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CYTOCHALASIN B AND THE KINETICS OF INHIBITION OF BIOLOGICAL TRANSPORT

A CASE OF ASYMMETRIC BINDING TO THE GLUCOSE CARRIER

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Summary

Cytochalasin B inhibits glucose transport in human erythrocytes by competing with glucose for the carrier on the inner surface of the cell membrane, but there is no cytochalasin site associated with the outward-facing form of the carrier. Such asymmetry may be demonstrated by zero trans exit and entry experiments, whereas Sen-Widdas exit experiments are not easily interpretable. The orientation of the transport system appears to be reversed in certain other cell types: chick embryo fibroblasts, Novikoff hepatoma cells and HeLa cells. Here the cytochalasin site is present in the external but not internal carrier form.

Cytochalasin B powerfully inhibits glucose flux in human erythrocytes. The mechanism by which it does so has been dispute. In entry [1] and in Sen-Widdas exit [1,2] experiments clear evidence of non-competitive inhibition has been presented. However, in studies with isolated membranes, the binding of cytochalasin B and glucose was demonstrated to be competitive [3]. Preliminary reports on equilibrium exchange flux claimed to show that here too the inhibition is of a competitive nature [4,5]. These apparent discrepancies suggested the need for a general kinetic analysis of reversible inhibition of transport [6]. From such an analysis, which was based on a kinetic model of a classical carrier type, several rules emerged: (i) a competitive mechanism can give rise to non-competitive kinetics, but not vice versa; (ii) the only unambiquous test distinguishing competitive from non-competitive mechanisms is equilibrium exchange. Accordingly the underlying mechanism of the inhibition is probably competitive.

The above observations could be taken to suggest, as Basketter and Widdas

[5] did, that cytochalasin B is exclusively bound on the inner surface of the cell membrane. This is not a necessary conclusion however, and all the results could be explained if the inhibitor has appreciable affinity on both surfaces (see below). In a brief reference to experimental strategies related to the problem of asymmetric inhibitor binding, Basketter and Widdas advocated the use of both equilibrium exchange and Sen-Widdas exit experiments. In this respect both these experiments are equivocal, whereas zero trans exit and entry experiments lend themselves to a straightforward interpretation [6]. For example if an inhibitor binds only to an inner carrier form, influx is inhibited in a non-competitive manner but efflux purely competitively. The latter observation would show that the inhibitor cannot be bound to the outward facing form of the carrier. Other experiments are of little value in establishing the symmetry of inhibitor binding. The basis for these assertions is discussed more fully below.

We now report efflux experiments on glucose and on a substrate of low affinity, xylose, which show that cytochalasin B is indeed bound only to the carrier form on the inner surface of the erythrocyte membrane. These results also provide a simple test for asymmetric inhibitor binding in this system, namely relative inhibitions of glucose and xylose exit. Application of the kinetic theory to other observations reported in the literature shows that depending on the cell type cytochalasin B may bind exclusively to either the inner or outer carrier forms. In other words the orientation of the binding site may be reversed in some tissues.

Materials and Methods

Human blood was obtained from an outdated blood bank supply. Two methods were employed to determine initial rates of sugar exit from loaded cells. One depended on changes in light scattering in the course of osmotic swelling and shrinking of cells, as previously described [7]. The exit time was the time required to achieve equilibrium calculated from the initial rate of sugar exit. In the other, the appearance of radioactivity in the suspending medium from cells preincubated with [^{14}C]glucose was followed. This technique was described by Miller [8] and involves sampling after 1 s or longer. Transport was stopped in a quenching solution prepared according to Levine and Stein [9]. Initial rates were determined from plots of radioactivity vs. time during the early linear period of exit when the accumulated concentration of glucose outside the cells was low (less than 0.13 mM with an initial internal concentration of 88 mM). The concentration of cells in the first assay procedure was 0.04%, and in the second 0.76%.

Assays were carried out at 25°C in solutions containing 0.9% NaCl and 5 mM sodium phosphate buffer, pH 7.0 [7]. Washed cells were preincubated with sugars in a shaking bath at 37°C.

