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SUGAR TRANSPORT ASYMMETRY IN HUMAN ERYTHROCYTES -- THE EFFECT OF BULK HAEMOGLOBIN REMOVAL AND THE ADDITION OF METHYLXANTHINES

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Summary

1. The glucose transport asymmetry of intact human red cells has been shown to be retained in pink erythrocyte ghosts (a preparation of membranes in which 95% of the red cell haemoglobin has been removed).

2. 3-Isobutyl-1-methylxanthine inhibits net glucose efflux in intact cells and ghosts and also net influx in cells. 5 mM theophylline inhibits net efflux in ghosts. The inhibition type is mixed. The major effect is a decrease in the V value for net flux but a small increase in K_m also occurs. 3-Isobutyl-1-methylxanthine binds the transport system from the external solution only.

3. Exchange flux of glucose shows virtually no inhibition by 3-isobutyl-1-methylxanthine.

4. The results are discussed in terms of models for sugar transport. A model consistent with the observed pattern of inhibition would be one in which transport is rate-limited by the membrane and in which net and exchange flux occur via separate transport cycles.

Introduction

Methylxanthines are well known phosphodiesterase inhibitors [1]. The observation of inhibition of sugar transport by these agents could be interpreted as being mediated via changes in intracellular cyclic AMP levels. Whitesell and Regen [2] have shown that 3-isobutyl-1-methylxanthine inhibits sugar transport in active, but not quiescent thymocytes. Taylor and Halperin [3]

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have shown that theophylline (1,3-dimethylxanthine) inhibits insulin-stimulated sugar transport in rat adipocytes. Holman and Naftalin [4] have shown that theophylline decreases sugar transport at the serosal border of rabbit small intestine. These systems all have an active adenyl cyclase and phosphodiesterases and it is therefore of interest to determine whether the methylxanthines have an effect on sugar transport independent of an action on cyclic AMP levels. The human red cell has very low levels of both adenyl cyclase [5] and phosphodiesterase [6] and both sugar and inhibitor concentrations either side of the cell membrane can be carefully controlled. Interpretation of the results of the inhibition produced by methylxanthines is thus only complicated by the complexity of the sugar-transport system itself. Removal of cytoplasmic haemoglobin was carried out in order to investigate the possibility of a major influence of haemoglobin on transport and inhibitor kinetics.

Materials and Methods

D-[1-^{3}H]Glucose and D-[U-^{14}C]glucose were obtained from the Radiochemical Centre, Amersham. Theophylline and 3-isobutyl-1-methylxanthine were obtained from Sigma. Phloretin was obtained from K and K Laboratories and cytochalasin B was from the Aldrich Chemical Company. Other reagents were obtained from B.D.H., Ltd.

Preparation of pink erythrocyte ghosts. 1 ml aliquots of outdated human erythrocytes (stored in citrate) were washed three times in 30 ml of phosphate/saline buffer (154 mM NaCl, 12.5 mM sodium phosphate, pH. 7.4). The cells were then lysed in 30 ml of ice-cold 12.5 mM sodium phosphate (pH 7.4) and the membranes were separated by centrifugation at 38 000 × *g* for 25 min. This single lysis removes approx. 95% of the cell haemoglobin. The membranes were then resealed at 30°C for 1 h in a phosphate/saline buffer with sufficient NaCl to return the final concentration to 154 mM. This is extensively the method of Dodge et al. [7].

Zero-trans entry experiments. These were carried out on washed erythrocytes and on pink ghosts. For the intact erythrocyte experiments, 200 μl of cells at 50% haematocrit were incubated at 20°C. 800 μl of radioactively labelled D-glucose solution were then injected rapidly to produce mixing. At an appropriate time interval, 8 ml of ice-cold stopping solution were added (154 mM NaCl, 1 mM $HgCl_2$, 0.14 mM NaI and 0.1 mM phloretin in 1% ethanol). Cells were spun rapidly through 500 μl of dibutylphthalate using a bench centrifuge. The supernatants were removed and after a further wash in stopping solution, 1 ml of 10% trichloroacetic acid was added to each pellet to extract the radioactively labelled glucose.

For pink-ghost experiments, 10 μl of packed ghosts were suspended in 40 μl of phosphate/saline buffer. 50 μl of a radioactively labelled D-glucose solution were injected rapidly to produce mixing. 1 ml of stopping solution was used to terminate transport at an appropriate time interval. Samples were immediately filtered through glass-fibre filters (Whatman GF/F). The trapped ghosts were then washed with a further 15 ml of stopping solution. The edges of the filters were cut off with a cork borer and the label was extracted in 1% trichloroacetic acid in scintillation vials.

