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A NEW APPROACH IN THE KINETICS OF BIOLOGICAL TRANSPORT

THE POTENTIAL OF REVERSIBLE INHIBITION STUDIES

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Summary

Kinetic equations are derived for reversible inhibition of both active and facilitated transport systems for seven common experimental arrangements. It is shown that the unique features of transport kinetics may be exploited to give new kinds of information. It is also shown that the familiar rules of enzyme kinetics, though often applied to transport, can be seriously misleading. The analysis leads to the following general conclusions: (1) A competitive mechanism frequently gives rise to non-competitive kinetics, depending on the experimental design, but a non-competitive mechanism never produces competitive kinetics. (2) Inhibition studies on exchange diffusion at equilibrium in non-active systems or in the final steady state in active systems are the only unambiguous kinetic tests to distinguish competitive from non-competitive mechanisms. (3) Substrate analogs that are bound to the carrier and transported are readily distinguished by inhibition kinetics from those not transported, even though both may rapidly enter the cell by another route. (4) Even in non-active systems competitive inhibitors commonly have far different affinities for the substrate sites on the two membranes faces: where sufficient non-polarity allows their penetration into the cell, inhibition kinetics readily establish such sidedness in their action. (5) Inhibition kinetics of the mixed competitive and non-competitive type result from moderately asymmetrical binding of inhibitor at the substrate site. (6) Asymmetry is a necessary feature of active transport; hence studies of inhibition kinetics should provide important insights into its mechanism.

Introduction

The kinetic treatment of reversible inhibition of biological transport systems has been adopted with little modification from the field of enzyme kinetics.

The result has not been altogether successful. According to the familiar rules of enzyme kinetics, when a substrate and inhibitor compete for the active site, inhibition is overcome by high substrate concentrations and is called competitive; when they are bound at different sites, inhibition persists at all substrate concentrations and is called non-competitive. This is not necessarily true of transport systems, where inhibition often persists despite addition of substrate and inhibitor to the same site. The reason is that the carrier operates between two aqueous compartments, outside and inside the cell. The substrate is normally present, for experimental purposes, in only one compartment initially. If the inhibitor is present in the order, the substrate may be unable to displace it from the carrier, in which case the behavior will be non-competitive.

These observations seem self-evident, but they have not always been appreciated. Our attention was forcibly drawn to the subject in the course of experiments with cytochalasin B. This substance reversibly inhibits glucose transport in erythrocytes in a manner which, according to one study, is purely competitive [1] and according to others purely non-competitive [2,3]. The discordant findings were based on different kinds of experiments, in which the procedures appeared to be thorough and the results convincing. There was no disagreement about specificity, in that other transport systems were unaffected, or about high affinity for the glucose carrier. Hence, a single physical mechanism could well underlie the behavior and the error may be one of interpretation. Re-examination of the kinetics of transport inhibition showed that this possibility was indeed feasible. Our subsequent studies of cytochalasin B, to be published separately, demonstrate that it adds exclusively to the form of the glucose carrier which is present on the inner surface of the erythrocyte membrane. This circumstance, together with the ability of cytochalasin B to penetrate into the cell, independent of the carrier, accounts for the anomalies.

Here we wish to present the general kinetic theory for reversible inhibition of transport, in order to provide simple tests for competitive and non-competitive inhibition and for asymmetric binding of inhibitors. The latter phenomenon is important for our understanding of biological transport and there is growing evidence that it may not be unusual. For example a number of alkyl and phenyl derivatives of glucose and galactose, which are non-transported inhibitors of the hexose system, have vastly higher affinity for carrier on one side of the membrane than the other [4,5], and in addition, they readily penetrate the cell [6,7].

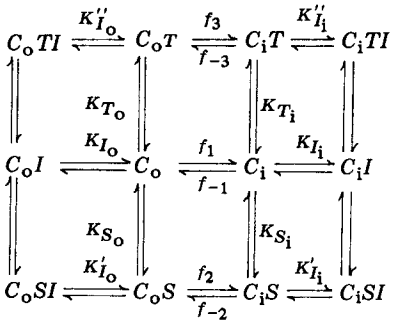
At the outset it is necessary to make a sharp distinction between a competitive or non-competitive mechanism, on the one hand, and competitive or non-competitive kinetics on the other. The former refers to the molecular mechanism involved, whether addition of substrate to the carrier excludes the binding of inhibitor (usually ascribed to involvement of the same binding site for both) or whether substrate and inhibitor may add simultaneously to the carrier (which must involve separate binding sites). The latter, the kinetic behavior, refers to experimental observations on rates, whether addition of sufficient quantities of substrate overcomes the effect of an inhibitor. Mechanism and kinetics do not necessarily agree, as indicated above, but it will be seen that appropriate kinetic experiments can establish the actual mechanism.

In the first section below we derive kinetic equations applying to a number

of common transport experiments. In later sections we deal with general conclusions which emerge and their application in transport studies. Finally, tables displaying the kinetic behavior in different experiments are provided. These may be used directly in planning and interpreting experiments without recourse to the mathematical derivation.

Kinetic theory

In order to analyze the kinetics of inhibition in various kinds of experiment, as well as the consequences of asymmetric inhibitor binding, we shall begin by deriving rate equations for the general transport scheme shown in scheme I. *



Scheme I. Transport scheme for two substrates S and T in the presence of an inhibitor I. Subscripts o and i refer to carrier forms on the outer and inner surfaces of the membrane respectively, and $f \pm$ i are rate constants for reorientation of carrier in the membrane. In purely competitive inhibition the ternary complexes C_oSI, C_iSI, etc. do not form ($K'_{Io} = K'_{Ii} = K''_{Io} = K''_{I_i} = \infty$). K_{S_o}, K_{T_o}, K_{I_o}, etc. are equilibrium constants.

