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TESTING TRANSPORT MODELS WITH SUBSTRATES AND RÉVERSIBLE INHIBITORS

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Summary

The kinetic behavior of five models for biological transport, only one of which is based on the classical carrier mechanism, is investigated. All give hyperbolic substrate saturation curves in accord with experimental observations on many systems. Several simple kinetic tests with substrates and competitive inhibitors serve to exclude or confirm proposed models. The tests involve measuring rates of efflux of radioactive substrate in the presence of (i) a competitive inhibitor outside the cell; (ii) inhibitor inside and outside; and (iii) unlabeled substrate outside. Rules for testing hypothetical mechanisms are presented in tables which may be consulted directly, disregarding the mathematical derivation.

Introduction

Studies of membrane transport have relied, for their interpretation, upon an almost unquestioned acceptance of some version of the classical carrier model, the essence of which is that a substrate binding site in an entity called the carrier is alternately, and not simultaneously, exposed at either surface of the cell membrane. Where other models are alluded to, they have usually been either variations on this theme, in which different physical devices produce the same effect, or simple pores, which probably lack the complexity required to explain many transport phenomena. It is possible to account for transport, however, by models distinctly different from these; and yet, perhaps surprisingly, no systematic attempt appears to have been made to define the alter-

natives and to design tests by which they may be recognized or eliminated. This we undertake here. We begin by considering hypothetical mechanisms and grouping them according to expected uniformities in their kinetic behavior. Then, through analysis of the classes arrived at in this way, we seek criteria by which each may be ruled out. Previously such criteria, when applied to the simple carrier or the pore, have depended solely upon the behavior substrates. We now investigate the behavior of reversible inhibitors, and find them to be as incisive a diagnostic tool as substrates, capable of providing information that substrates cannot give. Together, inhibitors and substrates serve to discriminate among a variety of possible mechanisms.

The potential usefulness of reversible inhibitors in such studies was suggested by a recent analysis of competitive and non-competitive inhibition of transport, based on a general form of the classical carrier model [1]. Earlier, Lieb and Stein [2,3] had presented a treatment which aimed at establishing rejection criteria for the simple pore and the simple carrier models, involving only the behavior of substrates.

In the following analysis, we have recourse to several simple kinetic tests, which taken together can distinguish among a number of transport mechanisms. These are (1) the inhibition pattern in a zero trans exit experiment when an inhibitor is present outside the cell; (2) the dependence of the transport rate upon the concentration of an inhibitor present both inside and outside; and (3) the effect of substrate on the trans side of the membrane upon unidirectional flux. Five different transport models are considered and appropriate rate equations are derived, from which it is shown how each model presents different kinetic behavior. The results are summarized in tables that should be useful in planning and interpreting experiments.

Theory

The representation of transport mechanisms

In discussing physical models for transport it will be convenient to use the following descriptive terms; carrier, pore, gated channel, shuttle and mobile carrier:

(i) *Carrier.* In view of its long usage in a general sense, a carrier will be taken to encompass all mechanisms in which there is specific, saturable transport of substrate.

(ii) *Pore.* A continuous, open passage extends through the membrane and is accessible to substrate from both sides. Transport involves no moving element other than the substrate itself, and does not necessarily depend on the presence of substrate binding sites.

(iii) *Gated channel.* A moveable barrier in a channel shifts back and forth past a fixed substrate binding site. Hence only bound substrate molecules can reach the far side of the barrier and in this way undergo transport.

(iv) *Shuttle.* The substrate binding site moves past a fixed barrier in the membrane. In consequence, binding to this site is required for passage.

(v) *Mobile carrier.* Any specific saturable transport system dependent on the operation of a mobile element. This definition includes both the gate and the shuttle as defined above.

A reading of the literature on transport reveals an unfortunate lack of agreement as to the meaning of these terms. Part of the difficulty stems from a seemingly clear mechanistic implication of the word 'carrier', which has detracted from its acceptance as a general term. In addition the actual mechanism, when fully understood, may not correspond to a single category. A mechanism involving elements of both a gate and a shuttle, for example, is easily envisaged; one such is shown in Model III below. There is a tendency also to designate a gated channel as a pore, even though its properties would be distinct from those of an open passage through the membrane.

Transport mechanisms may be represented in two different ways, which should be distinguished: (a) Physical models. The transport process is represented diagrammatically, for example as a shuttle or a gated channel. (b) Kinetic models. The key intermediates in transport and the rates of their conversion are represented mathematically. Distinguishable physical models may reduce to the same kinetic description, as is the case for certain shuttles and gated channels.