A requirement of the zero-*trans* entry experiment is that initial rates of uptake, at varying substrate concentrations, should be measured. To accomplish this, an electronic metronome was used for the short time intervals. Glucose influx could thus be measured accurately at 2 s at a range of glucose concentrations between 0.5 and 10 mM. Ginsburg and Stein [8] have used a similar system with incubation times as low as 1 s. Zero-time samples were obtained by adding stopping solution before the substrate. Equilibrium isotope levels were obtained by incubating for 20—30 min and were used in the calculation of entry rates:

$$V = \frac{(C_t - C_0)}{(C_\infty - C_0)} \cdot \frac{S}{t}$$

where C is the activity (in cpm) at times t, 0 and equilibrium. S is the sugar concentration. Zero-time activity was approx. 2% (for intact erythrocytes) and 5% (for ghosts) of the activity at equilibrium.

Zero-trans exit experiments. These were carried out on intact erythrocytes and pink ghosts. Washed erythrocytes were incubated for 1 h in 40 mM D-glucose. Labelled D-glucose was then added to a loosely packed suspension (approx. 80% haematocrit). 20 μl of the packed suspension were rapidly diluted in 5 ml of phosphate/saline buffer. At appropriate time intervals, 5 ml of stopping solution were added and the solutions centrifuged rapidly in a bench centrifuge. The pellets were then washed in a further 2 ml of stopping solution and then lysed in 1 ml of distilled water. 100 μl of this suspension were extracted in 20% trichloroacetic acid and the remaining solution (after spinning out membrane fragments) was used to estimate haemoglobin spectrophotometrically. This was used to correct exit rates for cell recovery. For pink ghost experiments, 10 μl of packed ghosts (resealed in the presence of radioactively labelled 40 mM D-glucose) were diluted in 1 ml of phosphate/saline buffer. The reaction was stopped at appropriate time intervals by the addition of 1 ml of ice-cold stopping solution and this was followed by rapid filtering through glass-fibre filters. The trapped ghosts were then washed with 15 ml of stopping solution and treated as described for the entry experiments.

The activity at equilibrium was approx. 2—5% of the activity at time zero for intact cells and for pink ghosts.

Exchange experiments. These were carried out on whole cells using the same method as zero-*trans* efflux except that efflux was studied with the glucose concentration outside equal to the glucose concentration inside. The results were analysed as described by Eilman and Stein [9].

Results

Fig. 1 shows a plot of the entry data obtained for intact erythrocytes (K_{zt}^{oi} = 1.60 ± 0.24 mM, V_{zt}^{oi} = 30.8 ± 2.2 mM · min^{-1}) and for pink ghosts (K_{zt}^{oi} = 1.56 ± 0.23 mM, V_{zt}^{oi} = 25.5 ± 3.2 mM · min^{-1}). Clearly, within experimental variation, there is no evidence for a change in the kinetic parameters for entry after removal of 95% of the cell haemoglobin. Further experiments showed that there was no significant leakage pathway in pink ghosts as 50 μM cytochalasin B gave 99% inhibition of the initial entry rate of 40 mM glucose. This