In this scheme S and T represent two different substrates; their inclusion makes it easy to derive rate expressions for equilibrium exchange (where S and T stand for radioactively labeled and unlabeled pools of the same substrate) as well as expressions for inhibition by competing substrates. The subscripts o and i designate the external and internal surfaces of the cell membrane: thus C_o and C_i are forms of the carrier in which the substrate binding site is exposed to the external or internal aqueous pool, and S_o and S_i represent substrate in these pools. Substrate or inhibition may add to the free carrier, forming the complex C_oS, C_oI, etc., and the possibility of non-competitive inhibition is allowd for by inclusion of a ternary complex C_oSI, C_oTI etc. Rate constants for movement of bound substrate through the membrane are designated $f \pm$ i, + standing for inward diffusion, −for outward. The binding constants for addition of sub-

* Neither this formal scheme for transport, nor the use of the word "carrier" denotes anything about the details of the physical mechanism, other than the appearance of a substrate binding site alternately at the inner and outer membrane face; i.e., a given site is not simultaneously accessible to substrate in the internal and external pools. In this sense a "mobile carrier" is represented, and any physical model having this property is described kinetically by Scheme I. Other transport schemes are possible, for example (i) a single form of free carrier and two forms of carrier · substrate complex [8]; (ii) two forms of free enzyme and one of the complex [8]; and (iii) the occurrence of a ternary complex, in which two molecules of substrate are bound simultaneously, one on either side of the membrane. The present model is the most common and has the required properties to explain the observations on cytochalasin. We shall, therefore, treat it in detail here and later investigate ways of distinguishing one model from another.

strate and inhibitors to carrier on either the outer or inner membrane face, K_{S_o}, K_{S_i}, K_{I_o}, K_{I_i} etc. are defined as dissociation constants, having units of concentration.

In order that the analysis be as useful as possible, the rate equations should apply to both active and non-active systems. In the latter the substrate moves down its concentration gradient to achieve equilibrium, and in accordance with this the principle of detailed equilibrium dictates the following relationship among the constants in Scheme I:

$$\frac{K_{So}}{K_{Si}} = \frac{f_{-1}f_2}{f_1 f_{-2}} \tag{1}$$

$$\frac{K_{T_o}}{K_{T_i}} = \frac{f_{-1}f_3}{f_1 f_{-3}} \tag{2}$$

In an active system cellular energy is made to impinge upon one or more of the steps designated by the constants, causing the relationship to deviate from equality and the ultimate substrate concentrations attained on either side of the membrane to become unequal. If constants α and β are defined as

$$\alpha = \frac{f_{-1}f_2 K_{S_i}}{f_1 f_{-2} K_{S_o}} \tag{3}$$

and

$$\beta = \frac{f_{-1}f_3 K_{T_i}}{f_1 f_{-3} K_{T_i}} \tag{4}$$

they are found to equal the ratio of final steady-state concentrations of substrate inside and outside the cell: $\{[S_i]/[S_o]\}_{(final)} = \alpha$ and $\{[T_i]/[T_o]\}_{(final)} = \beta$. In an equilibrating system α and β equal unity.

In deriving a rate equation we shall make one important simplifying assumption, that the slow step in the transport process is passage through the membrane itself rather than diffusion up to the carrier site or formation and dissociation of the carrier · substrate complex. This assumption is reasonable in view of the great speed of complex formation and dissociation of enzymes and their substrates [9]. It is also supported by the observation of accelerated exchange in leucine [10], choline (Devés, R. and Krupka, R.M., in preparation) and glucose [11] transport systems of erythrocytes *.

The general rate law for diffusion of substrate across the membrane is derived as follows. An equation is first written expressing the steady-state condition that the sum of movements of all forms of carrier in the inward direction must equal that in the outward:

$$f_1[C_o] + f_2[C_oS] + f_3[C_oT] = f_{-1}[C_i] + f_{-2}[C_iS] + f_{-3}[C_iT] \tag{5}$$

Next, the total concentration of carrier in all forms is a constant, C_t:

$$C_t = [C_o] + [C_oS] + [C_oT] + [C_oI] + [C_oSI] + [C_oTI] + [C_i] + [C_iS] + [C_iT] + [C_iI] + [C_iSI] + [C_iTI] \tag{6}$$

* Completely general rate equations which are not dependent on this assumption will be presented at a later date. It will then be shown that the observation of non-competitive kinetics, under conditions specified below, constitutes a simple test for equilibrium complex formation.

These equations, together with the dissociation constants for complex formation defined in the following manner, $[C_o][S_o]/[C_oS] = K_{S_o}$ etc., enable us to write down an expression for the rate of transport of substrate S:

$$\frac{d[S_i]}{dt} = f_2[C_oS] - f_{-2}[C_iS]$$

$$= \frac{\frac{f_1 f_{-2} C_t}{K_{S_i}}(\alpha[S_o] - [S_i]) + \frac{f_{-2} f_3 C_t}{K_{S_i} K_{T_o}}\left(\frac{\alpha}{\beta}[S_o][T_i] - [S_i][T_o]\right)}{\left(1 + \frac{[S_o]}{K_{S_o}} + \frac{[T_o]}{K_{T_o}} + \frac{[I_o]}{K_{I_o}} + \frac{[S_o]}{K_{S_o}}\frac{[I_o]}{K'_{I_o}} + \frac{[T_o]}{K_{T_o}}\frac{[I_o]}{K''_{I_o}}\right)M}$$