The selection of kinetically distinguishable mechanisms

In addition to the classical carrier model, alternative mechanisms involving either a simple pore or a mobile carrier having two substrate binding sites simultaneously accessible to substrate appear to call for close examination, and it would be helpful to have simple kinetic tests available by which they could be confirmed or excluded. Varous such mechanisms have been invoked in experimental studies. For example pore models have often been favored in explaining fluxes of inorganic ions [4] as well as of larger molecules. Systems which depend on more than one substrate binding site have also been suggested, and in the case of the dicarboxylate transporter of *Escherichia coli* there is clear evidence, both genetic and structural, for two separable membrane binding proteins involved in transport [5]. Striking differences in the affinities of substrate analogs on the internal and external membrane surfaces have led to the suggestion that different inner and outer components may exist in the glucose carrier of erythrocytes [6].

We shall limit our treatment to models with no more than one binding site exposed to substrate either inside or outside the cell, that is, no more than two when both membrane faces are counted. Such systems give rise to simple hyperbolic substrate saturation curves, in agreement with observations on many facilitated transport systems, e.g. the much studied glucose carrier of erythrocytes [7]. Models involving two or more sites accessible to substrate in one pool would exhibit more complex kinetics.

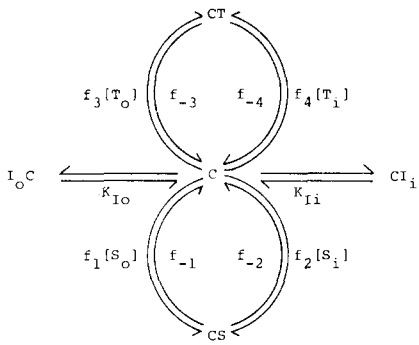
Transport systems may be static as in the case of a pore, or may involve a mobile element, as in a mobile carrier. Among those depending on a mobile element we shall deal with (a) mechanisms with two forms of free carrier and a substrate site alternately exposed to the two substrate pools; and (b) mechanisms with one form of free carrier, having substrate sites simultaneously accessible to both compartments. Among models based on the pore, in which the free carrier has only one form, those accommodating either one or two substrate molecules are considered.

Transport models

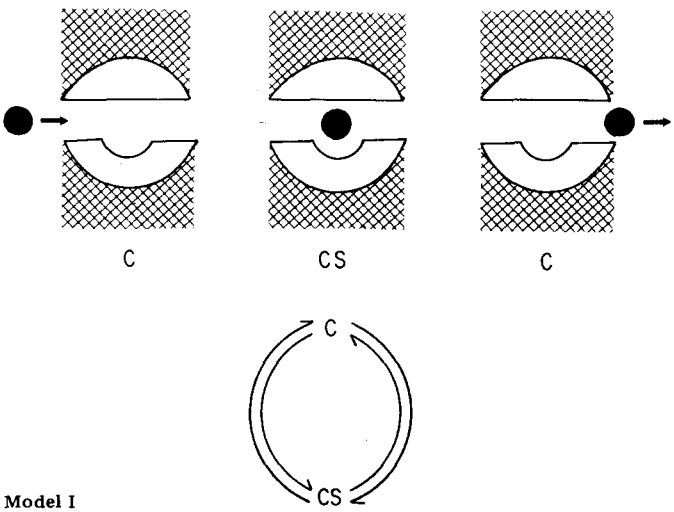
The models which we propose to analyze are described below. In each case the physical process is represented diagrammatically. The corresponding kinetic scheme is given in a simplified form, in which only those intermediates assumed to exist in significant amounts are shown. In general the physical representation chosen is not a unique determinant of the kinetic scheme.

Model I. A simple pore is represented which can be occupied by one substrate molecule at a time. The carrier exists in only one form and is accessible to substrate on both sides of the membrane.

