Fig. 4, Table I) and were similar to those obtained using D-glucose.

Infinite-trans uptake experiments

The uptake of 3-*O*-methyl-D-glucose into infinite-*trans* 3-*O*-methyl-D-glucose or infinite-*trans* D-glucose both revealed a K_m of about 6 mM (Fig. 3). The V_{max} for uptake was slightly greater when the inter-

TABLE I

THE KINETIC PARAMETERS FOR D-GLUCOSE EXCHANGE INHIBITION OF TRACER FLUXES IN HUMAN ERYTHROCYTES AT 20°C

Results from replicate observations are averaged before parameter fitting. The coefficient of variation of replicate observations was less than 10%. In each case the reciprocal of tracer permeability (calculated from replicate observations of tracer exchange) is significantly less ($P < 0.01$) than the ordinate intercept from Eqn. 6 obtained by linear regression of data using D-glucose concentrations from 10–100 mM.

Tracer	v_K_L (min^{-1})	v_K_H ($mM^{-1} \cdot min^{-1}$)	K_L (mM^{-1})	K_H (mM^{-2})	$1/K_L$ (mM)	K_L/K_H (mM)	$(v\_K_L \cdot K_L)/(v\_K_H)$
D-Glucose (n = 11)	26.2 ± 0.9	6.21 ± 6.8	0.44 ± 0.35	0.0168 ± 0.0193	2.27	26.0	1.8
3-*O*-Methyl-D-glucose (n = 10)	15.24 ± 1.08	3.88 ± 11.7	0.504 ± 1.06	0.0233 ± 0.074	1.98	21.6	1.97
D-Xylose (n = 9)	7.62 ± 0.51	1.74 ± 3.34	0.462 ± 0.612	0.0205 ± 0.0436	2.16	19.0	2.01
D-Fructose [a] (n = 6)	0.044 ± 0.002	0.0213 ± 0.023	0.844 ± 0.716	0.0245 ± 0.033	1.2	34.4	1.5

[a] 25°C.

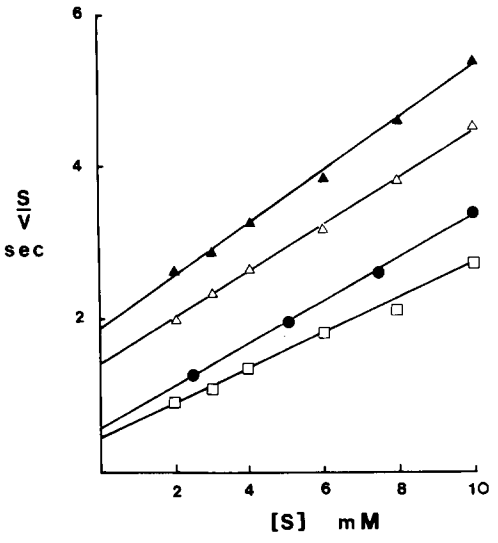


Fig. 3. D-Glucose uptake at 20°C into infinite-*trans* D-glucose (•) $K_m = 2.6 \pm 0.52$ mM; $V = 3.89 \pm 0.27$ mM/s and into infinite-*trans* 3-*O*-methyl-D-glucose (□) $K_m = 2.33 \pm 0.49$ mM; $V = 4.76 \pm 0.36$ mM/s. 3-*O*-Methyl-D-glucose uptake at 20°C into infinite-*trans* D-glucose (▲) $K_m = 6.0 \pm 0.60$ mM; $V = 3.03 \pm 0.17$ mM/s and infinite-*trans* 3-*O*-methyl-D-glucose (△) $K_m = 4.6 \pm 0.3$ mM; $V = 3.28 \pm 0.11$ mM/s. From two experiments with triplicate observations in each case.

nal sugar was 3-*O*-methyl-D-glucose.

The uptake of D-glucose into infinite-*trans* D-glucose or infinite-*trans* 3-*O*-methyl-D-glucose showed a K_m of about 2 mM (Fig. 3). Again, the V_{max} value for uptake was slightly greater when the internal sugar was 3-*O*-methyl-D-glucose. The parameter values for infinite-*trans* uptake of D-glucose reported here are similar to those described by Lacko et al. [14]. The V_{max} is slightly higher than the previously reported estimate but this may be because we use a higher internal D-glucose concentration. In view of the relative independence of the V_{max} values and particularly the K_m on the type of internal sugar it is not surprising that the results obtained for the mixed exchanges are consistent with either the carrier model Eqn. 5 or with the allosteric pore model. We have not carried out other tests of these equations using other sugars, but Miller [15] and Naftalin [16] and Baker and Naftalin [9] have noted heteroexchange rates that were considered to be inconsistent with the carrier model.