$$+ \left(1 + \frac{[S_i]}{K_{S_i}} + \frac{[T_i]}{K_{T_i}} + \frac{[I_i]}{K_{I_i}} + \frac{[S_i]}{K_{S_i}}\frac{[I_i]}{K'_{I_i}} + \frac{[T_i]}{K_{T_i}}\frac{[I_i]}{K''_{I_i}}\right)N \qquad (7)$$

where

$$M = f_{-1} + \frac{f_{-2}[S_i]}{K_{Si}} + \frac{f_{-3}[T_i]}{K_{Ti}} = \frac{f_{-1}}{f_1}\left(f_1 + \frac{f_2[S_i]}{\alpha K_{S_o}} + \frac{f_3[T_i]}{\beta K_{T_o}}\right)$$

and

$$N = f_1 + \frac{f_2[S_o]}{K_{S_o}} + \frac{f_3[T_o]}{K_{T_o}} = \frac{f_1}{f_{-1}}\left(f_{-1} + \frac{\alpha f_{-2}[S_o]}{K_{S_i}} + \frac{\beta f_{-3}[T_o]}{K_{T_i}}\right)$$

Application to experiment

The kinetics of transport may be studied either by measuring rates in the early stages of the reaction, initial rates under conditions specified at the very beginning of the process, or by following the changing distribution of substrate over the whole course of transport until no further net movement occurs. We shall deal with five different kinds of experiment depending on initial rate measurements and two covering the entire course of the reaction. Different experiments will be seen to supply different kinds of information, and their combination to allow a determination of the molecular nature of the inhibition, whether competitive or non-competitive, and of the symmetry or asymmetry of inhibitor binding.

I. Experiments involving initial rates

Initial rates of transport are most commonly studied under one of five different conditions of substrate distribution, as follows. (a) "Zero *trans* efflux", in which substrate is present inside the cell only and its initial rate of appearance outside is determined. (b) "Zero *trans* influx", the reverse of this, in which substrate is initially present only on the outside and its rate of penetration is measured. (c) "Sen-Widdas experiment", with substrate at a saturating concentration inside and at a lower, variable concentration outside. The net rate of exit is measured. (d) "Equilibrium exchange", where the substrate is present inside and outside the cell, one pool being labeled with radioactive substrate. The diffusion of the latter across the membrane is followed. (e) "Infinite *trans* influx", cells loaded with a saturating concentration of unlabeled substrate are

placed in a medium containing a lower concentration of labeled substrate. The initial rate at which label appears inside the cell is measured.

A. Non-transported competitive inhibitors

We shall consider each of these experiments in turn, confining our attention to a competitive mechanism, i.e., one in which the ternary complexes in Scheme I are not formed: $K_{I_o}' = K_{I_o}'' = K_{I_i}' = K_{I_i}'' = \infty$

(a) Zero trans efflux. We set $[T_o] = [T_i] = [S_o] = 0$:

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + \frac{f_{-2}}{f_1} + \frac{f_{-2}}{f_1}\frac{[I_o]}{K_{I_o}} + \frac{K_{S_i}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{I_i}} + \frac{f_{-1}}{f_1}\frac{[I_o]}{K_{I_o}}\right)\right\} \tag{8}$$

where v is the velocity at which the substrate S diffuses across the membrane. Rates of transport at varying substrate concentrations $[S_i]$ and at more than one inhibitor concentration may be analyzed by means of a reciprocal plot, $1/v$ againts $1/[S]$, i.e. a Lineweaver-Burke plot, as well as by any other linear transormation. The intercept in the reciprocal plot is the reciprocal of the maximal rate, and the ratio of slope to intercept is the half-saturation constant for substrate, $[S_i]_{1/2}$. Let us consider three different conditions of inhibitor distribution or affinity.

(i) Inhibitor is present inside the cell and outside, at equilibrium, and has affinity for carrier on both membrane surfaces. The substrate independent and dependent terms in Eqn. 8 are increased in the presence of inhibitor, i.e., both the intercept and slope. This is characteristic of non-competitive inhibition, since no matter how high the substrate concentration, the maximum velocity is reduced by the inhibitor *.

(ii) Inhibitor is restricted to the external pool because of inability to pass through the cell membrane; or equivalently, the inhibitor, though present on both sides, becomes bound only to those substrate sites exposed to the external solution, owing to asymmetry in carrier structure. Again both intercept and slope increase, as in non-competitive inhibition.

(iii) Inhibition is restricted to the internal membrane surface because of asymmetry in either the distribution or affinity of the inhibitor. The intercept in the reciprocal plot is now constant though the slope changes. In other words, V is constant while K_m increases, as in pure competitive inhibition.

(b) Zero trans influx. $[T_o] = [T_i] = [S_i] = 0$:

$$\frac{1}{v} = \frac{1}{f_2C_t}\left\{1 + \frac{f_2}{f_{-1}} + \frac{f_2[I_i]}{f_{-1}K_{I_i}} + \frac{K_{S_o}}{[S_o]}\left(1 + \frac{f_1}{f_{-1}} + \frac{[I_o]}{K_{I_o}} + \frac{f_1}{f_{-1}}\frac{[I_i]}{K_{I_i}}\right)\right\} \tag{9}$$

Under the above three conditions of inhibition on both sides of the membrane, outside only, or inside only, the kinetic form of the inhibition will be non-competitive, competitive or non-competitive, respectively, as inspection of Eqn. 9 shows.

