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ASYMMETRIC BINDING OF STEROIDS TO INTERNAL AND EXTERNAL SITES IN THE GLUCOSE CARRIER OF ERYTHROCYTES

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Summary

Steroids inhibit glucose transport in erythrocytes by binding to sites in the carrier which are exposed on both the outer and inner surfaces of the cell membrane. Some steroids are bound almost exclusively at inner sites (androstendione and androstandione), while others are bound about as firmly on one side as the other (corticosterone). Still others exhibit a moderate preference for the internal site (deoxycorticosterone). The inhibition is in all cases competitive with respect to a substrate which is bound at the same surface of the membrane as the inhibitor. However, in experiments on substrate entry, internally bound inhibitors act in an apparently non-competitive fashion, as expected if the carrier model is valid. This behaviour explains the appearance of competitive, non-competitive and mixed inhibitions with different steroids (Lacko, L., Wittke, B. and Geck, P. (1975) J. Cell Physiol. 86, 673–680).

Introduction

A number of steroids are potent, reversible inhibitors of glucose transport in erythrocytes, and what is remarkable is that their mechanism appears in some cases to be competitive and in other cases non-competitive [1]. The interpretation given by Lacko et al. [1], who reported these puzzling observa-

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tions, was that the carrier must possess two separate steroid binding sites. One of these overlaps the substrate site, and here the inhibition is competitive. The other is located outside the substrate site, allowing both the inhibitor and the substrate to add to the carrier at the same time. Steroids were assumed to differ in their relative affinities for the two sites, depending on their structure, some being bound predominantly at one site, and some predominantly at the other, with the result that the inhibition could be either competitive or non-competitive, or mixed if there is binding at both.

A far simpler explanation for these observations, however, has been overlooked [2]. We note that the substrate site in a carrier must in the course of transport be presented to both the external and internal surfaces of the cell membrane, in order to absorb the substrate on one side and later release it on the other. In consequence, an inhibitor which adds to the substrate site may do so at either of the two membranes faces, depending both upon the location of the inhibitor and upon its relative affinity for the substrate site or an associated inhibitor site as these appear on the inner and outer surfaces of the membrane. If the substrate is present in the suspending medium and its rate of entry into the cell is observed, and if an inhibitor is bound only at the external site, then the behaviour is simple and the inhibition is purely competitive. However, if the inhibitor binds on both the inner and outer surfaces of the cell membrane, the inhibition will be non-competitive, because the substrate in the external pool will not be in a position to compete at the internal site. The pattern of inhibition produced by a particular steroid would, if this interpretation applies, depend on its ability to become bound at the inner and outer sites. As steroids are known to penetrate the cell membrane (see for example Refs. 3 and 4), such an explanation for non-competitive inhibition is certainly feasible and ought to be tested. Should the mechanism prove to be correct, it carries with it certain implications for the physiological control of transport which are not found in the earlier interpretation. The reason is as follows. Where an inhibitor is bound, in an essentially competitive mechanism, on only one side of the membrane, the transport system acquires the properties of a specific valve, which allows the substrate to traverse the membrane more rapidly in one direction than in the other [5], and such a valve could function in regulating cell and plasma glucose levels.

Tests of asymmetric binding

A simple test for inhibitor binding at the inner and outer carrier sites in this system, as demonstrated earlier [6], is the relative inhibition of glucose and xylose efflux from cells preincubated with either sugar. The test depends on the unequal affinities of glucose and xylose for the carrier, their measured binding constants being 2.3 and 11.8 mM, respectively, in infinite *cis* exit experiments at 25°C [7]. On account of this difference the ability of the two substrates, when present inside the cell, to compete with an internal inhibitor differs. On the other hand, neither sugar, if present inside the cell and not outside, can compete with externally bound inhibitor. The relative effect on glucose and xylose exit is therefore a measure of the relative affinities of an inhibitor for carrier on the inner and outer faces of the membrane. For

example if the two sugars are affected equally, then either (i) the inhibitor is bound only on the outer surface of the membrane, or (ii) the mechanism is truly non-competitive. These alternatives are easily distinguished by varying the external substrate concentration, for example in the infinite *cis* net exit experiment of Sen and Widdas [8]. On the other hand if xylose exit is more inhibited than exit of glucose, then competitive binding of the inhibitor on the inner surface is indicated.