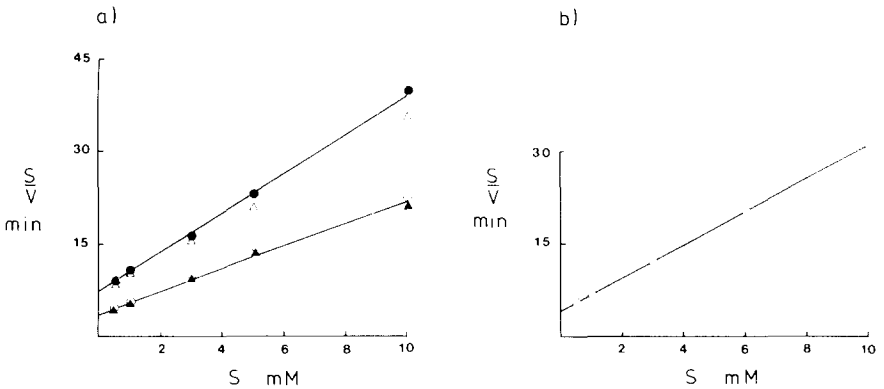


Fig. 1. (a) Zero-*trans* influx of D-glucose into human erythrocytes at 20°C (▲), K_{zt}^{oi} = 1.6 ± 0.24 mM, V_{zt}^{oi} = 30.8 ± 2.2 mM · min^{-1} (six observations at each point). In the presence of 0.75 mM isobutylmethylxanthine outside (●), K_{zt}^{oi} = 2.3 ± 0.15 mM, V_{zt}^{oi} = 18.8 ± 0.7 mM · min^{-1} (six observations at each point). Also shown is glucose uptake in the presence of isobutylmethylxanthine inside (○) (three observations at each point) and in the presence of isobutylmethylxanthine inside and outside (△) (three observations at each point). (b) Zero-*trans* influx of D-glucose into human erythrocyte ghosts at 20°C, K_{zt}^{oi} = 1.56 ± 0.23 mM, V_{zt}^{oi} = 25.5 ± 3.2 mM · min^{-1} (nine experiments).

comparison between whole cells and pink ghosts indicates that influx is rate-limited by the membrane and not by bulk association with haemoglobin within the cell [10]. This does not rule out the possibility that haemoglobin binding to membrane proteins influences their transport properties and more extensive removal of membrane haemoglobin would be needed to test this. Although sugar transport in extensively washed haemoglobin-free ghosts has been studied [11,12], the problem of whether the purified transport system is asymmetric has not been convincingly solved.

Fig. 1 also shows the effect of 3-isobutyl-1-methylxanthine on influx in intact erythrocytes. Inhibition occurs when 3-isobutyl-1-methylxanthine (0.75 mM) is added with the substrate. This inhibition produces mainly a decrease in the V value for entry although there is a 50% increase in the influx K_m (K_{zt}^{oi} = 2.3 ± 0.15 mM, V_{zt}^{oi} = 18.8 ± 0.7 mM · min^{-1}). If cells are preincubated with 3-isobutyl-1-methylxanthine for 1 h before the transport assay then no greater increase in transport inhibition is obtained. We have shown (by measuring the time-dependent increase in absorbance of cell lysates at 273 nm, the λ_{max} of 3-isobutyl-1-methylxanthine) that 3-isobutyl-1-methylxanthine equilibrates across the red cell membrane within 30 min. Hence, after 1 h incubation, 3-isobutyl-1-methylxanthine is inside the cells and this experiment indicates that 3-isobutyl-1-methylxanthine inhibition although noncompetitive is not progressive and not dependent on internal 3-isobutyl-1-methylxanthine. Also, when 3-isobutyl-1-methylxanthine is inside the cells only (Fig. 1), there is no inhibition of entry and this indicates that it must be interacting with the sugar-transport system from the outside only.

Fig. 2a shows the exit of 40 mM glucose in intact erythrocytes and can be compared with the exit in pink ghosts (Fig. 2b). There appears to be no significant change in the exit curve. An integrated rate-equation replot of this data gives the zero-*trans* exit parameters for cells (K_{zt}^{io} = 22.9 ± 6.3 mM, V_{zt}^{io} = 218 ±