(c) Sen-Widdas exit experiment (infinite cis). $[T_o] = [T_i] = 0$; $[S_i]K_{S_i} \gg 1$;

* This definition of non-competitive inhibition is adopted here for the sake of simplicity, but would include both pure non-competitive inhibition and mixed competitive and non-competitive inhibition.

$[S_i] \gg [S_o]$:

$$\frac{1}{v} = \frac{1}{f_1 C_t}\left\{1 + \frac{f_1}{f_{-2}} + \frac{[I_o]}{K_{I_o}} + \frac{f_1}{f_{-2}}\frac{K_{S_i}}{[S_i]}\frac{[I_i]}{K_{I_i}} + \frac{[S_o]}{K_{S_o}}\left(1 + \frac{f_2}{f_{-2}} + \frac{f_2}{f_{-2}}\frac{K_{S_i}}{[S_i]}\frac{[I_i]}{K_{I_i}}\right)\right\} \tag{10}$$

(i) Inhibitor binds on both sides of the membrane. Compared to the term in $[I_o]$, those in $[I_i]$ should be negligible because of being multiplied by $K_{S_i}/[S_i]$, which is very much smaller than unity. Hence in a plot of $1/v$ against $[S_o]$ the intercept increases but the slope is constant. The ratio of intercept to slope in such a plot is equal to the half-saturation constant for external substrate, and the intercept to the reciprocal of the maximum exit rate. The inhibitor reduces V and, by exactly the same factor, increases K_m. The inhibition has in this sense the features of both competitive and non-competitive inhibition, owing to the peculiar distribution of substrate across the membrane. Since it does not correspond to any case of enzyme inhibition, including "mixed competitive and non-competitive" in which V and K_m may change by different factors, we may for present purposes describe it as "competitive — non-competitive" meaning that it is competitive with respect to external substrate, non-competitive with respect to internal.

(ii) Inhibition outside. The kinetic behavior is identical to that above, where the inhibitor is capable of binding on either side.

(iii) Inhibition inside. Both the slope and intercept are increased, and by a similar factor. Hence V declines while K_m is nearly constant; inhibition is non-competitive.

(d) Equilibrium exchange (or exchange in the final steady state). We let S_i be labeled substrate, T_o the same substrate unlabeled; $[S_o] = [T_i] = 0$; $[S_i] = \alpha[T_o]$:

$$\frac{1}{v} = \frac{1}{f_{-2} C_t}\left\{1 + \frac{f_{-2}}{f_2} + \frac{K_{S_i}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{I_i}} + \frac{f_{-1}}{f_1}\frac{[I_o]}{K_{I_o}}\right)\right\} \tag{12}$$

In either case, active or non-active, inhibition is overcome at high substrate concentration and is therefore of the pure competitive type.

(e) Infinite trans influx (non-equilibrium exchange). Measurement of initial rates of zero *trans* entry may be difficult because the internal substrate concentration rapidly attains a level where efflux becomes important, diminishing the net rate of influx. However, if cells loaded with a high substrate concentration are placed in a medium containing labeled substrate, the label is diluted inside the cell and return flux is negligible. The rate equation for this case is found by setting $[T_i]$ as saturating, $[S_o]$ lower and variable, and $[T_o] = [S_i] = 0$:

$$\frac{1}{v} = \frac{1}{f_2 C_t}\left\{1 + \frac{f_2}{f_{-3}} + \frac{f_2}{f_{-3}}\frac{K_{T_i}}{[T_i]}\frac{[I_i]}{K_{I_i}} + \frac{K_{S_o}}{[S_o]}\left(1 + \frac{f_1}{f_{-3}} + \frac{[I_o]}{K_{I_o}} + \frac{f_1}{f_{-3}}\frac{K_{T_i}}{[T_i]}\frac{[I_i]}{K_{I_i}}\right)\right\} \tag{13}$$

If S and T represent molecules of the same substrate this may be rewritten as:

$$\frac{1}{v} = \frac{1}{f_2 C_t}\left\{1 + \frac{f_2}{f_{-2}} + \frac{f_2 K_{S_i}[I_i]}{f_{-2}[S_i]K_{I_i}} + \frac{K_{S_o}}{[S_o]}\left(1 + \frac{f_1}{f_{-2}} + \frac{[I_o]}{K_{I_o}} + \frac{f_1 K_{S_i}[I_i]}{f_{-2}[S_i]K_{I_i}}\right)\right\} \tag{14}$$

The system behaves in the same way as in the Sen-Widdas experiment: inhibi-

tion is competitive if the inhibitor adds to carrier on both sides or on the outside, non-competitive if only on the inside.

B. Competing substrates

Of the above experiments, only zero *trans* efflux and zero *trans* influx are of special interest here, for they enable us to distinguish substrate analogs which undergo transport from those that do not, even where both rapidly diffuse through the membrane without intervention of the carrier.

(a) Zero trans efflux. We determine the rate of efflux of S, with $[S_o] = 0$, in the presence of a second substrate, T:

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + f_{-2}\left[\frac{1 + \frac{[T_o]}{K_{T_o}}}{f_1 + f_3\frac{[T_o]}{K_{T_o}}}\right] + \frac{K_{S_i}}{[S_i]}\left[1 + \frac{[T_i]}{K_{T_i}} + \left(f_{-1} + \frac{f_{-3}[T_i]}{K_{T_i}}\right)\left[\frac{1 + \frac{[T_o]}{K_{T_o}}}{f_1 + \frac{f_3[T_o]}{K_{T_o}}}\right]\right]\right\} \quad (15)$$

(i) Analog on both sides. If the values of f_1 and f_3 are not too different, the slope but not the intercept increases, and inhibition is competitive. If the analog is not transported, and f_3 and f_{-3} are small relative to f_1 and f_{-1}, then as seen before, slope and intercept increase and inhibition is non-competitive.