Cytochalasin B was dissolved in dimethyl-sulfoxide at a concentration of 1 mg/ml and diluted as required in salt/buffer soltion.

Uniformly labeled D-[^{14}C]glucose was supplied by New England Nuclear, and cytochalasin B by Sigma Chem. Co. Other chemicals were of reagent grade.

TABLE I

EFFECT OF PRE-INCUBATION OF CELLS IN CYTOCHALASIN B (1.16 μM) ON EXIT RATES MEASURED IN THE PRESENCE OR ABSENCE OF CYTOCHALASIN (1.0 μM) IN THE EXTERNAL ASSAY MEDIUM

Preincubation at 37°C for at least 30 min. Light scattering assay.

Cytochalasin B concentration (μM)		Exit time (min)
Preincubation	Assay medium	
0	0	0.67 ± 0.056
1.16	1.00	1.35 ± 0.030
0	1.00	1.37 ± 0.042
1.16	0	0.70 ± 0.017

Results

The inhibition of glucose exit seen when cells were placed in a medium containing cytochalasin B was not enhanced by preincubation of the glucose-loaded cells with the inhibitor, as the following experiment shows. Cells equilibrated with 122 mM glucose were suspended for at least 30 min at 37°C in the presence or absence of cytochalasin, and rates of glucose exit into sugar-free media with or without cytochalasin were determined. The results are given in Table I. In view of this finding later experiments were performed without preincubating cells with the inhibitor.

Non-competitive inhibition in a Sen-Widdas experiment, as reported by Taverna and Langdon [1] and by Bloch [2], was confirmed here. Results of

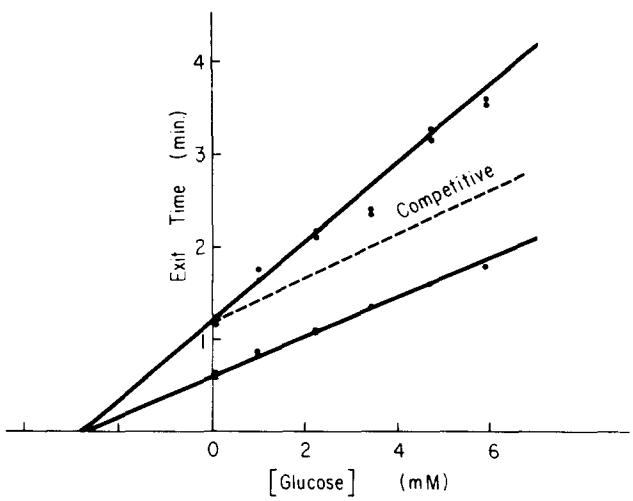

Fig. 1. Inhibition of glucose transport by cytochalasin B in a Sen-Widdas experiment. Light scattering assay, 0.04% cells. Exit times (in minutes) calculated from initial rates of net glucose loss from cells preloaded with 122 mM glucose. The external medium was salt-buffer solution containing glucose at varying concentrations, as shown. In some runs 0.85 μM cytochalasin B was included in the external medium (upper line). Apparent K_i for cytochalasin B, 0.9 μM; affinity constant for glucose, 2.3 mM. The dotted line represente exit times in the case of competitive inhibition.

such an experiment are shown in Fig. 1, where the K_i value for cytochalasin was found to be 0.85 μM.

If the inhibitor binds competitively on the inner surface of the membrane and if addition on the external surface is unimportant, inhibition of sugar exit should be highly dependent on the affinity, as well as concentration, of the substrate inside the cell. This prediction was tested with D-glucose, 2-deoxy-D-glucose and D-xylose, whose half-saturation constants on the outer surface of the membrane are 2.31 ± 0.30, 1.84 ± 0.29, and 11.83 ± 0.81 mM respectively [7]. With cells loaded with 125 mM sugar, exit times in the presence of 1.0 μM cytochalasin relative to those in the absence of inhibitor, were 2.1 ± 0.08, 2.1 ± 0.09 and 4.9 with glucose, 2-deoxyglucose and xylose, respectively. With only 0.2 μM cytochalasin the value for xylose was 1.8 ± 0.16. As expected therefore xylose movement is more sensitive to the inhibitor than movements of glucose or deoxyglucose, which are similarly retarded. The effects of increasing cytochalasin concentration on the exit of glucose and xylose are shown in Fig. 2. In a Sen-Widdas experiment the exit of xylose is inhibited non-competitively, just as in the case of glucose (Fig. 3).