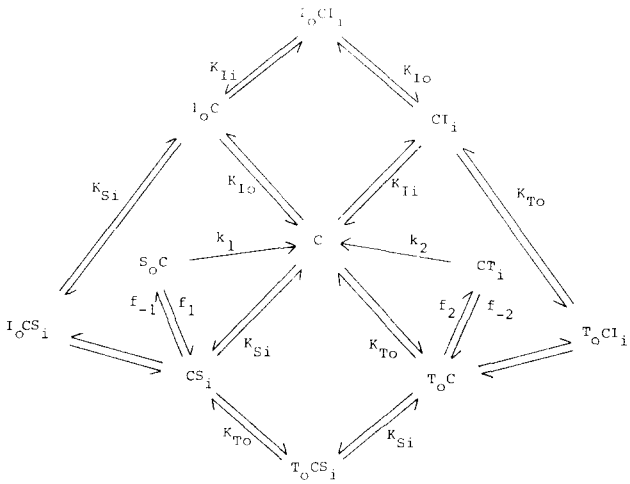
Occupancy applies only to that region of an opening through the membrane which limits the rate of substrate diffusion. This region could be very short even though a pore of uniform diameter extending across the whole membrane would accommodate many substrate molecules. To account for substrate specificity, there must be severe restrictions upon the molecular structures



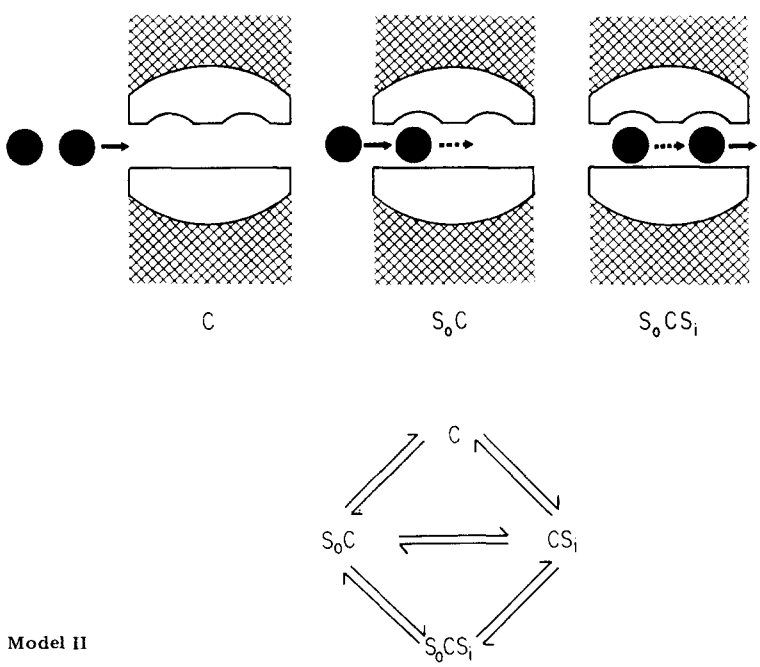
Scheme I. General transport scheme for the one-site, simple pore model (I), showing two substrates, S and T. The same substrate complex, CS, is formed whether substrate enters the pore from the external or internal solutions (S_o or S_i, respectively). This is also true for substrate T. An inhibitor, being unable to pass through the pore, forms a complex on one side of the membrane distinguishable from that on the other (I_oC and CI_i).



Model I

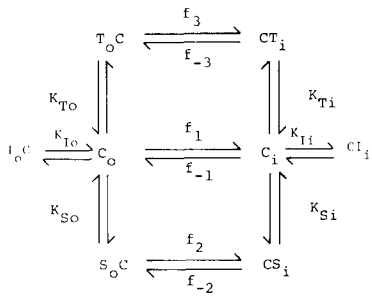


Scheme II. Transport scheme for the two-site, simple pore model (II), showing two substrates S and T and an inhibitor I. A ternary complex of carrier with two substrate molecules or one substrate and one inhibitor may be formed. The subscript i denotes a complex formed from substrate or inhibitor inside the cell, and o from outside. To simplify the full kinetic scheme as far as possible for our purpose, it is assumed that substrate T is present only externally (T_o) and substrate S only internally (S_i). The inhibitor is present in both compartments (I_o and I_i).