(ii) Analog outside. There is now little or no inhibition, and the rate of exit of substrate S may actually increase.

(iii) Analog inside. The slope but not the intercept increases, giving pure competitive inhibition.

(b) Zero trans influx. $[S_i] = 0$:

$$\frac{1}{v} = \frac{1}{f_2C_t}\left\{1 + f_2\left[\frac{1 + \frac{[T_i]}{K_{T_i}}}{f_{-1} + f_{-3}\frac{[T_i]}{K_{T_i}}}\right] + \frac{K_{S_o}}{[S_o]}\left[1 + \frac{[T_o]}{K_{T_o}} + \left(f_1 + \frac{f_3[T_o]}{K_{T_o}}\right)\left[\frac{1 + \frac{[T_i]}{K_{T_i}}}{f_{-1} + \frac{f_{-3}[T_i]}{K_{T_i}}}\right]\right]\right\} \quad (16)$$

With analog on both sides or only on the outside, inhibition is competitive; if only on the inside there is little or no inhibition, or rate enhancement

II. Studies over the course of transport; the use of integrated rate equations

The changing internal substrate concentration is usefully studied over the whole approach to equilibrium, with the substrate initially confined either in the cell interior or the suspending medium, a subject recently explored (Miller, D.M., in preparation). To derive appropriate rate expressions we first write down the complete rate equation for the condition $[T_i] = [T_o] = 0$:

$$\frac{d[S_i]}{dt} = \frac{A(\alpha[S_o] - [S_i])}{g_1(I) + \frac{[S_o]}{K_{S_o}}g_2(I) + \frac{[S_i]}{K_{S_i}}g_3(I) + \frac{[S_o][S_i]}{K_{S_o}K_{S_i}}g_4(I)} \quad (17)$$

where

$$A = f_1 f_{-2}\frac{C_t}{K_{S_i}} = \frac{f_2 f_{-1}C_t}{\alpha K_{S_o}}$$

$$g_1(I) = f_1\left(1 + \frac{[I_i]}{K_{I_i}}\right) + f_{-1}\left(1 + \frac{[I_o]}{K_{I_o}}\right)$$

$$g_2(I) = f_2\left(1 + \frac{[I_i]}{K_{I_i}}\right) + f_{-1}\left(1 + \frac{[I_o]}{K'_{I_o}}\right)$$

$$g_3(I) = f_1\left(1 + \frac{[I_i]}{K'_{I_i}}\right) + f_{-2}\left(1 + \frac{[I_o]}{K_{I_o}}\right)$$

$$g_4(I) = f_2\left(1 + \frac{[I_i]}{K'_{I_i}}\right) + f_{-2}\left(1 + \frac{[I_o]}{K'_{I_o}}\right)$$

In the experiments to be analyzed the concentration of substrate in the external solution $[S_o]$ is kept constant. Eqn. 17 may then be rewritten as

$$\frac{d[S_i]}{dt} = \frac{A(\alpha[S_o] - [S_i])}{B + D[S_i]} \tag{18}$$

where

$$B = g_1(I) + \frac{[S_o]}{K_{S_o}} g_2(I)$$

$$D = \frac{1}{K_{S_i}}\left(g_3(I) + \frac{[S_o]}{K_{S_o}} g_4(I)\right)$$

Integration of Eqn. 18 for the condition when $t = 0$, $[S_i] = [\overline{S_i}]$, gives the following equation:

$$D([\overline{S_i}] - [S_i]) + (B + \alpha D[S_o]) \log\left(\frac{\alpha[S_o] - [\overline{S_i}]}{\alpha[S_o] - [S_i]}\right) = At \tag{19}$$

(a) The rate of exit. When cells loaded with substrate are placed in a large volume of substrate-free solution, transport proceeds down the concentration gradient until internal and external concentrations are equal. If the extracellular volume is made sufficiently large this final concentration is effectively zero, and the substrate concentration in the external medium remains so during the course of the experiment. Eqn. 19 may then be written as

$$\frac{[\overline{S_i}] - [S_i]}{t} = \frac{A}{D} - \frac{B}{D} \cdot \frac{1}{t} \log\left(\frac{[\overline{S_i}]}{[S_i]}\right) \tag{20}$$

From Eqn. 18 the half-saturating concentration of substrate within the cell may be shown to be:

$$[S_i]_{1/2} = \widetilde{K}_{S_i} = B/D = K_{S_i} g_1(I)/g_3(I) = K_{S_i}\left(\frac{f_1 + f_{-1} + \frac{f_1[I_i]}{K_{I_i}} + \frac{f_{-1}[I_o]}{K_{I_o}}}{f_1 + f_{-2} + \frac{f_1[I_i]}{K_{I_i}} + \frac{f_{-2}[I_o]}{K_{I_o}}}\right) \tag{21}$$

The maximum velocity of exit equals A/D (neglecting the minus sign which indicates the outward direction of transport):

$$\widetilde{V} = A/D = f_1 f_{-2} C_t / \left(f_1 + f_{-2} + \frac{f_1[I_i]}{K'_{I_i}} + \frac{f_{-2}[I_o]}{K_{I_o}}\right) \tag{22}$$

Hence, Eqn. 20 may be rewritten as:

$$\frac{[\overline{S_i}] - [S_i]}{t} = \widetilde{V} - \widetilde{K}_{S_i} \cdot \frac{1}{t} \log\left(\frac{[\overline{S_i}]}{[S_i]}\right) \tag{23}$$

An inhibitor's effect on $\widetilde{V}$ and $\widetilde{K}_{S_i}$ are now easily seen. Consider first a competitive mechanism, where $K'_{I_i} = \infty$. If the inhibitor binds at sides on both sides of the membrane $\widetilde{V}$ falls while $\widetilde{K}_{S_i}$ is little changed: kinetically inhibition is of the non-competitive type. If inhibitor adds solely to the external site the result is the same. If it binds exclusively on the internal surface inhibition is competitive, for $\widetilde{V}$ is unchanged while $\widetilde{K}_{S_i}$ gets bigger. If the mechanism is truly non-competitive, and substrate and inhibitor simultaneously add to the carrier, then in all cases the kinetic pattern is non-competitive.