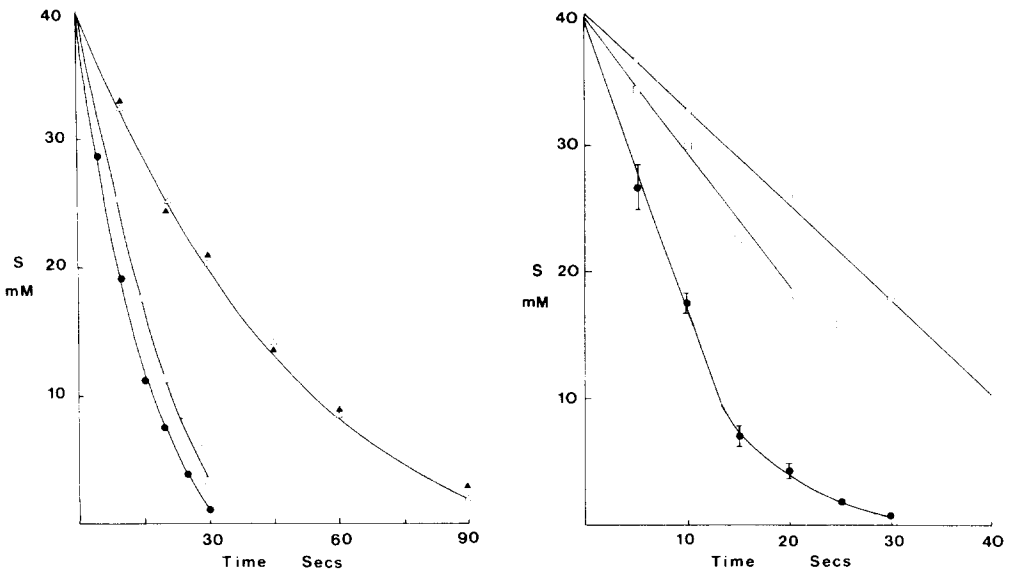


Fig. 2. (a) A time course for the exit of D-glucose (40 mM) at 20°C from human erythrocytes (●) (four experiments) in the presence of 0.75 mM isobutylmethylxanthine inside (□) (two experiments), isobutylmethylxanthine inside and outside (△) (three experiments) and isobutylmethylxanthine outside (▲) (two experiments). (b) A time course for the exit of D-glucose (40 mM) at 20°C from human erythrocyte ghosts (●) (four experiments) in the presence of 0.5 mM isobutylmethylxanthine inside and outside (○) (two experiments) and 5 mM theophylline inside and outside (□) (two experiments).

33.5 mM · min^{-1}) and for ghosts (K_{zt}^{io} = 28.4 ± 10.4 mM, V_{zt}^{io} = 262 ± 59 mM · min^{-1}). The equation used [13,14] is:

$$-\frac{\ln S_t/S}{S-S_t} = \frac{V}{K_M} \cdot (1 + S/\pi) \cdot \frac{t}{S-S_t} - \left(\frac{1}{K_M}(1 + S/\pi) + \frac{1}{\pi}\right)$$

where S is the internal concentration at time zero and S_t is the internal concentration at time t. Karlish et al. [15] obtained a similar K_m for glucose exit, though the reported V was lower than that found in the present investigation. The experiment has been repeated here to provide a direct comparison with the pink-ghost data.

The effect of 3-isobutyl-1-methylxanthine (0.75 mM) on glucose exit is shown in Fig. 2a. 3-Isobutyl-1-methylxanthine inside the cells had virtually no effect on exit, while when present in the exit buffer or inside and outside the cells it produced a marked inhibition of exit. The transformed data (Fig. 3) showed a large decrease in the V value for exit and a small increase in the K_m value (V_{zt}^{io} = 83 ± 11 mM · min^{-1}, K_{zt}^{io} = 32.9 ± 7.3 mM). Further evidence that the lack of inhibition by 3-isobutyl-1-methylxanthine preincubated with the cells for 1 h (3-isobutyl-1-methylxanthine inside) is not due to poor access of 3-isobutyl-1-methylxanthine to internal sites comes from experiments on ghosts where 3-isobutyl-1-methylxanthine can be added directly inside and outside by resealing ghosts in the presence of 3-isobutyl-1-methylxanthine. This treatment produces similar effects on exit to those found in cells (Fig. 2b). Theophylline (1,3-dimethylxanthine) at 5 mM also inhibits glucose exit