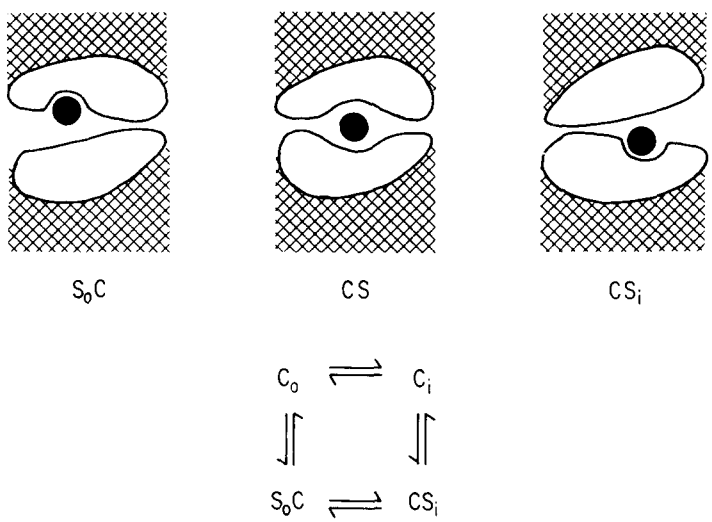


Model II

permitted to enter this passage, which for this reason would be the point of slowest movement, i.e. the rate-limiting step in diffusion. In addition, it would be impossible for two substrate molecules to pass one another at this point, with the result that flux in one direction would oppose flux in the other. Competitive inhibition is not easily explained, but would occur if an inhibitory sub-



Scheme III. The classical mobile carrier model (Model III), involving one substrate binding site and two forms of free carrier, one in which the site is exposed to the external, the other to the internal, solution. Transport depends on a carrier re-orientation step in which there is interconversion of the two carrier forms. The significance of subscripts i and o is as in the previous schemes.



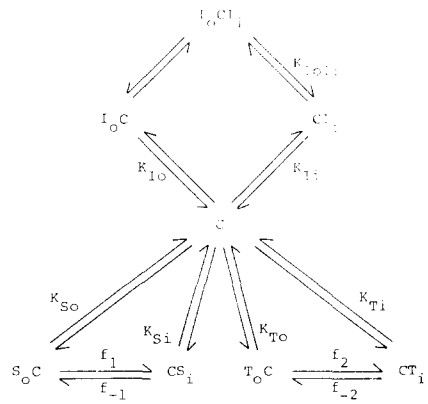
Model III

strate analog is bound outside the restrictive channel or if it partly enters this region but owing to steric restraints does not proceed further.

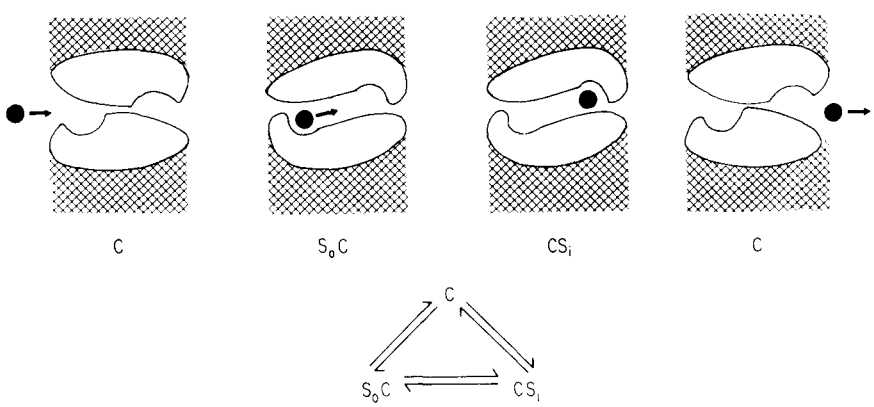
Model II. This is a simple pore with room for two substrate molecules, which in other respects is like the pore in Model I. Because the substrate must fit the pore closely there is interference between molecules moving in opposite directions.

The kinetic treatment of this model should apply as well to longer pores which accommodate more than two substrate molecules, for in any case the predicted behavior depends on the ability of molecules to enter the pore simultaneously from opposite compartments.

Model III. The carrier exists in two forms, one in equilibrium with substrate in the external pool, the other with substrate in the internal pool, and their interconversion is the essential step in translocation of substrate through the membrane. The nature of this reorientation step is necessarily left undefined. It may involve operation of a gate or a shuttle or a combination of the two, as in



Scheme IV. Mechanism involving a mobile element and one form of free carrier, as in Model IV, with substrate sites exposed to both pools, external and internal (subcripts o and i, respectively). Substrate may add to the external or internal site but not to both at the same time, since a conformational change related to transport occludes the second site following binding of substrate. Inhibitors, not undergoing transport, do not induce this conformational change. Hence a ternary complex may form, a carrier with two inhibitor molecules.