It is also of interest to derive the exit equation for the case of a competing substrate. We shall consider only the simplest condition, where the latter is present at equal concentrations inside and outside the cell ($[T_o] = [T_i] = [T]$ in Eqn. 7). Now

$$\widetilde{K}_{S_i} = B/D = K_{S_i} \frac{\left\{ \frac{1 + [T]}{K_{T_i}} + \left(f_{-1} + \frac{f_{-3}[T]}{K_{T_i}}\right) \left[\frac{1 + \frac{[T]}{K_{T_o}}}{f_1 + \frac{f_3[T]}{K_{T_o}}} \right] \right\}}{1 + f_{-2} \left[\frac{1 + \frac{[T]}{K_{T_o}}}{f_1 + f_3 \frac{[T]}{K_{T_o}}} \right]} \tag{24}$$

$$\widetilde{V} = A/D = \frac{f_{-2} C_t}{1 + f_{-2} \left[\frac{1 + [T]/K_{T_o}}{f_1 + \frac{f_3[T]}{K_{T_o}}} \right]} \tag{25}$$

In the presence of T, $\widetilde{K}_{S_i}$ increases but there is comparatively little effect on V. The inhibition is therefore competitive, in contrast to the case of a non-transported analog.

(b) The rate of entry. The extracellular volume is ordinarily made much larger than that of the cells, so that $[S_o]$ may be treated as a constant. The initial concentration of substrate within the cells, $[\overline{S_i}]$, is zero. Eqn. 19 may now be written as

$$\frac{[S_i]}{t} = \frac{-A}{D} + \left(\frac{B}{D} + \alpha[S_o]\right) \cdot \frac{1}{t} \log\left(\frac{\alpha[S_o]}{\alpha[S_o] - [S_i]}\right) \tag{26}$$

A/D, as before, is a rate of transport; at sufficiently low $[S_o]$, in the absence of inhibitor it has the same value as V for exit: $f_{-2}C_t/(1 + f_{-2}/f_1)$. At saturating $[S_o]$ its value becomes $f_{-1}C_t K_{S_i}/(1 + f_{-2}/f_2)[S_o]\alpha$. B/D is a substrate affinity constant, as in the exit experiment, and its exact value also depends upon $[S_o]$.

The analysis of the type of inhibition seen with a competitive inhibitor under our three conditions is now as follows. With inhibitor capable of binding on both surfaces of the membrane the rate A/D declines at low values of $[S_o]$, but is constant at high values. The affinity constant B/D is little affected at low $[S_o]$ but increases at high $[S_o]$. In the former circumstance inhibition may be termed non-competitive, but in the latter, competitive. With inhibition restricted to the outside surface the transport rate represented by A/D declines and $\widetilde{K}_{S_i}$ remains about the same, regardless of $[S_o]$: inhibition is non-competitive. With inhibition solely on the inside the intercept A/D is unaffected but $\widetilde{K}_{S_i}$ gets bigger, at all levels of $[S_o]$: competitive inhibition.

III. Non-competitive mechanisms

When the mechanism is non-competitive there is no ambiguity; for in all experiments, as may be demonstrated by means of Eqn. 7, the kinetic behaviour is also non-competitive. As a result, such inhibitors are likely to provide little information about transport mechanisms. Depending on the symmetry of the system in binding inhibitor or substrate, inhibition may not be of the pure non-competitive type, where slope and intercept in a reciprocal plot are raised to exactly the same degree.

IV. Studies of active or passive transport

The forms of the rate expressions are identical whether $\alpha = 1$ (in non-active transport) or $\alpha \gg 1$ (in active transport).

V. Rules for interpreting inhibition kinetics

The detailed conclusions of the preceding sections are summarized in Table I. Several more general rules follow.

(i) A competitive mechanism can give rise to non-competitive kinetics, but not vice versa. Hence, if a substance inhibits non-competitively in some experiments but competitively in another the mechanism must be competitive.

(ii) The only unambiguous test of a competitive as against a non-competitive mechanism is provided by equilibrium exchange or exchange in the final steady state. If conclusions are to be drawn from a single type of experiment this one should be chosen. Otherwise it is the best foundation for interpreting other experiments.

(iii) If the mechanism is competitive, the inhibition pattern in zero *trans* exit and zero *trans* entry experiments establishes the sidedness of inhibition (whether binding occurs on both sides of the membrane, exclusively outside, or exclusively inside); whether the inhibitor undergoes transport on the carrier (i.e., is a competing substrate) and, with one exception where doubt remains, whether it penetrates into the cell either with or without assistance from the carrier. The expected patterns of inhibition in these experiments and in a third, infinite *trans* entry, are summarized in Table II. The latter experiment serves to add confirmation to the conclusions reached from the first two, and could as well be replaced by a Sen-Widdas exit experiment, which has essentially the same properties.