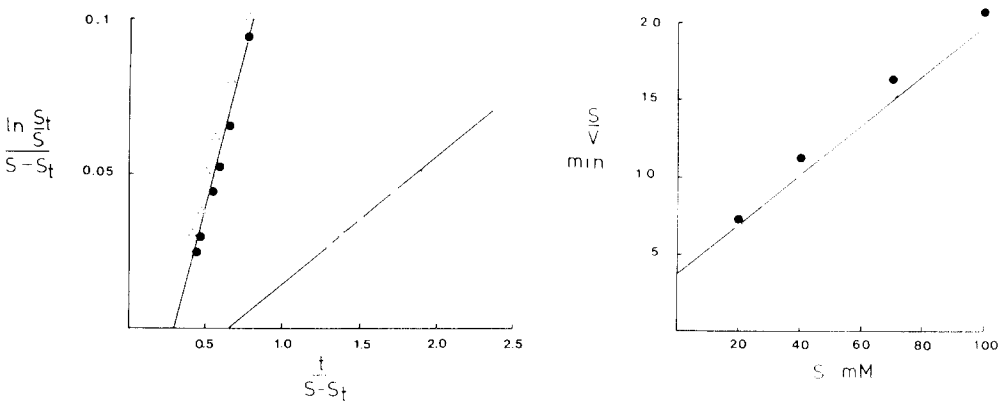


Fig. 3. An integrated rate-equation replot of zero-*trans* glucose exit in erythrocytes (●), K_{zt}^{io} = 22.9 ± 6.3 mM, V_{zt}^{io} = 218 ± 33.5 mM · min^{-1} (four experiments), and in the presence of 0.75 mM isobutylmethylxanthine inside and outside the erythrocytes (○), K_{zt}^{io} = 32.9 ± 7.3 mM, V_{zt}^{io} = 83 ± 11 mM · min^{-1} (three experiments). The integrated rate-equation replot of zero-*trans* glucose exit in ghosts is also shown (△), K_{zt}^{io} = 28.4 ± 10.4 mM, V_{zt}^{io} = 262 ± 59 mM · min^{-1} (four experiments).

Fig. 4. Equilibrium exchange of D-glucose at 20°C in the presence (●) and in the absence ( ) of 0.75 mM isobutylmethylxanthine (three to nine observations at each point).

in ghosts. The ghost-exit data are insufficiently accurate to determine the 3-isobutyl-1-methylxanthine and theophylline inhibition type.

The inhibition of exit appears to be greater than the inhibition of entry. This may be partly due to the difference in methodology necessarily adopted for measuring exit and entry. In entry, the inhibitor-dependent reduction in percentage filling will decrease with time, while in exit the inhibitor-dependent increase in percentage remaining will increase with time. The argument that influx is less inhibited than efflux, because the former process is rate-limited by association with haemoglobin, is inappropriate because of the demonstration of a low V value for entry in erythrocyte ghosts. However, a real asymmetric inhibition is a possibility. This would occur if the inhibitor had a greater tendency to combine with unliganded transport subunits outside than unliganded transport subunits inside. This possibility is considered further in Discussion.

Although net flux is strongly inhibited by 3-isobutyl-1-methylxanthine, there is very little inhibition of exchange flux (Fig. 4) (K_{ee} = 21.5 ± 2.6 mM, V_{ee} = 362 ± 15 mM · min^{-1} in the absence and K_{ee} = 22.5 ± 4.4 mM, V_{ee} = 339 ± 23 mM · min^{-1} in the presence of the inhibitor).

To determine whether cyclic AMP had a direct effect similar to that of methylxanthines on sugar exit, exit of 40 mM glucose was measured at 10 s from cells with and without 3 mM cyclic AMP in the efflux buffer. In the absence of cyclic AMP the decrease in sugar concentration inside was 18.65 ± 1.53 mM in 10 s (n = 3), in the presence of cyclic AMP the decrease in sugar concentration inside was 18.84 ± 0.35 mM in 10 s (n = 3).

Discussion

The experiments presented in this report were carried out to obtain information on the reasons for kinetic asymmetry in net sugar transport.

The asymmetric carrier model proposed by Geck [21] accounts well for the low K_m, low V and the high K_m, high V found in sugar-transport net entry and exit, respectively. It also readily accounts for the high K_m and high V found in equilibrium exchange. We have therefore attempted to account for the asymmetric effect of methylxanthines in terms of this model. The explanation that methylxanthines combine with the free carrier outside and with free carrier inside and hence reduce net sugar flux by a reduction in carrier backflux can be considered.

The net flux equation has been given by Geck [21] and by Stein and Lieb [19]. The effect of 3-isobutyl-1-methylxanthine (Scheme Ia) on this equation is shown in Appendix. Here, it is shown that V and K_m are both affected (but V will be more affected than K_m) by an inhibitor such as 3-isobutyl-1-methylxanthine which may combine with free carrier. Greater inhibition of exit than of entry will be expected if the inhibitor has greater affinity for the carrier outside than for carrier inside (if K_i is greater than K_o). K/V will be increased by this type of inhibitor because K/V for net flux contains resistance terms for return of the free carrier both in the inward and outward direction and these resistances are both increased by the inhibitor. The equation for exchange is also given in Appendix. In the presence of the inhibitor, the V value for exchange is unaffected by the inhibitor because it does not contain terms for carrier backflux. However, K/V for exchange in the presence of the inhibitor should be the same as K/V for net flux in the presence of the inhibitor. The experimental data show that in the absence of the inhibitor $K_{zt}^{io}/V_{zt}^{io} = 0.105$ min while $K_{ee}/V_{ee} = 0.059$ min. If the asymmetric carrier model is to apply then these values should be equal. The problem of inconsistencies in the net and exchange K/V ratios has been noted previously by Ginsburg and Ram [13]. In the presence of 3-isobutyl-1-methylxanthine, the inconsistency is greater ($K_{zt}^{io}/V_{zt}^{io} = 0.397$ min, $K_{ee}/V_{ee} = 0.066$ min). It should be noted that the major objection to the carrier model as an explanation for human erythrocyte sugar transport comes from a consideration of the infinite-*cis* entry K_m and the infinite-*trans* exit K_m for sugars. These experiments give an additional estimate of the internal K_m which should be compatible with the zero-*trans* exit K_m. A K_m at the internal surface which is lower than predicted for the asymmetric carrier has been demonstrated by Hankin et al. [16]. This finding has been supported by Foster et al. [17], Lieb and Stein [18], Ginsburg and Stein [13], Baker and Naftalin [14] and the present authors (unpublished results).