In interpreting experiments of this kind it is important to have reference inhibitors whose behaviour is understood. These are available. Reversible competitive inhibitors which do not enter the cell, such as maltose [9] and phloretin [10], could only bind at the external surface, while cytochalasin B was shown to enter the cells and bind only on the inner surface of the membrane, even though it was dissolved in the suspending medium [6,11]. These inhibitors therefore provide the reference points of exclusive binding either internally or externally.

Kinetic theory

The general rate equation for zero *trans* exit of substrate from preloaded cells may be written directly from the general equations derived previously [12] for the transport of a substrate by a system whose kinetic behaviour is that of the familiar carrier mechanism:

$$v = \frac{\bar{V}_{S_i}}{1 + \frac{[I_o]}{\tilde{K}^S_{I_o}} + \frac{\bar{K}_{S_i}}{[S_i]}\left(1 + \frac{[I_o]}{\bar{K}_{I_o}} + \frac{[I_i]}{\bar{K}_{I_i}}\right)} \tag{1}$$

where $[I_o]$ and $[I_i]$ are the external and internal inhibitor concentrations, and $\bar{K}_{I_o}$, $\bar{K}_{I_i}$ and $\tilde{K}^S_{I_o}$ are experimental inhibition constants * for the addition of inhibitor externally or internally, as denoted by the subscripts I_o and I_i, respectively; $[S_i]$ is the internal substrate concentration. From Eqn. 1 the ratio of rates in the absence of an inhibitor and in its presence is given by:

$$\frac{v_{(-I)}}{v_{(+I)}} = 1 + \frac{[I_o]\left(\frac{1}{\tilde{K}^S_{I_o}} + \frac{\bar{K}_{S_i}}{\bar{K}_{I_o}[S_i]}\right) + [I_i]\frac{\bar{K}_{S_i}}{\bar{K}_{I_i}[S_i]}}{1 + \bar{K}_{S_i}/[S_i]} \tag{2}$$

If we assume that in the absence of inhibitor the substrate concentration is saturating ($\bar{K}_{S_i}/[S_i] << 1$ and $\tilde{K}^S_{I_o}\bar{K}_{S_i}/\bar{K}_{I_o}[S_i] << 1$) then Eqn. 2 yields

$$\frac{v_{(-I)}}{v_{(+I)}} - 1 = \frac{[I_o]}{\tilde{K}^S_{I_o}} + \frac{[I_i]\bar{K}_{S_i}}{\bar{K}_{I_i}[S_i]} \tag{3}$$

For two substates X and G (standing for xylose and glucose) with different affinity constants $\bar{K}_{X_i}$ and $\bar{K}_{G_i}$, the ratio of inhibitions is given by

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* The full definitions of these constants were given before [12]. $\bar{K}_{I_o}$ and $\bar{K}_{I_i}$ are determined in the absence of substrate in the compartment which is *trans* with respect to the inhibitor, and $\tilde{K}^S_{I_o}$ with a saturating concentration of substrate in the *trans* compartment.

$$R = \frac{\left(\frac{v_{(-I)}}{v_{(+I)}} - 1\right)_X}{\left(\frac{v_{(-I)}}{v_{(+I)}} - 1\right)_G} = \frac{\frac{[I_o]}{\tilde{K}^X_{I_o}} + \frac{[I_i]\bar{K}_{X_i}}{\bar{K}_{I_i}[X_i]}}{\frac{[I_o]}{\tilde{K}^G_{I_o}} + \frac{[I_i]\bar{K}_{G_i}}{\bar{K}_{I_i}[G_i]}} \tag{4}$$

In the case of phloretin, which can bind only on the external surface of the membrane, R is found from Eqn. 4 to be

$$R_{(I_o)} = \tilde{K}^G_{I_o}/\tilde{K}^X_{I_o} \tag{5}$$

Cytochalasin B adds only at the internal surface and with such an inhibitor, assuming that the xylose and glucose concentrations are equal, the ratio R is

$$R_{(I_i)} = \bar{K}_{X_i}/\bar{K}_{G_i} \tag{6}$$

If an inhibitor permeates through the cell membrane, so that $[I_o] = [I_i]$, a measure of its relative affinity on the outer and inner sufaces of the membrane may be calculated for any given value of R by the use of Eqns. 4—6. In order to derive the required relationship, the numerator and denominator of the left-hand side of Eqn. 4 are multiplied by $\tilde{K}^G_{I_o}$, yielding

$$R = \frac{\frac{\tilde{K}^G_{I_o}}{\tilde{K}^X_{I_o}} + \frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}} \cdot \frac{\bar{K}_{X_i}}{[X_i]}}{1 + \frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}} \cdot \frac{\bar{K}_{G_i}}{[G_i]}} = \frac{R_{(I_o)} + \frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}} \frac{\bar{K}_{X_i}}{[X_i]}}{1 + \frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}} \frac{\bar{K}_{G_i}}{[G_i]}} \tag{7}$$