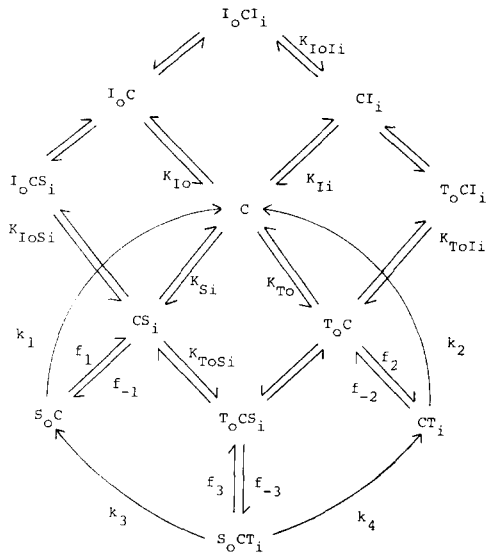


Model IV

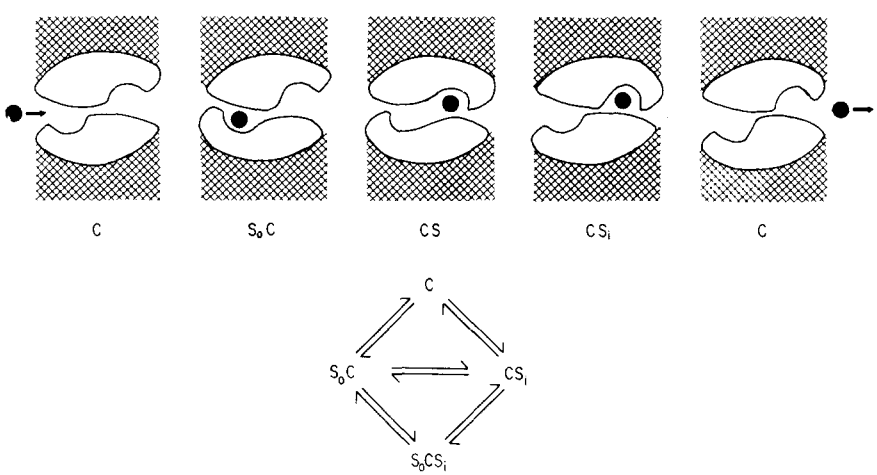
the diagram. The intermediate form of the carrier (CS) is assumed to be kinetically unimportant.

Model IV. A gated channel mechanism is represented having only one form of free carrier, with binding sites simultaneously available to substrate in both internal and external pools. Addition of a substrate molecule on either side induces a conformational change associated with the transport process which occludes the other site (S_oC and CS_i); in consequence a ternary complex of carrier and two substrate molecules is not formed. This conformational change is the essential step in transport and therefore should not occur with a competitive inhibitor. For this reason addition of an inhibitor at one site does not occlude the other; hence two inhibitor molecules may be bound simultaneously. The simplified kinetic scheme omits the initial complex since this is assumed to form transiently.

Model V. This is a gated channel mechanism similar to the previous one,



Scheme V. Mechanism involving a mobile element and one form of free carrier with a site exposed in both pools, as in Model V, where a ternary complex may form with two substrate molecules, two inhibitor molecules, or one substrate and one inhibitor. Movement of substrate may occur in the complex of two substrate molecules (f_3 and f_{-3}). For the sake of simplicity it is assumed that substrate T is present only externally (T_o) and substrate S only internally (S_i), as in the treatment of Scheme II.



Model V

except that two substrate molecules may add simultaneously to sites on opposite sides of the membrane. The kinetic scheme appears to be the same as that for the second pore model (Model II), but on account of the difference in mechanism, substrate molecules moving in opposite directions may now assist one another rather than interfere.

The dominant properties of the five models are summarized in Table I.

TABLE I

CHARACTERISTICS OF TRANPORT MODELS

Model	Number of sites per free carrier form	Number of free carrier forms	Involvement of carrier rearrangement step	Number of substrate molecules bound
I	1	1	–	1
II	2	1	–	2
III	1	2	+	1
IV	2	1	+	1
V	2	1	+	2

Tests of the Models

(1) Selection of inhibitors

A word should be said about the properties of the inhibitors to be used in these experiments. First, inhibitor and substrate, when present in the same compartment, must compete for binding to the carrier. Second, some experiments depend on an inhibitor present in the external medium only, while others involve an inhibitor inside the cell as well. The way in which these requirements may be fulfilled is now considered.

Our previous analysis of the kinetics of reversible inhibition for the classical carrier model [1] showed that the only unambiguous test of whether substrate and inhibitor can add to the same binding site is inhibition of equilibrium exchange (or exchange in the final steady state in active systems). The reason is obvious, since in this case substrate is always in a position to compete with the inhibitor. In experiments in which substrate is initially present on only one side of the membrane it may be unable to reach sites accessible to an inhibitor on the other side. Whatever the mechanism the kinetics of inhibition must be competitive in an equilibrium exchange experiment if the inhibitor binds at the substrate site.