The key experiment, inhibition of exit into a sugar-free solution, was carried out by means of two different techniques. Rates were determined by following either changes in light-scattering with cells loaded with various glucose concentrations, or the appearance of radioactivity in the suspending medium from cells preincubated in [^{14}C]glucose. Reciprocal plots for both assays are presented in Figs. 4 and 5. In both, inhibition is of the competitive type.

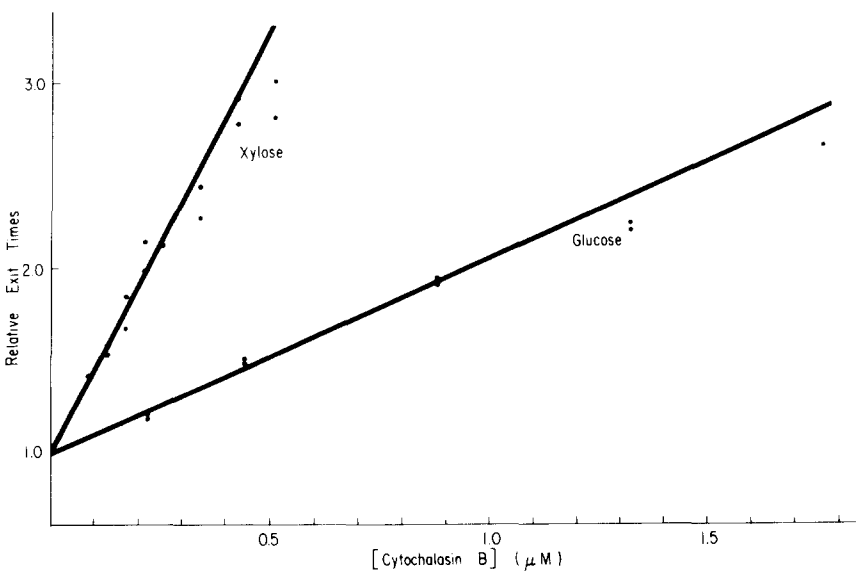

Fig. 2. Inhibition of glucose and xylose efflux in the presence of varying concentrations of cytochalasin B. Light-scattering assay in salt buffer solution. The relative exit time is calculated as the exit time in the presence of the inhibitor divided by that in its absence. Cells were preincubated with 125 mM glucose or 97 mM xylose. Cytochalasin B concentration for 50% inhibition: with glucose, 0.96 μM; with xylose, 0.22 μM.