Cross-multiplying Eqn. 7 and gathering the terms in $\tilde{K}^G_{I_o}/\bar{K}_{I_i}$ then gives

$$R - R_{I_o} = \frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}}\left(\frac{\bar{K}_{X_i}}{[X_i]} - \frac{R\bar{K}_{G_i}}{[G_i]}\right) = \frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}}\left(\frac{\bar{K}_{X_i}}{\bar{K}_{G_i}}\frac{[G_i]}{[X_i]} - R\right)\frac{\bar{K}_{G_i}}{[G_i]} \tag{8}$$

Since under the conditions of the experiment $[X_i]$ is to be made equal to $[G_i]$, Eqn. 8 may be rewritten as

$$\frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}}(R_{(I_i)} - R)\frac{\bar{K}_{G_i}}{[G_i]} = R - R_{I_o} \tag{9}$$

The ratio of external and internal affinities is now given by

$$\frac{\tilde{K}^G_{I_o}}{\bar{K}_{I_i}} = \frac{[G_i]}{\bar{K}_{G_i}}\left(\frac{R - R_{(I_o)}}{R_{(I_i)} - R}\right) \tag{10}$$

The values of the binding constant for glucose in zero *trans* exit experiments ($\bar{K}_{G_i}$) has been reported in the literature, and since $R_{(I_o)}$ and $R_{(I_i)}$ are also experimental constants (Eqns. 5 and 6), $\tilde{K}^G_{I_o}/\bar{K}_{I_i}$ may be estimated.

Experimental methods

Rates of sugar exit from preloaded cells were determined by the light-scattering technique, as previously described [7]. The volume of the assay solution was 25 ml and the temperature was maintained at 25°C. The assay medium

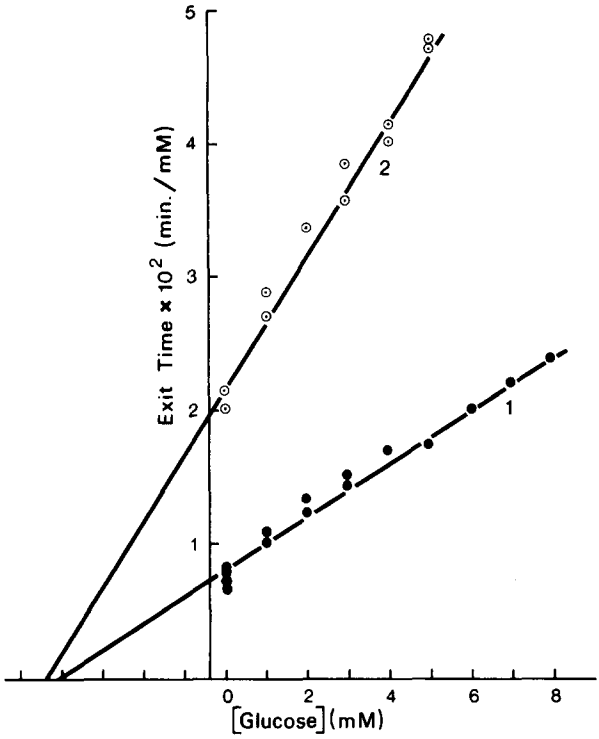

Fig. 1. Infinite *cis* net exit experiment with 117 mM internal glucose and varying concentrations of external glucose, showing inhibition by 73 μM androstandione present in the assay solutions, together with 0.2% ethanol. The lines drawn through the experimental points were calculated by a least-squares treatment. See Table II for analysis.

contained 17 mM sodium phosphate, pH 7.5, made isotonic (310 mosM) by the addition of NaCl. The inhibitors were dissolved beforehand in the assay medium, which in some cases contained a small volume of ethanol (0.2—1.0%), as indicated in Figs. 1—4. Substrate exit began with the injection of 50 μl of a 20% cell suspension, previously loaded with substrate, into the assay medium.