Fluorodinitrobenzene inactivation

In view of the clear evidence for multiple components involved in the sugar transport mechanism we wished to examine whether these components were equally susceptible to inactivation by fluorodinitrobenzene (FDNB). Sen and Widdas [17] showed that net efflux of D-glucose was more inhibited than D-glucose exchange, while net entry of D-glucose was only poorly inhibited by FDNB. An explanation for these findings in terms of the allosteric pore is that FDNB treatment structurally modifies the internal pore gate so that sugars are less effective in causing a substrate induced destabilization of the internal subunit interface (that is, K_{CC} is increased). In the absence of a modification by FDNB treatment it is the instability of the internal gate which gives rise to asymmetry so that a relatively selective effect on this subunit interface stability constant would reverse the asymmetry in net fluxes. The occupancy of the pore in equilibrium exchange at high D-glucose concentrations is dependent upon the interface stability factor $(K_{BB} + K_{CC})K_{BC}$ and since $K_{BB} > K_{CC}$ a relatively selective effect on K_{CC} would be expected to have a smaller effect on exchange than on net efflux. It is not suggested that FDNB is completely selective for the inner pore gate, since long exposure to the reagent at

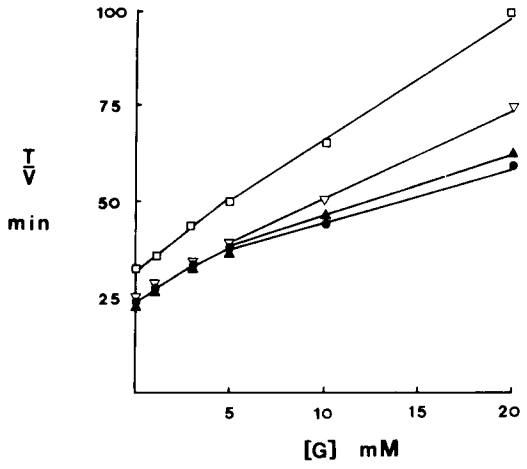


Fig. 4. D-Glucose inhibition of tracer (100 μM) D-fructose exchange uptake at 25°C after treatment with no (•), 0.75 mM (▲), 1.5 mM (▽) and 3 mM (□) FDNB. Single experiments with duplicate observations in the presence of FDNB and three experiments in the absence of FDNB.

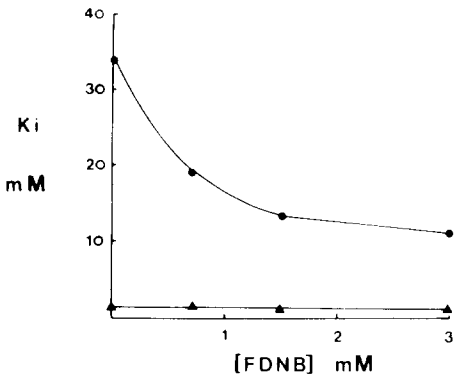


Fig. 5. The effect of FDNB pretreatment for 1 h at 25°C on K_L/K_H (•) and $1/K_L$ (▲) measuring using D-fructose as the tracer substrate and D-glucose as the exchange inhibitor.

high concentrations gives total inhibition of the transport system.

If the suggested mechanism for FDNB action is correct, then FDNB might be expected to have a smaller effect on exchange inhibition than on net efflux but it might also be expected to lower the apparent K_i due to the increase in K_{CC}. Fig. 4 shows that this is the case. Here quite high concentrations of FDNB give rather small inhibition of tracer D-fructose flux. 3 mM FDNB treatment gives much greater inhibition of net D-glucose efflux [17]. Nevertheless, in the inhibited condition the apparent K_i for D-glucose is lowered and this appears to be due to a lowering of the high K_i, which is reduced by about one-half (Fig. 5) with no change in the low K_i. It seems likely that the internal pore gate stability constant K_{CC} is increased to a value similar to that of the outer pore gate stability constant, K_{BB}. Thus in FDNB-treated cells $K_H \approx K_S^2 2K_{BB}K_{BC}$, which is about twice K_H in untreated cells, which equals $K_S^2(K_{BB} + K_{CC})K_{BC}$. FDNB also reduces the component ratio $\nu\_K_L \cdot K_L/\nu\_K_H$ from about 2 to about 1 and the inhibition plots in FDNB-treated cells become more linear.