Another model to consider is that proposed by Naftalin and coworkers [4,14]. This model is important because it can account for the asymmetry in net fluxes as well as giving a low K_m in the infinite-*cis* influx experiment. The model proposes that the membrane transport system is a symmetric gating pore and that the operational asymmetry arises because uptake is rate-limited by bulk association with haemoglobin inside the cell. To test this model we have removed 95% of the cell heamoglobin and show that asymmetry is unaffected. Taverna and Langdon [11] have compared sugar uptake into

ghosts with uptake into inverted membrane vesicles using internalised glucose oxidase to measure uptake. They suggested that the sugar-transport system was symmetric. Symmetry in erythrocyte ghosts was also suggested by Benes et al. [22]. Their evidence was based on phloretin inhibition and low-affinity sugar transport at 37°C. Jung et al. [12] also showed sugar transport in erythrocyte ghosts and demonstrated at high K_m for exchange. Jung et al. [12] also pointed out that extensively purified membrane ghosts are much more leaky than intact erythrocytes. We consider that the difference in the extent of leakage accounts for the differences between our results and other results on erythrocyte ghosts. A single lysis step and incomplete removal of haemoglobin was chosen for the experiments reported here as we were able to show that an increased leakage pathway could thus be avoided (see Results). The present experiments demonstrate that the possibility of bulk association of sugar with haemoglobin as a mechanism for asymmetry can be eliminated although, as mentioned in Results, the possibility of an effect of tightly bound residual haemoglobin cannot be eliminated.

An alternative model (an allosteric pore model) which can account for asymmetric net flux and also symmetric infinite-*cis* transport entirely in terms of membrane parameters, has recently been proposed [20]. The model is unfortunately more cumbersome than the carrier model but it may well be important to examine the possibility that some transport systems are kinetically more complicated than other just as some enzymes, particularly control enzymes, are kinetically more complicated than others. The allosteric pore model is based on Koshland's [24] consideration of the allosteric enzyme and the equations relevant to the present experiments are given in Appendix and in Scheme Ib. The model has the following features. The pore can be simultaneously occupied at outside and inside sites. Substrate binding induces a conformational change which opens gates to the pore (interfaces between subunits). Monovalent occupancy of the pore results in low K_m, low V transport parameters because *trans* gates are not fully open. However, monovalent occupancy exposes a high K_m site (negative cooperativity) and if the substrate concentration is sufficiently high, binding to this site can destabilise the *trans* gate and result in a high V for transport. This effect is greater from inside than outside and transport asymmetry (in zero-*trans* experiments) results. The K_m values measured in an infinite-*cis* experiment (the K_m values at the *trans* surface) are low because both internal and external pore gates are maintained in a partially destabilised (opened) state by the high *cis* concentration. Hence, only a low *trans* concentration is required to complete the pore opening process.

An asymmetric effect of inhibitors of transport may be accounted for by this model. If the inhibitor combines preferentially with an external site, but only when the pore is doubly occupied from inside with a vacant site outside, then uncompetitive inhibition of exit may be expected. Both V and K_m for exit will be decreased equally with no effect on entry. Such an effect has recently been reported by Baker and Naftalin [23] who showed that ^2H_2O substitution for H_2O resulted in selective inhibition of net exit of glucose from human erythrocytes.