In experiments with 1,2-*O*-isopropylidene-D-glucofuranose, the cells were preincubated as usual at 37°C for 1 h with xylose or glucose, and then sufficient isopropylidene glucose was dissolved in the suspension to produce the same final concentration as was used in the assay medium (75 mM). Incubation of this suspension continued for 1 h at 37°C before measurement of glucose or xylose efflux.

All chemicals were of reagent grade, and water was glass distilled. The steroids were obtained from Sigma Chem. Co. Blood was obtained from an outdated blood-bank supply.

Results and Discussion

The experimental values of R found for a number of inhibitors are listed in Table I. As expected, all the steroids produce R values which lie within the

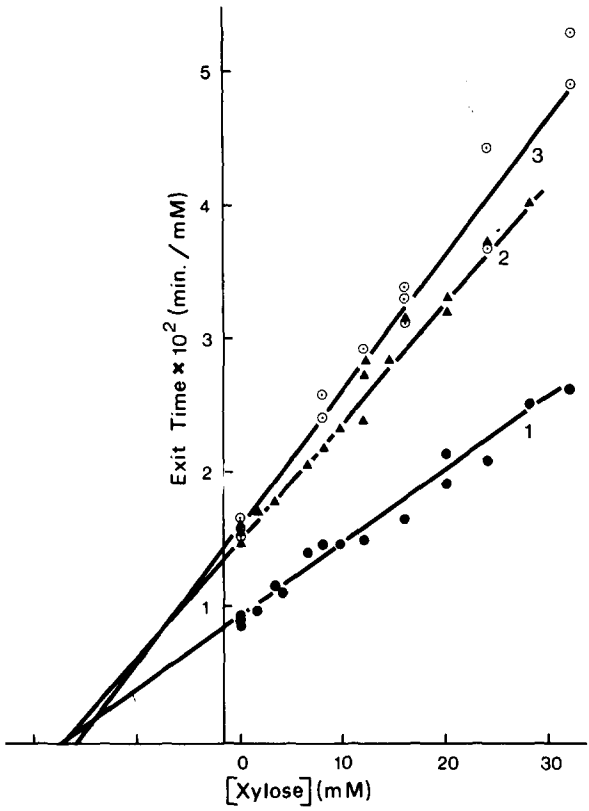

Fig. 2. Infinite *cis* exit experiment with 117 mM internal xylose and varying concentrations of external xylose. (1) Control with 1% ethanol; (2) 16 μM androstendione with 1% ethanol; (3) 24 μM androstandione. Control in the absence of ethanol is not shown. See Table II.

TABLE I

RELATIVE INHIBITIONS OF XYLOSE AND GLUCOSE EXIT

As given by R (Eqn. 4). With cytochalasin B, which is bound only on the internal surface of the membrane [6, 11], $R = R_{(I_i)} = 2.98 \pm 0.04$ (Eqn. 6). With an inhibitor bound on the external surface only (maltose [9], phloretin [10] and phlorizin), $R = R_{(I_o)}$ has an average value of 0.93 ± 0.04 (Eqn. 5). The estimated ratio of affinities on the internal and external surfaces, $\tilde{K}^G_{I_o}/\bar{K}_{I_i}$, is calculated from Eqn. 10, on the assumption that the internal half-saturation constant for glucose, $\bar{K}_{G_i}$, is 14 mM (see text).

Inhibitor	Half-saturation concentration in xylose assay (μM)	R	Estimated $\tilde{K}^G_{I_o}/\bar{K}_{I_i}$
Cytochalasin B	0.28 ± 0.003	2.98 ± 0.04	>100
Androstendione	24 ± 4	2.95 ± 0.43	>100
Androstandione	18 ± 2	2.64 ± 0.49	40
5-β-Androstane-3,17-dione	71 ± 5	1.84 ± 0.21	6.4
Deoxycorticosterone	38 ± 3	1.68 ± 0.23	4.5
Testosterone	43 ± 4	1.40 ± 0.19	2.4
Hydrocortisone	284 ± 60	1.38 ± 0.18	2.3
Cortisone	292 ± 15	1.35 ± 0.15	2.0
Corticosterone	88 ± 5	1.17 ± 0.15	1.1
1,2-*O*-Isopropylidene-D-glucofuranose	45 ± 3 (mM)	1.14 ± 0.14	0.9
Phlorizin	132 ± 5	0.98 ± 0.07	0
Phloretin	0.56 ± 0.025	0.90 ± 0.08	0
Maltose	13.1 ± 0.5 (mM)	0.91 ± 0.07	0