Discussion

We have previously shown [4] that the allosteric pore model is more compatible with the information that is available on erythrocyte sugar transport than are other models. In particular, there can no longer be any doubt that a single species of monovalent carrier is an inadequate description of the process because of the demonstration of both high and low affinity constants at both membrane surfaces [18]. Much of the evidence supports the possibility of substrate-induced changes in the transport system and is therefore consistent with the allosteric pore model and with negative cooperativity in substrate binding and permeability.

The results of the present paper add further support to the possibility of negative cooperatively between components of the sugar transport system. There appears to be a shift from transporter involved in low K_m exchange to transporter involved in high K_m exchange as the concentration of D-glucose is increased. This is thought to be due to a substrate induced destabilization of interfaces between subunits (the pore gates) at high D-glucose concentrations. The evidence that D-glucose shows negative cooperativity is confirmed by the use of other substrates at tracer concentrations. Additional substrates were used because there appeared to be a difference between our results and some work from other laboratories. The evidence that the inhibition of the flux of tracer concentrations of 3-*O*-methyl-D-glucose, D-xylose and D-fructose by D-glucose also show both a low and a high K_m eliminates the possibility that the nonlinearity of our reciprocal plots of D-glucose exchange is due either to errors involved in measuring the very fast transport of D-glucose, or to errors due to a slight metabolism of tracer concentrations of D-glucose. This is because the additional tracers are transported more slowly than D-glucose and show no evidence for a metabolic breakdown of the radioactive label. The literature has previously suggested that the apparent K_i for inhibition of the uptake of low-affinity sugars, such as D-fructose, is lower than the apparent exchange K_m for D-glucose. Our results show that this anomaly occurs because the former measurement is usually made at too low a range of D-glucose concentrations, while the latter measurement is usually made at too high a range of D-glucose concentrations. A large range of both low and high D-glucose concentrations reveals both the low and the high K_m for both experiments.

In FDNB-treated cells there is a progressive lowering of the high K_m and the component ratio falls to 1. This thought to be due to an effect on the inner pore gate which in normal cells has a lower interface

stability constant. An increase in this constant thus reduces the negative cooperativity between subunits and reverses the asymmetry of the transport system.

A model in which some carriers are antiparallel [11] would give a single affinity constant in exchange experiments. However, the evidence presented for two affinity constants for D-glucose in the exchange inhibition experiment could be compatible with a model in which two different species of carrier were arranged in parallel [11] (see also Ref. 19). The coefficient form of the equation for the two-carrier model (Eqn. 7) is equivalent to the equation derived from the allosteric pore model:

$$V/S = \frac{((V_1/K_1) + (V_2/K_2)) + (V_1 + V_2)/(K_1K_2)S}{1 + ((1/K_1) + (1/K_2))S + (1/K_1K_2)S^2} \quad (7)$$

where V_1 and V_2 are the exchange V_{max} values for the two carriers and K_1 and K_2 are their respective exchange K_m values. This model, however, predicts a higher K_m in infinite-*cis* D-glucose uptake and infinite-*trans* D-glucose efflux than is observed [9]. It is also inconsistent with the evidence that the K/V ratios for net efflux and exchange (determined at high D-glucose concentrations) show different susceptibility to methylxanthines [10].