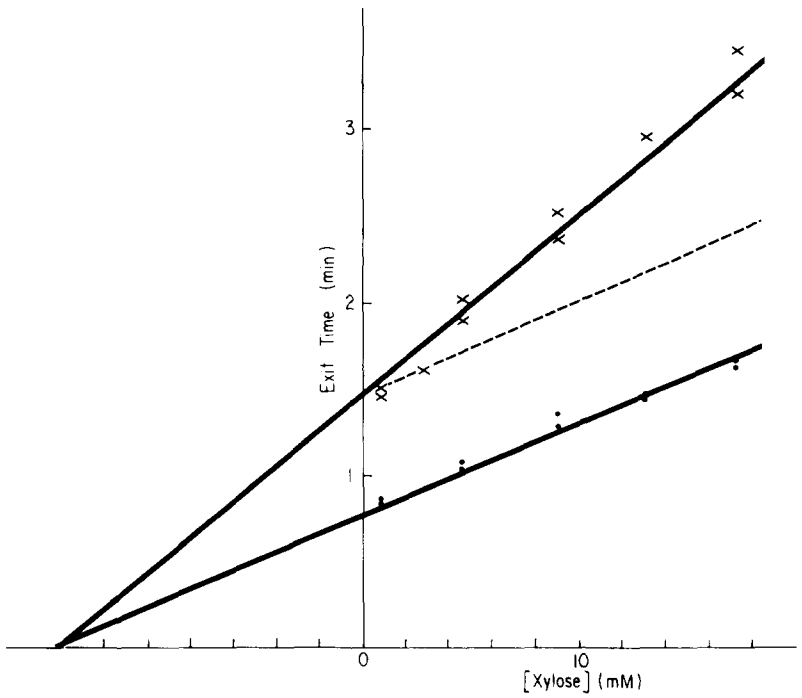

Fig. 3. Inhibition of xylose transport by cytochalasin B in a Sen-Widdas experiment. Light scattering assay, 0.04% cells. Exit times (in min) calculated from initial rates of net xylose loss from cells pre-loaded with 97 mM xylose. The external medium contained xylose at varying concentrations either in the presence or absence of cytochalasin B (0.17 μM). Apparent K_i for cytochalasin, 0.20 μM; affinity constant for xylose, 15 mM. The dotted line is for the case of competitive inhibition.

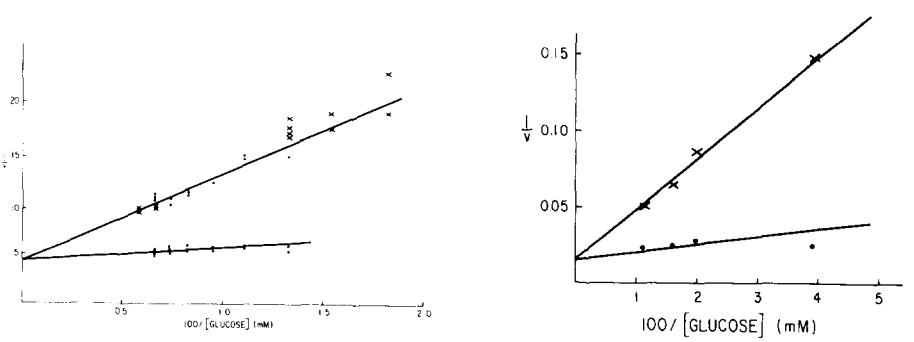

Fig. 4. Initial rates (v) of glucose exit in the presence or absence of 0.85 μM cytochalasin B determined by light-scattering method (upper and lower lines respectively). The external medium contained 60 mM mannitol, except at the 2 lowest glucose concentrations, as well as 0.9% NaCl and 5 mM sodium phosphate, pH 7.0, 25°C. The rate is calculated as exit time per mole of glucose (min · M^{-1}). Cell concentration, 0.04%.

Fig. 5. Initial rates of glucose exit in the presence or absence of 4.2 μM cytochalsin B determined from the appearance of uniformly labeled [^{14}C]glucose in the external medium (upper and lower lines respectively). Rates are given as mmol · l^{-1} · min^{-1}. Exit medium 0.9% NaCl + 5 mM sodium phosphate, pH 7.0, 25°C. Quenching medium: cold 2 mM $HgCl_2$ and 1.25 mM KI and 2% NaCl, with centrifugation at 1°C. Cell concentration, 0.76%. Radioactivity determined by scintillation counting in Aquasol.

Discussion

The preliminary reports of competitive inhibition of equilibrium exchange with either glucose or 3-*O*-methyl glucose [4,5] indicate, according to the rules already cited, that the mechanism, and not only kinetics, is competitive. The present observations, competitive inhibition of glucose exit and stronger inhibition of the exit of xylose, a substrate of low affinity, confirm this conclusion.

The question of the relative affinities of cytochalasin B on the inner and outer membrane surfaces was not answered by previously available experiments: entry, equilibrium exchange, and Sen-Widdas exit. This is apparent from general equations for rates under these conditions *, which were shown to be [6]:

Zero trans entry:

$$\frac{1}{v} = \frac{1}{f_2 Ct}\left\{1 + \frac{f_2}{f_{-1}} + \frac{f_2[I_i]}{f_{-1}K_{Ii}} + \frac{K_{So}}{[S_o]}\left(1 + \frac{f_1}{f_{-1}} + \frac{[I_o]}{K_{Io}} + \frac{f_1[I_i]}{f_{-1}K_{Ii}}\right)\right\} \tag{1}$$