TABLE I

INHIBITION BEHAVIOR IN VARIOUS TRANSPORT EXPERIMENTS AS DEPENDENT ON THE SYMMETRY OF BINDING

The inhibitor or substrate analog can compete with the substrate for the carrier site, and may become bound to the carrier forms (a) on both the external and internal membrane surfaces, (b) only on the external surface, or (c) only on the internal surface (columns 3—5). The competitive analog may or may not undergo transport on the carrier (column 2). C/N (competitive/non-competitive) is a designation peculiar to the Sen-Widdas experiment, since the behavior indicates competition with substrate in the external medium, non-competition with that inside the cells. C, competitive kinetics; N, non-competitive or mixed competitive and non-competitive kinetics.

Experiment	Competitive analog	Site of inhibition		
		Both membrane surfaces	Outer membrane surface	Inner membrane surface
Zero *Trans* efflux	Non-transported	N	N	C
	Transported	C	No inhibition	C
Zero *Trans* influx	Non-transported	N	C	N
	Transported	C	C	No inhibition
Sen-Widdas exit	Non-transported	C/N	C/N	N
Equilibrium exchange or steady-state exchange	Non-transported	C	C	C
Infinite *Trans* influx	Non-transported	C	C	N
Efflux, integrated rate equation	Non-transported	N	N	C
	Transported	C		
Influx, integrated rate equation	Non-transported	N (low[S_o]) C (high[S_o])	N	C

(iv) A competing substrate which is transported at a rate comparable to that of the substrate under investigation never inhibits non-competitively. However, a very poorly transported substrate would resemble a non-transported analog in giving non-competitive, or partially non-competitive, inhibition.

(v) Efflux experiments over the whole course of transport show the same

TABLE II

INHIBITION KINETICS OBSERVED IN THREE DIFFERENT TRANSPORT EXPERIMENTS AS RELATED TO THE PROPERTIES OF THE INHIBITOR: WHETHER IT UNDERGOES TRANSPORT ON THE CARRIER, WHETHER IT ADDS TO CARRIER FORMS ON BOTH MEMBRANE SURFACES OR ONLY ON THE INNER OR OUTER FACE, AND WHETHER IT ENTERS THE CELLS (EITHER BY PASSIVE DIFFUSION OR VIA THE CARRIER)

Inhibitor and substrate add to the same carrier site except in the last case (bottom) where different sites are involved and the kinetics are non-competitive in all experiments. C, competitive; N, non-competitive; O, no inhibition.

Experiment			Characteristics of inhibitor		
Zero *trans* exit	Zero *trans* entry	Infinite *trans* entry	Carrier transport	Sidedness of inhibition	Penetration into cell
N	N	C	No	Both	Yes
N	C	C	No	Outer	?
C	N	N	No	Inner	Yes
C	C	C	Yes	Both	Yes
O	C	C	Yes	Outer	Yes
C	O	O	Yes	Inner	Yes
N	N	N	Non-competitive mechanism		

pattern of inhibition in relation to sidedness as initial rate studies. The corresponding influx experiments involve a unique inhibition pattern.

VI. Asymmetry in carrier systems

Strong asymmetry even in simple equilibrating transport systems may not be unusual, judging by observations on cytochalasin B and other inhibitors of glucose transport in erythrocytes. The observations on cytochalasin B are as follows. (i) Competitive binding of D-glucose and cytochalasin B to membrane preparations was demonstrated [1]. (ii) Non-competitive inhibition of transport was found in Sen-Widdas experiments [2,3]. (iii) Non-competitive inhibition of glucose entry was shown [2]. Rules summarized above indicate that (a) where both competitive and non-competitive behavior is seen, the mechanism cannot be non-competitive; (b) where a competitive mechanism produces non-competitive kinetics in entry and Sen-Widdas experiments, the inhibitor must enter the cell and become bound exclusively to sites on the inner membrane surface. The theory predicts pure competitive inhibition in zero *trans* exit experiments, and this has now been found [12].

As noted earlier, various alkyl derivatives of hexoses have been shown to be far more strongly bound to carrier on one side of the membrane than the other [4,5]. These compounds readily enter the cells, though not by means of the carrier [6,7]. Another interesting example, this time of structural asymmetry in the carrier rather than in its binding site, is that of the choline transport mechanism in erythrocytes, which undergoes reaction with *N*-ethylmaleimide (and inactivation) only when located on the inner surface of the membrane [11].

Asymmetry is requisite in active transport systems, where substrate disequilibrium is imposed through the agency of the carrier and at the expense of metabolic energy. An element of asymmetry impressed in the carrier may appear as a shift in any of the constants in the equation for α (Eqn. 3), relative to the non-energized state of the system. Non-transported substrate analogs able to pass through the cell membrane could be useful in studying such problems, since such shifts should be manifest in altered inhibition behavior.

So far we have spoken only of extreme asymmetry, where the inhibitor is bound far more strongly on one side of the membrane than the other. With moderate asymmetry different affinity constants are found on the two membrane surfaces. Table III summarizes the experimental parameters which are determined in initial rate experiments. One parameter is obtained in purely competitive inhibition (or in the competitive–non-competitive inhibition of a Sen-Widdas experiment) and two parameters in non-competitive inhibition, one obtained from ratios of slopes in plots of $1/v$ against $1/[S]$ and one from ratios of intercepts. In non-competitive inhibition which is formally pure, slope and intercept are raised by the inhibitor to exactly the same degree, and the two parameters are equal. In mixed competitive and non-competitive inhibition the parameters are unequal. It is seen that in nearly all cases the apparent inhibition constants are combinations of binding constants and ratios of flux constants, as are substrate binding constants.