Several models for sugar transport with in series components have been described. A carrier in series with an unstirred layer [20]would give a linear equation for exchange inhibition [12]. A model in which the transporter is in series with a compartment in which a heamoglobin-sugar complex is formed has been suggested by Naftalin and coworkers [9,21]. This model would have be modified to account for large asymmetries at low haemoglobin concentrations [10]. It would also have to be modified to account for the fast tracer fluxes that occur in the absence of D-glucose induced conformational changes that we have demonstrated in this report. An interesting new model has been described by Foley et al. [22] to account for sugar transport in adipocytes. It has been proposed that in series microcarriers are separated by a membrane pore. It can readily be shown that the equation for the exchange inhibition experiment derived from this model is

$$\frac{T}{V} = (1/P_1) + (1/P_2) + ((1/P_1K_1) + (1/P_2K_2))S$$

where P_1 and P_2 are the tracer V/K values at the inner and outer microcarriers and K_1 and K_2 are their respective exchange K_m values for S. The equation predicts that different tracers may give different K_i values for D-glucose inhibition but also that the inhibition should be linear and only a single K_m should be evident for each tracer. Hence the model cannot account for erythrocyte sugar transport since it is inconsistent with the evidence for negative cooperativity that we have presented.

It is of interest to note that the adipocyte and erythrocyte sugar transport systems show many similarities particularly in their specificity requirements [7]. A major difference is that there is no evidence for asymmetry for 3-*O*-methyl-D-glucose in adipocytes [23,24] but this sugar, like D-glucose, shows asymmetric net fluxes in erythrocytes (Holman, unpublished results). Also, for adipocytes, there is less evidence for nonlinearity in plots of 3-*O*-methyl-D-glucose exchange at low concentrations [24] or at concentrations up to 60 mM (Holman and Rees, unpublished data). Also, the adipocyte system is less susceptible to inhibition by FDNB [25]. Thus we conclude that all these lines of evidence suggest that the allosteric pore model can be applied to both systems and that the mentioned differences between the two systems are due to a greater subunit interface instability at the inner surface of the erythrocyte system.

Appendix

The allosteric pore equation for the flux of S_o in the presence of another substance, T_o, and to the flux of S_o in the presence of internal sugar (S_i and T_i) is

$$U^S_{oi} = \frac{\text{Tot} \sum_{m=1}^{2}\sum_{n=0}^{2}\sum_{p=0}^{2}\sum_{q=0}^{2} S_o^m S_i^n T_o^p T_i^q \, v\_K_{mnpq}}{\sum_{m=0}^{2}\sum_{n=0}^{2}\sum_{p=0}^{2}\sum_{q=0}^{2} S_o^m S_i^n T_o^p T_i^q \, K_{mnpq}} \quad (A1)$$

where $m + p$ and $n + q \leqslant 2$ and $m + n + p + q \leqslant 3$, $K_{oooo} = 1$ and Tot is the concentration of all forms of the pore. The association constants K_{mnpq} are the products of monomer association constants (K_s and K_T for S and T, respectively), the statistical factor 2 when $m = p$ or $n = q$ and the relevant subunit interface stability constants. Multivalent occupancy

(when $m + p$ or $n + q = 2$) may result in unstable interfaces between subunits. Negatively cooperative interactions (when the interface stability constants are less than 1) will result in low affinity but high translocation rates. The translocation rates for each occupancy state are

$$V_{mnpq} = v\_K_{mnpq}/K_{mnpq}$$

In the efflux of S or in the influx or efflux of T the Tot/denominator of Eqn. A1 is unchanged as it is equal to the concentration of unoccupied pores. This is independent of the direction in which flux is measured.

For the efflux of S_i

$$U^{S}_{io} = \frac{Tot}{D} \sum_{m=0}^{2} \sum_{n=1}^{2} \sum_{p=0}^{2} \sum_{q=0}^{2} S_o^m S_i^n T_o^p T_i^q \, v\_K_{mnpq}$$

For the influx of T_o

$$U^{T}_{oi} = \frac{Tot}{D} \sum_{m=0}^{2} \sum_{n=0}^{2} \sum_{p=1}^{2} \sum_{q=0}^{2} S_o^m S_i^n T_o^p T_i^q \, v\_K_{mnpq} \qquad (A2)$$

For the efflux of T_i

$$U^{T}_{io} = \frac{Tot}{D} \sum_{m=0}^{2} \sum_{n=0}^{2} \sum_{p=0}^{2} \sum_{q=1}^{2} S_o^m S_i^n T_o^p T_i^q \, v\_K_{mnpq}$$

Because the system is nonaccumulating

$$\sum_{m=1}^{2} \sum_{n=0}^{2} \sum_{p=0}^{2} \sum_{q=0}^{2} v\_K_{mnpq} = \sum_{m=0}^{2} \sum_{n=1}^{2} \sum_{p=0}^{2} \sum_{q=0}^{2} v\_K_{mnpq}$$

$$\sum_{m=0}^{2} \sum_{n=0}^{2} \sum_{p=1}^{2} \sum_{q=0}^{2} v\_K_{mnpq} = \sum_{m=0}^{2} \sum_{n=0}^{2} \sum_{p=0}^{2} \sum_{q=1}^{2} v\_K_{mnpq}$$

The flux equations for two sugars can be greatly simplified for certain experimental situations such as exchange inhibition and infinite-*trans* uptake and efflux.