An inhibitor restricted to the external solution is easily found. Inhibitors able to penetrate the cell are probably less common, but are known, as in the example of a number of glucose derivatives [8,9]. It is sometimes found that such inhibitors, though having access to both sides of the membrane, bind preferentially to carrier on one side [6,10]. A combination of two inhibitors may then be employed in the proposed experiments, one adding to external, the other to internal, sites. Where inhibitors with the required properties are unavailable it may be possible to seal a non-penetrating inhibitor inside cell ghosts or membrane vesicles.

(2) Kinetic analysis

Kinetic equations are now derived for the above models. The kinetic schemes are expanded to include two substrates, S (radioactive) and T (unlabeled), and a reversible competitive inhibitor I. The rate-limiting step is assumed to be movement of substrate through the membrane rather than dissociation from the binding site in all schemes except the first, where this would not be possible. Rate equations are given for initial rates (v) of unidirectional efflux of

substrate S under the conditions of inhibitor and substrate distribution required in the proposed experimental tests:

(a) Inhibitor is present outside (trans). $[I_i] = 0$; $[S_o] = [T_o] = [T_i] = 0$.

(b) Inhibitor is present on both sides. $[I_i] = [I_o] = [I]$; $[S_o] = [T_o] = [T_i] = 0$.

(c) Unlabeled substrate is present outside (trans). Inhibitor is absent. $[I_o] = [I_i] = 0$; $[S_o] = [T_i] = 0$. The equation derived for this third condition is applied to three different experimental situations: (i) The internal substrate concentration is held constant and the external concentration varied; (ii) the external concentration is constant and the internal varied; and (iii) the external and internal concentrations, which are equal, are varied.

Scheme I (one-site, pore)

Condition a: Inhibitor outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{\overline{K}_{S_i}}{[S_i]}\left(1 + \frac{[I_o]}{K_{I_o}}\right)\right\} \tag{1}$$

where $\overline{K}_{S_i} = (f_{-1} + f_{-2})/f_2$. Inhibition is competitive, since only the slope of a reciprocal plot ($1/v$ against $1/[S_i]$) is increased by the inhibitor.

Conditions b: Inhibitor both sides.

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

Inhibition is again competitive.

Condition c: Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{\overline{K}_{S_i}}{[S_i]}\left(1 + \frac{[T_o]}{\overline{K}_{T_o}}\right)\right\} \tag{3}$$

where $\overline{K}_{T_o} = (f_{-4} + f_{-3})/f_3$.

(i) $[S_i]$ constant, $[T_o]$ varied: When $[T_o] \gg [S_i]$, $v \to 0$.

(ii) $[T_o]$ constant, $[S_i]$ varied: Unidirectional efflux of substrate S is inhibited by external substrate, T_o, in a purely competitive manner.

(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$. As S and T represent the same substrate, $\overline{K}_{T_o} = \overline{K}_{S_o}$; and Eqn. 3 becomes

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{f_1}{f_2} + \frac{\overline{K}_{S_i}}{[S]}\right) \tag{4}$$

The relationship is linear. The maximum rate of equilibrium exchange is less than the maximum rate of zero trans efflux by the factor $(1 + f_1/f_2) = (1 + \overline{K}_{S_i}/\overline{K}_{S_o})$, as comparison of Eqns. 4 and 1 shows.

Scheme II (two-site, pore)

Condition a: Inhibitor outside.

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{[I_o]}{K_{I_o}} + \frac{K_{S_i}}{[S_i]}\left(1 + \frac{[I_o]}{K_{I_o}}\right)\right\} \tag{5}$$

Inhibition is purely non-competitive since slope and intercept are raised equally by the inhibitor.

Condition b: Inhibitor both sides.

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{[I]}{K_{I_o}} + \frac{K_{S_i}}{[S_i]}\left[1 + [I]\left(\frac{1}{K_{I_o}} + \frac{1}{K_{I_i}}\right) + \frac{[I]^2}{K_{I_i}K_{I_o}}\right]\right\} \tag{6}$$

Inhibition is mixed competitive and non-competitive, in that slope and intercept are both increased, but the slope more than the intercept. Dependence on the inhibitor concentration involves a square term, which is apparent in plots of $1/v$ against $[I]$.