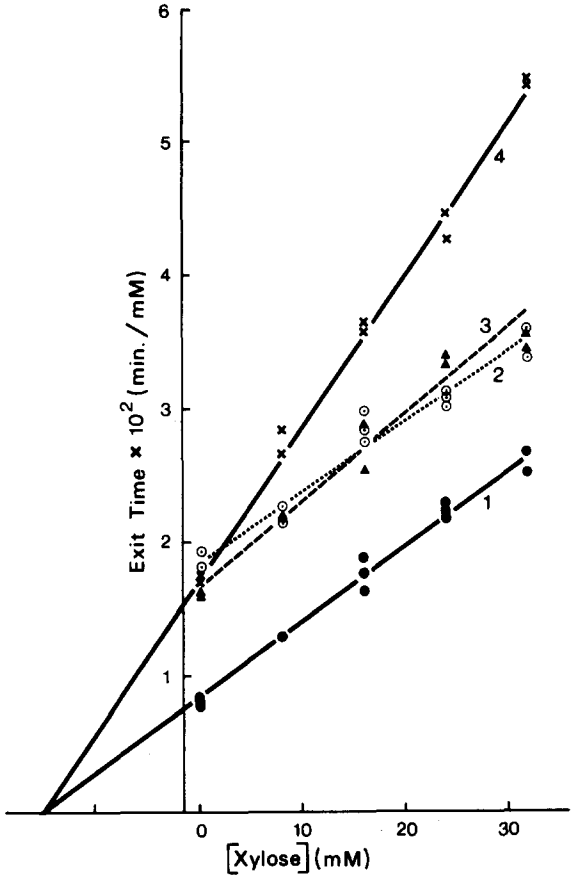

Fig. 3. Infinite *cis* exit experiment with xylose, as in Fig. 2. (1) Control, with 0.25% ethanol; (2) 100 μM corticosterone with 0.25% ethanol; (3) 290 μM hydrocortisone with 0.25% ethanol; (4) 40 μM deoxycorticosterone.

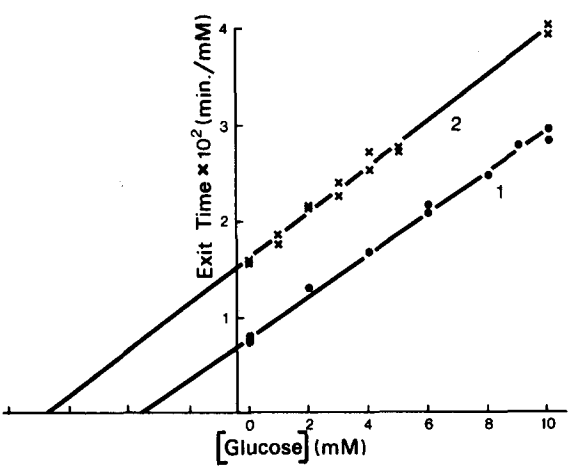

Fig. 4. Infinite *cis* exit experiment with glucose, as in Fig. 1. Lower line, control; upper line, exit times in the presence of 49 μM deoxycorticosterone; 0.2% ethanol in both cases.

limits of $R_{(I_i)}$ and $R_{(I_o)}$. The former is the experimental value for cytochalasin B, which as noted above binds only to the internal carrier form, and the latter is the value for phloretin, phlorizin or maltose, which add only to the outer form of the carrier.

The steroids previously reported to act non-competitively in infinite *trans* entry experiments [1] are now seen to inhibit the exit of xylose more than glucose, showing that they are in fact bound competitively; they add, however, on the inner rather than outer surface of the membrane, and this makes their inhibition of entry non-competitive. The highest value of R is found for androstendione, and is very close to that for cytochalasin B. This steroid must therefore be bound largely on the inner surface of the membrane. Near the other extreme is corticosterone with a value higher, but not much higher, than that for phloretin; corticosterone must therefore add at the outer site. The glucose analogue 1,2-*O*-isopropylidene-D-glucose has a similar value. The other steroids have binding ratios between these, so that some, such as deoxycorticosterone, add more readily to the inner site, but are not exclusively bound there.