The full equation for equilibrium exchange of S is derived from Eqn. A1

$$U^{S}_{oi} = \frac{Tot \sum_{m=1}^{2} \sum_{n=0}^{2} S^m S^n \, v\_K_{mn00}}{\sum_{m=0}^{2} \sum_{n=0}^{2} S^m S^n K_{mn00}} \qquad (A3)$$

To simplify this equation for the routine analysis of negative cooperativity in equilibrium exchange we return to a consideration of subunit interactions. If there is negative cooperativity between subunits then

$$1 + K_{BB} + K_{CC} \approx 1 + K_{BC}(K_{BB} + K_{CC}) \qquad (A4)$$

$$1 + \frac{V_H}{V_L} K_{BB} \approx 1 + \frac{V_{HE}}{V_L} K_{BC}(K_{BB} + K_{CC})$$

where K_{BB}, K_{CC} and K_{BC} are interface stability constants. These are dimensionless ratios for the stability between B and C subunits compared with A subunits as described in [4] and in the text. V_H and V_L are defined in the text in the description of net flux. V_{HE} is the V_{max} for the high K_m exchange components. Hence

$$\frac{U^{S}_{oi}}{S} = \frac{Tot(v\_K^{S}_{L} + v\_K^{SS}_{HE} S)}{1 + SK_{LE} + S^2 K^{SS}_{HE}} \qquad (A5)$$

where $v\_K^{S}_{L} = V^{S}_{L} K_S$

$$v\_K^{SS}_{HE} = V^{SS}_{HE} K_S^2 (K^{SS}_{BB} + K^{SS}_{CC}) K^{SS}_{BC}$$

$$K^{SS}_{HE} = K_S^2 (K^{SS}_{BB} + K^{SS}_{CC}) K^{SS}_{BC}$$

The parameter subscripts S and T are introduced to distinguish between the possible different destabilization factors that may arise if neighbouring subunits are occupied by different sugars (S or T). For $1/K_{LE}$ the subscripts are used to distinguish this high-affinity exchange constant from the sugar/monomer dissociation constant $1/K_S$. We make this distinction because the value obtained for the constant $1/K_{LE}$ is higher than previously anticipated [4]. This may be because the form of the pore involved in high-affinity exchange is doubly occupied, albeit from opposite sides of the membrane, and some slight subunit destabilization may occur which increases the apparent $1/K_{LE}$ value above the value for $1/K_S$. This destabilization would be much less than that involved when pores are doubly occupied from the same side of the membrane. $1/K_{LE}$ may also be higher than $1/K_S$ because of the approximation involved in Eqn. A4 which has eliminated some forms of the pore which have low affinity. Otherwise, the approximation involved in Eqn. A4 is adequate for D-glucose

and D-galactose transport [4]. The approximation is unfortunate but has to be made if least-squares curve fitting is to be performed because Eqn. A3 contains too many parameters.

If a different sugar T is used as a tracer at a concentration well below its K_m then the chances of more than one molecule of tracer entering the same pore are negligible, hence

$$U_{oi}^{T} = \frac{\text{Tot} \sum_{m=0}^{2} \sum_{n=0}^{2} S_o^m S_i^n T_o \, \upsilon\_K_{mn10}}{\sum_{m=0}^{2} \sum_{n=0}^{2} S_o^m S_i^n K_{mn00}} \quad \text{(A6)}$$