Condition c: Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{K_{S_i}}{[S_i]}\right)\left(1 + \frac{[T_o]}{K_{T_o}}\right) \tag{7}$$

(i) $[S_i]$ constant, $[T_o]$ varied: As $[T_o] \to \infty$, $v \to 0$.
(ii) $[T_o]$ constant, $[S_i]$ varied: Efflux of substrate S is non-competitively inhibited by T_o.
(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$; $K_{T_o} = K_{S_o}$:

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{K_{S_i}}{K_{S_o}} + \frac{K_{S_i}}{[S]} + \frac{[S]}{K_{S_o}}\right) \tag{8}$$

Equilibrium exchange is subject to substrate inhibition, and as $[S] \to \infty$, $v \to 0$ *.

Scheme III (one-site, mobile)

Condition a: Inhibitor outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + \frac{f_{-2}}{f_1}\left(1 + \frac{[I_o]}{K_{I_o}}\right) + \frac{K_{S_i}}{[S_i]}\left[1 + \frac{f_{-1}}{f_1}\left(1 + \frac{[I_o]}{K_{I_o}}\right)\right]\right\} \tag{9}$$

This model can account for a variety of inhibition patterns. Pure non-competitive behavior results when $f_{-2} = f_{-1}$. If $f_{-2} > f_{-1}$, as in the case of accelerated exchange, inhibition tends towards the uncompetitive type. If $f_{-1} > f_{-2}$, where exchange is slower than zero trans flux, inhibition becomes partly competitive. A further complexity, as we shall demonstrate elsewhere, is that if the rate-limiting step in transport is dissociation of the carrier · substrate complex, inhibition will always be competitive. However slow dissociation was ruled out in one study [11], and is probably unlikely.

Condition b: Inhibitor both sides.

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

* In treating this model Lieb and Stein [2] neglected to take into account the formation of a complex with two substrate molecules, and consequently found the kinetic properties of the one-site and the two-site pores to be identical.

Depending on the values of the kinetic constants, the inhibitor may show various degrees of non-competitiveness.

Condition c: unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + f_{-2}\left(\frac{1 + [T_o]/K_{T_o}}{f_1 + f_3[T_o]/K_{T_o}}\right) + \frac{K_{S_i}}{[S_i]}\left[1 + f_{-1}\left(\frac{1 + [T_o]/K_{T_o}}{f_1 + f_3[T_o]/K_{T_o}}\right)\right]\right\} \tag{11}$$

(i) $[S_i]$ constant, $[T_o]$ varied: At low values of $[S_i]$, where $K_{Si}/[S_i] \gg 1$, relative rates in the presence of saturating levels of T_o and in the absence of T_o are given by:

$$\frac{v_{[T_o]\to\infty}}{v_{[T_o]=0}} = \frac{(1 + f_{-1}/f_1)}{(1 + f_{-1}/f_3)} \tag{12}$$

This ratio may be greater or less than unity, depending on the relative sizes of f_3 and f_1; when f_3 is the larger, exchange is more rapid than zero trans flux, and when smaller, less rapid.

(ii) $[T_o]$ constant, $[S_i]$ varied. Let T and S represent the same substrate, so that $f_3 = f_2$. The characteristic pattern of acceleration or inhibition by the unlabeled trans substrate, as exhibited in reciprocal plots, depends on the ratio of f_{-1} and f_{-2} (whereas the occurrence of acceleration as against inhibition is determined by f_1/f_2). When $f_{-2} > f_{-1}$, the most probable circumstance in the case of acceleration ($f_2 > f_1$), the pattern tends toward the uncompetitive, the intercept falling more than the slope (in the case of inhibition, rising more). When $f_{-1} > f_{-2}$ which is probable in inhibition, the pattern is competitive, the slope being altered more than the intercept. If $f_{-1} = f_{-2}$ the effect is of the non-competitive type, with slope and intercept identically altered.

(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$; $K_{T_o} = K_{S_o}$, $f_3 = f_2$, $f_{-3} = f_{-2}$.

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + \frac{f_{-2}}{f_2} + \frac{K_{S_i}}{[S]}\left(1 + \frac{f_{-1}}{f_1}\right)\right\} \tag{13}$$

The relationship between $1/v$ and $1/[S]$ is now linear. The ratio of V for exchange to that for zero trans efflux (from Eqns. 9 and 13) is equal to $(1 + f_{-2}/f_1)/(1 + f_{-2}/f_2)$, a quantity which we shall find useful in discriminating among the models.

Scheme IV (two-site, mobile, with site occlusion)

Condition a: Inhibitor outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{K_{S_i}}{[S_i]}\left(1 + \frac{[I]_o}{K_{I_o}}\right)\right\} \tag{14}$$

Competitive inhibition.