The ratio of affinities for the internal and external carrier forms is given roughly in the last column in Table I. These estimates depend not only upon R, which is determined in the present experiment, but upon the affinity constant for internal glucose, $\bar{K}_{G_i}$. This constant was reported by Miller [13] to be 7.4 ± 1.4 mM, and by Karlish et al. [14] to be 25.4 ± 6.7 mM, both at 20°C. The reason for the disagreement is not known, and we have used an intermediate value of 14 mM which arbitrarily is the mean of the lower figure plus 1 S.D. and the upper figure minus 1 S.D. As the internal concentration of sugar in our experiments was 117 mM, it follows that very approximately $[G_i]/\bar{K}_{G_i} = 8$. Obviously little confidence can be placed in the precise value given in Table I for $\tilde{K}^G_{I_o}/\bar{K}_{I_i}$, but the ratios should indicate trends in the relative affinities of different steroids for the carrier exposed on the inner surface of the membrane.

These conclusions about the relative affinities of different steroids lead to certain expectations about the type of inhibition they should produce in infinite *cis* exit experiments, where the external substrate concentration is varied. In such experiments the net rate of substrate exit from cells loaded with a saturating concentration of substrate is measured in the presence of relatively low, and variable concentrations of substrate in the surrounding medium [8]. If binding is exclusively inside, the inhibition should be non-competitive, and if exclusively outside, purely competitive [2,6]. Where the inhibitor binds on both sides of the membrane a mixture of competitive and non-competitive inhibitions may be seen. It must be remembered, however, that in this experiment the inhibitor bound internally competes with a high concentration of substrate, while the inhibitor bound externally competes with a very low concentration. Internal binding should therefore go undetected unless it is considerably stronger than external binding. We expect the inhibitors which are bound far more firmly to the inner than to the outer carrier form to exhibit non-competitive behaviour in such an experiment, and inhibitors which are bound as strongly on the outside as on the inside to inhibit competitively.

The expectations are realized. Thus the inhibitors which, judged by their effects on the exit of internal glucose and xylose, are competitive (androstendione and androstandione), are seen to be non-competitive with respect to external substrate (Figs. 1 and 2); while the steroids that were most nearly non-competitive in their effects on the exit of glucose and xylose, that is, those that inhibited nearly equally despite the difference in affinity of the two sugars, are found to be competitive with external substrate, as in the cases of corticosterone and hydrocortisone (Fig. 3).

Deoxycorticosterone was found to inhibit competitively when glucose was the substrate (Fig. 4), but to inhibit non-competitively with xylose (Fig. 3). These striking observations are consistent with the ideas developed here. The figures in Table I indicate that this steroid has significantly greater affinity for the inner than for the outer carrier form, but inhibition at the inner site is presumably overcome by competition with internal glucose, which has a relatively high affinity for the carrier. The inhibition seen in the experiment with glucose is for this reason the result of deoxycorticosterone addition at the outer surface of the cell membrane, and in consequence is competitive. Xylose on the other hand has considerably lower affinity for the carrier than glucose, and therefore does not overcome inhibition at the internal site. Hence in the experiment with xylose, the inhibition is non-competitive.

A summary of the inhibition patterns seen in infinite *cis* exit experiments is given in Table II. The observed patterns are consistent with the predictions given in Table I regarding relative affinities for the inner and outer carrier forms.

In the experiments described above, the cells were not preincubated with the steroids, and first came into contact with the inhibitors when the exit rates were measured. It seemed possible that the steroids might require a period of time to enter the cell, during which internal binding, and inhibition, would increase. This possibility was tested by preincubating glucose- and xylose-loaded cells with steroids at 25°C for periods of 0.5 h or longer. After an initial incubation period of about 10 min with an inhibitor, the cells were sedimented and resuspended in the same solution of substrate plus inhibitor. Exit rates were determined in salt solution and in a solution containing the inhibitior at the same concentration as in the preincubation. Tests were carried out with testosterone, corticosterone, deoxycorticosterone, and androstendione. In no case was the inhibition increased by preincubation, and no inhibition was observed when cells preincubated with an inhibitor were assayed in salt solution. We conclude therefore that penetration must be very rapid.