If the negative cooperativity assumption (Eqn. A4) is applied then Eqn. A6 becomes

$$\frac{U_{oi}^{T}}{T} = \frac{\text{Tot}(\upsilon\_K_L^T + \upsilon\_K_{HE}^{ST} S)}{1 + SK_{LE} + S^2 K_{HE}^{SS}} \quad \text{(A7)}$$

where

$$\upsilon\_K_L^T = V_L^T K_T$$

$$\upsilon\_K_{HE}^{ST} = V_{HE}^{ST} K_S K_T (K_{BB}^{ST} + K_{CC}^{SS}) K_{BC}^{ST}$$

In infinite-*trans* influx the influx of S_o into an infinite concentration of T_i or S_i is measured. Setting T_i and T_o to 0 and S_i to ∞ in Eqn. A1 gives

$$\frac{U_{oi}^{S}}{S_o} = \frac{\text{Tot}\, \upsilon\_K_{1200}/K_{1200}}{\frac{K_{0200}}{K_{1200}} + S_o}$$

In terms of interface stability constants this becomes

$$\frac{U_{oi}^{S}}{S_o} = \frac{\text{Tot}\, V_{HE}^{SS}}{\frac{1}{K_S K_{BC}^{SS}} + S_o} \quad \text{(A8)}$$

Setting S_i and T_o to 0 and T_i to ∞ in Eqn. A1 gives

$$\frac{U_{oi}^{S}}{S_o} = \frac{\text{Tot}\, \upsilon\_K_{1002}/K_{1002}}{\frac{K_{0002}}{K_{1002}} + S_o}$$

or

$$\frac{U_{oi}^{S}}{S_o} = \frac{\text{Tot}\, V_{HE}^{ST}}{\frac{1}{K_S K_{BC}^{ST}} + S_o} \quad \text{(A9)}$$

Acknowledgements

This work is supported by a grant from the British Diabetic Association, for which we are grateful. We also thank Mrs. Pat Cooper for her skilled typing of the manuscript.

References

1 Naftalin, R.J. (1970) Biochim. Biophys. Acta 211, 65–78
2 Lieb, W.R. and Stein, W.D. (1970) Biophys. J. 10, 587–609
3 Le Fevre, P.G. (1973) J. Membrane Biol. 11, 1–19
4 Holman, G.D. (1980) Biochim. Biophys. Acta 599, 202–213
5 Klingenberg (1981) Nature 290, 449–454
6 Stein, W.D. and Lieb, W.R. (1974) Israel. J. Chem. 11, 325–339
7 Rees, W.D. and Holman, G.D. (1981) Biochim. Biophys. Acta 646, 251–260
8 Devés, R. and Krupka, R.M. (1979) Biochim. Biophys. Acta 556, 533–547
9 Baker, G.F. and Naftalin (1979) Biochim. Biophys. Acta 550, 474–484
10 Challiss, J.R.A., Taylor, L.P. and Holman, G.D. (1980) Biochim. Biophys. Acta 602, 155–166
11 Eilam, Y. (1975) Biochim. Biophys. Acta 401, 364–369
12 Edwards, P.A.W. (1974) Biochim. Biophys. Acta 345, 373–386
13 Levine, M., Levine, S. and Jones, N.M. (1971) Biochim. Biophys. Acta 225, 291–300
14 Lacko, L., Wittke, B. and Kromphardt, H. (1972) Eur. J. Biochem. 25, 447–454
15 Miller, D.M. (1968) Biophys. J. 8, 1329–1338
16 Naftalin, R.J. (1971) Biochim. Biophys. Acta 233, 635–643
17 Sen, A.K. and Widdas, W.F. (1962) J. Physiol. 160, 404–416
18 Ginsburg, H. and Stein, W.D. (1975) Biochim. Biophys. Acta 382, 353–368
19 Bloch, R. (1974) J. Biol. Chem. 249, 3543–3550
20 Regen, D.M. and Tarpley, H.L. (1974) Biochim. Biophys. Acta 339, 218–233
21 Naftalin, R.J. and Holman, G.D. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 257–299, Academic Press, New York

22 Foley, J.E., Foley, R. and Gliemann, J. (1980) J. Biol. Chem. 255, 9674–9677
23 Whitesell, R.R. and Gliemann, J. (1979) J. Biol. Chem. 254, 5276–5283
24 Taylor, L.P. and Holman, G.D. (1981) Biochim. Biophys Acta 642, 325–335
25 Czech, M.P., Pillion, D.J. and Shanahan, M.F. (1978) J Supramol Struct. 9, 363–371