Equilibrium exchange:

$$\frac{1}{v} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_2} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{Ii}} + \frac{f_{-1}[I_o]}{f_1 K_{Io}}\right)\right\} \tag{2}$$

Sen-Widdas exit:

$$\frac{1}{v} = \frac{1}{f_1 Ct}\left\{1 + \frac{f_1}{f_{-2}} + \frac{I_o}{K_{Io}} + \frac{K_{Si}}{f_{-2}[S_i]}\left[f_1\left(1 + \frac{[I_i]}{K_{Ii}}\right) + f_{-1}\left(1 + \frac{[I_o]}{K_{Io}}\right)\right]\right.$$
$$\left. + \frac{[S_o]}{K_{So}}\left[1 + \frac{f_2}{f_{-2}} + \frac{K_{Si}}{f_{-2}[S_i]}\left(f_{-1} + f_2\left(1 + \frac{[I_i]}{K_{Ii}}\right)\right)\right]\right\} \tag{3}$$

where the various constants refer to the general kinetic scheme in Fig. 6 for transport in the presence of an inhibitor both inside and outside the cell (I_i and I_o respectively). These equations are to be compared with that for zero trans exit:

$$\frac{1}{v} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_1} + \frac{f_{-2}[I_o]}{f_1 K_{Io}} + \frac{K_{Si}}{[Si]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{Ii}} + \frac{f_{-1}[I_o]}{f_1 K_{Io}}\right)\right\} \tag{4}$$

The entry, Sen-Widdas exit and exchange experiments, where inhibition is non-competitive, non-competitive and competitive respectively, combine to tell us that addition of cytochalasin B on the inner surface of the membrane must be an important factor in inhibition (i.e. the terms in $[I_i]$ contribute significantly to the forms of Eqns. 1 and 3). The relative importance of binding on the outside is difficult to determine from these experiments and, in fact, entry and exchange are completely uniformative in this regard. Even the Sen-Widdas

* The equations are derived on the assumption that dissociation of substrate from the carrier is rapid compared to movement through the membrane, an assumption supported by the observation of accelerated exchange in this system [10]. It may be shown that if dissociation is rate-limiting, inhibition by a competitive inhibitor of both zero trans exit and entry will be competitive, regardless of the distribution of the substrate and inhibitor. With cytochalasin B both competitive and non-competititve inhibitions are seen, so that the assumption of rapid dissociation is required by the data.

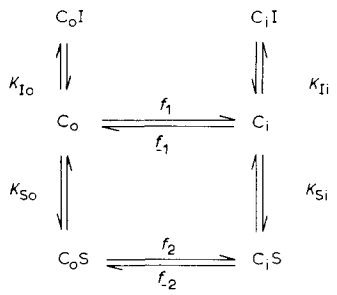

Fig. 6. Kinetic scheme for transport of substrate in the presence of a competitive inhibitor on both sides of the cell membrane. Subscripts o and i refer to forms of the carrier which combine with substrate in the external and internal solutions respectively (S_o and S_i). I_o and I_i represent inhibitor outside and inside the cell. K_{So}, K_{Io} etc. are dissociation constants, and f_1, f_2 etc. rate constants for carrier reorientation steps in the membrane.

experiment is not subject to an unambiquous interpretation. Experimentally the ratio of zero efflux to exchange at saturating concentrations is approximately 3 [10]. In terms of Fig. 6 this ratio (Eqns. 2 and 4) equals $(1 + f_{-2}/f_1)/(1 + f_{-2}/f_2)$, and consequently f_2/f_1 cannot be smaller than 3. Such a value would diminish the importance of the term in $[I_i]$ in the intercept compared to that in the slope of a Sen-Widdas plot (Eqn. 3). If f_{-2}/f_{-1} is also large, K_{Si} could be greater than the experimental value for internal affinity, for Eqn. 4 shows this constant to be equal to $K_{Si}\ (1 + f_{-1}/f_1)/(1 + f_{-2}/f_1)$. At 20°C the apparent affinity was 25 mM (11) and would presumably by higher at 25°C. As a result of these factors, the inhibitor might raise slope and intercept equally (Eqn. 3) only if $[I_o]/K_{Io}$ is not significant relative to $[I_i]/K_{Ii}$. The experiment must therefore leave us in doubt about the importance of cytochalasin addition on the external surface of the membrane (see Appendix).