If an inhibitor is less specific than this (as may be the case for methyl-

xanthines) it may combine with external sites in the pore both when the internal sites are unoccupied and also when the internal sites are occupied by substrate. It may also combine with or inactivate vacant internal sites in the presence and in the absence of substrate bound outside. If the inhibitor affinity for external sites is greater than the inhibitor affinity for internal sites, then greater inhibition of exit than of entry will result. For this less specific type of inhibition the inhibitor may combine with or inactivate internal and external sites simultaneously providing neither site is occupied by substrate. This will result in mixed inhibition kinetics as bound substrate will reduce the number of inhibitor molecules bound per pore and some protection from inhibition will occur at high substrate concentrations.

Both ^2H_2O [23] and the methylxanthines give much greater inhibition of net than of exchange influx. In terms of the allosteric pore, this is because a large fraction of both internal and external sites are simultaneously occupied by substrate at all substrate concentrations in the equilibrium exchange experiment. At low substrate concentrations a low K_m, low V exchange predominates while at high substrate concentrations a high K_m, high V exchange predominates. There is alternation between these conformational states but no return to the empty transport system as an intermediate occurs and therefore there are very few sites with which an inhibitor of this type can combine.

The results presented in this report indicate that transport in human erythrocytes is not rate-limited by bulk association of substrate and haemoglobin at the inner membrane surface. Also, 3-isobutyl-1-methylxanthine is shown to produce mixed inhibition of net sugar fluxes with little effect on exchange flux. This type of inhibition is consistent with the allosteric pore model and consistent with a direct combination of 3-isobutyl-1-methylxanthine with the transport system from the external solution. As human erythrocytes lack adenyl cyclase and phosphodiesterase activities an effect of 3-isobutyl-1-methylxanthine mediated through cyclic AMP is unlikely. In systems with a more active adenyl cyclase and phosphodiesterase, experiments on sugar transport attempting to demonstrate an involvement of cyclic AMP by the use of phosphodiesterase inhibitors may have to be treated with caution.

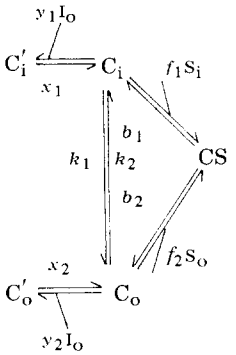
Appendix

Analysis of the asymmetric carrier was carried out by Geck [21] and Stein and Lieb [19]. They showed that the net efflux equation is:

$$u_{io}^{zt} = \frac{T \cdot S_i}{KR_{oo} + R_{12}S_i}$$

where S_i is the substrate concentration inside, $K = [(k_1/f_1) + (k_2/f_2)]$, $R_{oo} = [(1/k_1) + (1/k_2)]$, $R_{12} = [(1/b_2) + (1/k_2)]$ and T is the total carrier. k_1 is the rate constant for carrier movement from inside to outside, k_2 is the rate constant for carrier movement form outside to inside, b_1 and b_2 are the rate constants for the breakdown of the carrier substrate complex inside and outside, respectively, and f_1 and f_2 are the rate constants for the formation of carrier substrate complex inside and outside, respectively [19].

(a)



(b)

$$A\,3 + I_o \overset{K_o}{\rightleftharpoons} A\,2\,I$$

$$A\,3 + I_o \overset{K_i}{\rightleftharpoons} A\,2\,I$$

$$A\,3 + 2\,I_o \overset{K_o K_i}{\rightleftharpoons} AI\,2$$

$$A\,2\,B + I_o \overset{K_i}{\rightleftharpoons} ABI$$

$$A\,2\,C + I_o \overset{K_o}{\rightleftharpoons} ACI$$

$$AB\,2 + I_o \overset{K_i}{\rightleftharpoons} B\,2\,I$$

$$AC\,2 + I_o \overset{K_o}{\rightleftharpoons} C\,2\,I$$

Scheme I (a) The carrier model with free carrier inside and free carrier outside combining with inhibitor outside; $K_i = x_1/y_1$, $K_o = x_2/y_2$. (b) The allosteric pore model with subunits simultaneously available to substrate at internal and external surfaces. The accessibility of a third subunit is dependent on a substrate-induced conformational change and is not available to the inhibitor. A subunits are unoccupied, B subunits are occupied by substrate from outside and C subunits are occupied by substrate from inside. Inhibitor outside can block one A subunit outside in A3, A2C and AC2 pores with dissociation constant K_o. Inhibitor outside can block one A subunit inside in A3, A2B and AB2 pores with dissociation constant K_i. There are no subunits accessible to inhibitor in ABC, B2C and C2B pores (those forms of the pore predominantly involved in exchange) because outside and inside sites are simultaneously occupied by substrate. The possibility that substrate can change the affinity for inhibitor binding on the other side of the membrane is considered to be insignificant in the case of methylxanthines.