Where an inhibitor binds preferentially, but not exclusively, on one side of

TABLE III

HALF-SATURATION CONSTANTS IN VARIOUS EXPERIMENTS FOR INHIBITORS HAVING DIFFERENT CHARACTERISTICS AND FOR SUBSTRATE (SEE TEXT FOR EQUATIONS)

In competitive inhibition only one constant is measured. In non-competitive inhibition there are two, one constant obtained from the change in slope in a reciprocal plot ($1/v \times 1/S$), the so-called competitive component, and another from the change in intercept, the so-called non-competitive component. When these are equal inhibition is described as purely non-competitive, but as 'mixed competitive and non-competitive' when the non-competitive component is considerably smaller than the competitive. The influence of the relative values of reorientation constants (f_1, f_{-1}, f_2 and f_{-2}) upon the ratio of the inhibition parameters will not be dealt with here, but must be taken into consideration in testing for predominant, but not exclusive, inhibitor addition on one side of the membrane.

Experiment	Substrate constant	Measured inhibition parameter	Site of inhibition			
			Both sides		Outside	Inside
			$K_{I_o} = K_{I_i}$	$K_{I_o} \neq K_{I_i}$	$[I_i] = 0$ or $K_{I_i} = \infty$	$[I_o] = 0$ or $K_{I_o} = \infty$
Zero *trans* efflux	$\frac{K_{S_i}(1 + f_{-1}/f_1)}{1 + f_{-2}/f_1}$	Competitive	K_{I_o}	$\frac{1 + f_{-1}/f_1}{1/K_{I_i} + f_{-1}/f_1 K_{I_o}}$	$K_{I_o}(1 + f_1/f_{-1})$	$K_{I_i}(1 + f_{-1}/f_1)$
		Non-competitive	$K_{I_o}(1 + f_1/f_{-2})$	$K_{I_o}(1 + f_1/f_{-2})$	$K_{I_o}(1 + f_1/f_{-2})$	—
Zero *trans* influx	$\frac{K_{S_o}(1 + f_1/f_{-1})}{(1 + f_2/f_{-1})}$	Competitive	K_{I_o}	$\frac{1 + f_1/f_{-1}}{1/K_{I_o} + f_1/f_{-1}K_{I_i}}$	$K_{I_o}(1 + f_1/f_{-1})$	$K_{I_i}(1 + f_{-1}/f_1)$
		Non-competitive	$K_{I_o}(1 + f_{-1}/f_2)$	$K_{I_i}(1 + f_{-1}/f_2)$	—	$K_{I_i}(1 + f_{-1}/f_2)$
Sen-Widdas exit	$\frac{K_{S_o}(1 + f_1/f_{-2})}{(1 + f_2/f_{-2})}$	Competitive	$K_{I_o}(1 + f_1/f_{-2})$	$K_{I_o}(1 + f_1/f_{-2})$	$K_{I_o}(1 + f_1/f_{-2})$	$\frac{K_{I_i}[S_i]}{K_{S_i}}(1 + f_{-2}/f_1$
		Non-competitive	—	—	—	$\frac{K_{I_i}[S_i]}{K_{S_i}}(1 + f_{-2}/f_2)$
Equilibrium exchange ($\alpha = 1$)	$\frac{K_{S_i}(1 + f_{-1}/f_1)}{(1 + f_{-2}/f_2)}$	Competitive	K_{I_o}	$\frac{(1 + f_{-1}/f_1)}{1/K_{I_i} + f_{-1}/f_1 K_{I_o}}$	$K_{I_o}(1 + f_1/f_{-1})$	$K_{I_i}(1 + f_{-1}/f_1)$
		Non competitive	—	—	—	—
Infinite *trans* entry	$\frac{K_{S_o}(1 + f_1/f_{-2})}{(1 + f_2/f_{-2})}$	Competitive	$K_{I_o}(1 + f_1/f_{-2})$	$K_{I_o}(1 + f_1/f_{-2})$	$K_{I_o}(1 + f_1/f_{-2})$	$\frac{K_{I_i}[S_i]}{K_{S_i}}(1 + f_{-2}/f_1)$
		Non-competitive	—	—	—	$\frac{K_{I_i}[S_i]}{K_{S_i}}(1 + f_{-2}/f_2)$

the membrane we expect mixed competitive and non-competitive inhibition, to a degree that varies with the experiment. Such behavior can be explained by postulating two different kinds of binding site, one competitive with substrate and the other non-competitive, though asymmetric addition is the simpler hypothesis and is easily tested. This hypothesis provides a simple explanation for observations on series of structurally related inhibitors with similar physical properties in which some members inhibit in a competitive, others in a mixed and still others in a non-competitive fashion, as was the case for inhibition of glucose transport in erythrocytes by steroid [12] and pyridine [13] derivatives. The relative affinities for the glucose carrier or the two membrane surfaces has in fact, been shown to be very sensitive to small changes in substrate structure [4,5].

VII. Reversible inhibitors as tools for discriminating among transport mechanisms

The occurrence of non-competitive kinetics with an inhibitor whose mechanism is competitive (as established by competitive binding studies or inhibition of exchange flux) can be expected of certain transport models, the present case being one, but not of others. For example, if there is only one form of free carrier and that form has sites on both sides of the membrane, then substrate on one side may actually compete with inhibitor on the other side. Further, with certain models only one inhibitor molecule may add to the carrier at a time, while with others the availability of sites on both sides of the membrane could enable two to bind. Such behavior is readily demonstrated in kinetic studies, and, hence, reversible competitive inhibitors capable of penetrating the cell should be of great value in confirming or excluding particular mechanisms. This aspect of the problem will be treated in a later publication.

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