In the report by Lacko and coworkers [1] inhibition was determined in infinite *trans* entry experiments, in which cells were preloaded with 200 mM glucose and then placed in a solution of radioactive glucose at various concentrations, and the rate at which the latter entered the cell was measured. In the present experiments the net efflux from cells containing 117 mM sugar is observed in the presence of relatively low, and varied, concentrations of substrate in the suspending medium. Though these experiments do not give the rate of the same process, they are essentially alike in that they involve a high and unvaried internal substrate concentration and a low and varied external concentration, and the dependence on the sidedness of inhibitor addition is

TABLE II

THE NATURE OF THE INHIBITION PRODUCED BY VARIOUS INHIBITORS IN SEN-WIDDAS EXPERIMENTS (COMPETITIVE, NON-COMPETITIVE OR MIXED)

The indicator of the type of inhibition (second last column) is equal to unity in pure competitive inhibition and to zero in pure non-competitive inhibition; in mixed competitive and non-competitive inhibition it lies between zero and one. This indicator is calculated from intersection points on the horizontal and vertical axes (third and fourth columns) in Sen-Widdas plots (see Figs. 1—4). The rationale is as follows. In competitive inhibition the straight lines drawn through the experimental points determined in the presence and absence of inhibitor are parallel, and in consequence the ratio of intercepts on the two axes are equal. In non-competitive inhibition the two lines intersect on the horizontal axis. The indicator of the type of inhibition is calculated from the following formula:

$$\text{Indicator} = \frac{\text{Ratio (abscissa)} - 1}{\text{Ratio (ordinate)} - 1}$$

Inhibitor	Substrate	Experimental ratio		Indicator of type of inhibition	Type
		Abscissa	Ordinate		
Cytochalasin B	Glucose	0.95 ± 0.01	1.88 ± 0.11	≈0	Non-competitive *
	Xylose	0.72 ± 0.02	1.68 ± 0.05	≈0	Non-competitive
Androstendione	Xylose	1.00 ± 0.02	1.63 ± 0.09	≈0	Non-competitive
Androstandione	Glucose	1.08 ± 0.02	2.72 ± 0.14	0.05 ± 0.01	Non-competitive
	Xylose	1.12 ± 0.03	1.70 ± 0.15	0.18 ± 0.05	Non-competitive
Deoxycorticosterone	Glucose	1.87 ± 0.03	2.06 ± 0.09	0.82 ± 0.07	Mixed (mainly competitive)
	Xylose	0.97 ± 0.01	1.99 ± 0.09	≈0	Non-competitive
Testosterone	Glucose	1.88 ± 0.06	2.82 ± 0.22	0.47 ± 0.06	Mixed
Corticosterone	Xylose	1.79 ± 0.05	2.03 ± 0.02	0.76 ± 0.05	Mixed (mainly competitive)
Hydrocortisone	Xylose	1.77 ± 0.04	1.99 ± 0.13	0.78 ± 0.11	Mixed (mainly competitive)
Phloretin	Glucose			≈1.0	Competitive **
Maltose	Glucose			≈0.1	Competitive **

* Ref. 6.
** Ref. 7.

the same [2]. The only significant difference is that the internal glucose concentration was higher in the experiments of Lacko and his coworkers, and as a result the internal component of inhibitor binding should be less evident than in our experiments, because of competition from the substrate. In agreement with this, deoxycorticosterone, corticosterone and hydrocortisone are reported to be competitive by these workers, but exhibit a small non-competitive component (though mainly competitive) in our experiments (see Table II). In both cases androstendione and androstandione produce purely non-competitive inhibition.

Finally, a word may be said about the half-saturation constants for the inhibitors, listed in Table I. Where the inhibitor adds only on the external surface of the membrane, this constant is a true measure of the inhibition constant $\tilde{K}_{I_o}^{S}$ [12], but whatever component of binding involves the inner surface will be masked by competition from the high internal substrate concentration,

as noted, and the constants will therefore underestimate the true strength of internal binding. With this in mind, the constants in Table I, measured with a substrate of relative low affinity, xylose at 117 mM, may be compared with the constants reported by Lacko and coworkers [1], which were determined with a substrate of higher affinity present at a higher concentration: glucose at 200 mM. With the most nearly competitive of the steroids (in infinite *cis* net exit experiments or in infinite *trans* entry experiments) the reported inhibition constants are similar. On the other hand with androstendione and androstandione the constants given by Lacko and coworkers are between 100 and 200 μM, while we find them to be approx. 20 μM. These differences are expected, and the true values must actually be several times lower than this figure of 20 μM. In view of the bias in the estimates of affinity, which varies with different steroids, any attempted correlation of inhibition with the affinity of the steroids for membrane lipids [1] must be viewed with caution.

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