$$V = T \cdot (1/R_{12})$$

$$K_m = \frac{KR_{oo}}{R_{12}}$$

$$K/V = KR_{oo}/T$$

In the presence of 3-isobutyl-1-methylxanthine (Scheme Ia):

$$u_{io}^{\prime t} = \frac{T \cdot S_i}{K\left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{k_1}(1 + I/K_i)\right) + \left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{b_2}\right) S_i}$$

Where I is the 3-isobutyl-1-methylxanthine concentration and K_i is the inhibitor-carrier inside dissociation constant and K_o is the inhibitor-carrier outside dissociation constant.

$$V = T \cdot \left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{b_2}\right)$$

$$K_m = \frac{\left(K\,\frac{1}{k_2}(1 + I/K_o) + \frac{1}{k_1}(1 + I/K_i)\right)}{\left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{b_2}\right)}$$

$$K/V = K\left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{k_1}(1 + I/K_i)\right)/T$$

For exchange:

$$u_{io} = \frac{T \cdot S_i}{KR_{oo} + R_{ee}S_i}$$

where

$$R_{ee} = \left(\frac{1}{b_1} + \frac{1}{b_2}\right),$$

$$V = T \cdot (1/R_{ee}),$$

$$K_m = \frac{KR_{oo}}{R_{ee}}$$

and

$$K/V = KR_{oo}/T$$

In the presence of 3-isobutyl-1-methylxanthine:

$$u_{io} = \frac{T \cdot S_i}{K\left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{k_1}(1 + I/K_i)\right) + R_{ee} \cdot S_i}$$

$$V = T \cdot (1/R_{ee})$$

$$K_m = \frac{K\left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{k_1}(1 + I/K_i)\right)}{R_{ee}}$$

$$K/V = K\left(\frac{1}{k_2}(1 + I/K_o) + \frac{1}{k_1}(1 + I/K_i)\right)/T$$

The kinetic analysis of the allosteric pore has recently been given [20]. Net efflux under zero-*trans* exit conditions is:

$$u_{io}^{zt} = \frac{T(kS_i + k_1S_i^2 \cdot K_{ii})}{1/K_x + S_i + S_i^2 K_{ii}}$$

where k is the rate constant for translocation through a singly occupied pore and k_1 is the rate constant for translocation through the pore doubly occupied from inside. K_x is the association constant for binding of substrate to an isolated subunit times the equilibrium constant for the transition of an unoccupied subunit into an occupied subunit. K_{ii} is K_x times the internal subunit interface stability constant. The interfaces between subunits can be regarded as gates to the pore.

If it is assumed that 3-isobutyl-1-methylxanthine binds to accessible unoccupied subunits within the pore (Scheme Ib) the equation becomes:

$$u_{io}^{zt} = \frac{T(kS_i + k_1S_i^2K_{ii})}{\frac{1}{K_x}\left(1 + \frac{I}{K_i} + \frac{I}{K_o} + \frac{I^2}{K_iK_o}\right) + S_i\left(1 + \frac{I}{K_o}\right) + S_i^2K_{ii}\left(1 + \frac{I}{K_o}\right)}$$

with a similar equation for entry except that the subscripts i and o are reversed.

In the case of ^2H_2O, it is assumed that inhibitor binds only in the presence of substrate inside and only to pore sites which are doubly occupied from inside and have a vacant site outside:

$$u_{io}^{zt} = \frac{T \cdot (kS_i + k_1 S_i^2 K_{ii})}{\frac{1}{K_x} + S_i + S_i^2 K_{ii}\left(1 + \frac{I}{K_o}\right)}$$

In the case of 3-isobutyl-1-methylxanthine, the percentage inhibition will be less as the substrate concentration is increased. The inhibition type will be mixed with the major effect on V. In the case of ^2H_2O, the percentage inhibition will rise as the substrate concentration is increased. The K_m and V will be reduced to that of the singly occupied pores (low K_m, low V) and the inhibition type will be uncompetitive. The exchange equation for the allosteric pore has been previously given [20]. In the presence of the inhibitors the denominator of the flux equation has the same terms as those given above but with additional terms due to the exchange components. It is these exchange components which predominate and these are unaffected by inhibitor.

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