The result given by a zero trans exit experiment is, by contrast, unequivocal, as inspection of Eqn. 4 shows. The ratio f_{-2}/f_1 must be greater than unity in order that exchange be three times more rapid than zero trans efflux (see above). It follows that this ratio, which multiplies $[I_o]K_{Io}$ in the intercept, cannot work to diminish the effect of inhibitor in the external solution at saturating substrate concentrations, i.e. cannot reduce the $[I_o]$ term to insignificance. Only if $[I_o]/K_{Io}$ is altogether negligible is the intercept a constant and the inhibition kinetics purely competitive, as found experimentally. Hence this observation proves that cytochalasin B has far higher affinity for the inner than for the outer carrier form.

Inhibition studies with cytochalasin B have been reported for several other types of cell. Horse leucocytes also show non-competitive inhibition of influx [12], demonstrating that here too the inhibitor is bound internally. This single observation does not exclude binding on the external surface as well [6]. However in chick embryo fibroblasts [13], Novikoff hepatoma cells [14,15], and HeLa cells [16,17] inhibition of uptake was purely competitive. As shown previously [6], this behavior results only if the inhibitor cannot add to the inward facing form of the carrier. It appears therefore that in all cases the glucose carrier exhibits extreme asymmetry towards cytochalasin B, but that the oientation of the transport system may be inward or outward in different cell types.

A competitive mechanism does not prove that substrate and inhibitor actually combine at the same site, and in the case of cytochalasin B there is reason for doubt. Glucose is reported to protect the carrier against the irreversible inhibitor 1,2-butanedione, but cytochalasin B slightly accelerates destruction of the transport system [18]. The reverse is true for another inhibitory reagent, 1-fluoro-2,4-dinitrobenzene. Here inactivation is accelerated by substrate [7], while cytochalasin B gives almost complete protection [2]. Other inhibitors only partially protect [7,19]. In addition, the structures of glucose and cytochalasin B have little in common, and a specific site for glucose is unlikely to have a vastly greater affinity (by a factor in the order of 10^4) for an unrelated structure. The present finding adds weight to these arguments, for the glucose transport site must obviously make its apperance on both sides of the membrane while that for cytochalasin is restricted to one side. This is not easily explained if the same site binds the substrate and the inhibitor. It suggests that an allosteric inhibition site may be present on either the internal or external surface of the membrane, depending on the type of cell, distinct from the substrate site and closely associated with the site of fluorodinitrobenzene reaction.

Appendix

Equilibrium exchange is the only kinetic experiment that unfailingly tells us whether the inhibition mechanism (as opposed to the kinetic pattern) is competitive or non-competitive [6]. However it is completely ambiguous regarding the symmetry of inhibitor addition to the internal and external forms of the carrier. Assuming the mechanism is competitive, different combinations of experiments, such as zero trans exit and entry, may be required to determine the latter question, depending both on the sidedness of inhibitor addition itself and on the relative rates of reorientation (movement) of free and loaded carrier. To illustrate the problem and its possible solution we shall deal with Sen-Widdas and zero trans exit experiments, giving special attention to the case of the glucose system, for which the loaded carrier reorientates more rapidly than free carrier (as discussed above). It will be seen that zero trans exit is especially suitable, and Sen-Widdas exit unsuitable, where internal binding of the inhibitor is important but where an external component may also occur. If the sidedness of inhibitor addition or the relative reorientation rates of free and loaded carrier differ from the case above, other combinations of experiments may be required.

1. Sen-Widdas exit experiment

(a) The inhibitor binds exclusively to the inner carrier form: The kinetics of inhibition is most simply indicated by the relative effects of the inhibitor upon the intercept and slope of the conventional rate plot. Reference to Eqn. 3 shows that in the presence of an internal inhibitor the intercept of a plot of exit time versus external substrate concentration is increased by the following factor:

$$1 + \frac{\dfrac{K_{Si}\ [I_i]}{[S_i]\ K_{Ii}}}{1 + \dfrac{f_{-2}}{f_1} + \dfrac{K_{Si}}{[S_i]}\left(\dfrac{f_1 + f_{-1}}{f_1}\right)} \tag{5}$$

and the slope by another factor:

$$1 + \frac{\dfrac{K_{Si}\ [I_i]}{[S_i]\ K_{Ii}}}{1 + \dfrac{f_{-2}}{f_2} + \dfrac{K_{Si}}{[S_i]}\left(\dfrac{f_{-1} + f_2}{f_2}\right)} \tag{6}$$

When the kinetics of inhibition is purely competitive the slope is constant and the intercept rises (with added inhibitor); when non-competitive, the slope and intercept increase equally. The dependence of the kinetics of inhibition upon the relative values of reorientation constants for free (f_1 and f_{-1}) and loaded carrier (f_2 and f_{-2}) are most readily seen from the ratio of the second terms in expressions 5 and 6, which indicates the relative effects of the inhibitor on the intercept and on the slope:

$$R = \frac{1 + \frac{f_{-2}}{f_2} + \frac{K_{Si}}{[S_i]}\left(\frac{f_{-1} + f_2}{f_2}\right)}{1 + \frac{f_{-2}}{f_1} + \frac{K_{Si}}{[S_i]}\left(\frac{f_1 + f_{-1}}{f_1}\right)} \tag{7}$$

(i) $f_1 = f_{-1} = f_2 = f_{-2}$:

$R = 1$ (Pure non-competitive inhibition)

(ii) $f_2 = f_{-2} >> f_1 = f_{-1}$:

$R << 1$ (uncompetitive * inhibition)

(iii) $f_1 = f_{-1} >> f_2 = f_{-2}$

$R >> 1$ (competitive inhibition)

Cases (i), (ii) and (iii) demonstrate the difficulty of interpretation in the absence of knowledge of the ratios of flux constants. In case (ii), which the glucose system may approach, inhibition is strengthened as the external substrate concentration rises. On the other hand inhibitor added outside acts competitively (1.b) and so is less effective at higher substrate concentrations. The combination of internal and external inhibitor would therefore tend to make inhibition independent of substrate concentration, in other words non-competitive. The same conclusion emerges upon examination of Eqn. 3: addition of inhibitor to the outer carrier form, in the presence of inhibitor inside, would raise the intercept term, thereby increasing tha value of R and drawing the inhibition pattern toward the non-competitive form. Without knowing the relative sizes of the various constants exactly we cannot be certain about the importance of these effects. Herein lies the source of ambiquity in our Sen-Widdas experiments.

(b) The inhibitor binds exclusively to the outer carrier form: Eqn. 3 now reduces to a form in which the inhibitor concentration appears in the intercept but not the slope term. In consequence the kinetics of inhibition must be purely competitive in every case.

2. Zero trans exit

(a) The inhibitor binds exclusively to the inner carrier form: Eqn. 4 reduces to a form in which the inhibitor concentration appears in the slope but not in the intercept term. The kinetics of inhibition therefore must be purely competitive regardless of the relative rates of reorientation steps.

(b) The inhibitor binds exclusively to the outer carrier form: By following a similar procedure to that above (1.a) the relative effect of the inhibitor on the intercept and the slope is found to be

$$R = \frac{1 + \frac{f_1}{f_{-1}}}{1 + \frac{f_1}{f_{-2}}} \tag{8}$$

(i) $f_1 = f_{-1} = f_2 = f_{-2}$:

$R = 1$ (Pure non-competitive inhibition)

* Uncompetitive: Inhibition increases as the external substrate concentration increases.

(ii) $f_2 = f_{-2} >> f_1 = f_{-1}$

$R = 2$ (Uncompetitive inhibition)

(iii) $f_1 = f_{-1} >> f_2 = f_{-2}$

$R << 1$ (Competitive inhibition)

In case (ii) addition of inhibitor externally raises the intercept more than the slope, and for this reason, where the inhibitor also binds internally the experiment is especially sensitive to external binding.

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