Condition b: Inhibitor both sides.

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{K_{S_i}}{[S_i]}\left[1 + [I]\left(\frac{1}{K_{I_i}} + \frac{1}{K_{I_o}}\right) + \frac{[I]^2}{K_{I_i}K_{I_oI_i}}\right]\right\} \tag{15}$$

Competitive inhibition, non-linear in [I].

Condition c: Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{K_{S_i}}{[S_i]}\left(1 + \frac{[T_o]}{K_{T_o}}\right)\right\} \tag{16}$$

(i) $[S_i]$ constant, $[T_o]$ varied: When $[T_o] >> [S_i]$, $v \to 0$.
(ii) $[T_o]$ constant, $[S_i]$ varied: Competitive inhibition.
(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$; $K_{T_o} = K_{S_o}$:

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{f_{-1}}{f_1} + \frac{K_{S_i}}{[S]}\right) \tag{17}$$

The ratio of the maximum rates of exchange and zero trans efflux (from Eqn. 13) is now $1/(1 + f_{-1}/f_1)$.

Scheme V (two-site, mobile, without site occlusion)

Condition a: Inhibitor outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{\left(1 + \frac{[I_o]}{K_{I_oS_i}}\right) + \frac{K_{S_i}}{[S_i]}\left(1 + \frac{[I_o]}{K_{I_o}}\right)\right\} \tag{18}$$

Non-competitive inhibition.

Condition b: Inhibitor both sides.

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{[I]}{K_{I_oS_i}} + \frac{K_{S_i}}{[S_i]}\left[1 + [I]\left(\frac{1}{K_{I_o}} + \frac{1}{K_{I_i}}\right) + \frac{[I]^2}{K_{I_i}K_{I_oI_i}}\right]\right\} \tag{19}$$

Non-competitive inhibition, non-linear in [I].

Condition c: Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1 + \frac{[T_o]}{K_{T_oS_i}} + \frac{K_{S_i}}{[S_i]}\left(1 + \frac{[T_o]}{K_{T_o}}\right)}{C_t(f_{-1} + f_{-3}[T_o]/K_{T_oS_i})} \tag{20}$$

(i) $[S_i]$ constant, $[T_o]$ varied. When $[S_i]/K_{Si} \ll 1$ the ratio of rates with saturating trans substrate ($[T_o] \to \infty$) and in the absence of T_o is equal to f_{-3}/f_{-1}, which could vary from zero to greater than one.
(ii) $[T_o]$ constant, $[S_i]$ varied. Inhibition or acceleration by trans substrate is non-competitive, provided that K_{To} and K_{SiTo} are of similar size.
(iii) Equilibrium exchange. $[S_i] = [T_o] = [S]$; $K_{To} = K_{So}$; $K_{ToSi} = K_{SoSi}$:

$$\frac{1}{v} = \frac{1 + \frac{[S]}{K_{S_oS_i}} + \frac{K_{S_i}}{[S]}\left(1 + \frac{[S]}{K_{S_o}}\right)}{C_t(f_{-1} + f_{-3}[S]/K_{S_oS_i})} \tag{21}$$

This relationship is obviously non-linear in [S] (unless $f_{-3} = f_{-1}$ and $K_{S_oS_i} = K_{S_o}$, when the presence of trans substrate has no effect on the rate).

Discussion

(1) Inhibitors

The usefulness of competitive inhibitiors in testing transport models is apparent from Table II, which summarizes the behavior of the five systems. It

TABLE II

INHIBITION PATTERNS IN SUBSTRATE EXIT EXPERIMENTS

Model	Inhibitor outside		Inhibitor both sides	
	Type	Concentration dependence	Type	Concentration dependence
I	competitive	[I]	competitive	$[I]^2$
II	non-competitive	[I]	mixed	$[I]^2$
III	non-competitive *	[I]	non-competitive **	$[I]^2$
IV	competitive	[I]	competitive	$[I]^2$
V	non-competitive	[I]	mixed	$[I]^2$

* See discussion under Eqn. 9.
** See discussion under Eqn. 10.

is seen that a trans inhibitor (i.e. inhibitor and substrate occupying opposite sides of the membrane) produces non-competitive inhibition in some systems, and competitive inhibition in others. In all cases the dependence of the rate on the inhibitor concentration is linear. With inhibitor present on both sides, inhibition may be competitive, non-competitive, or a combination of the two (mixed). The dependence on the inhibitor concentration now reveals the number of substrate binding sites available at a single moment on either membrane surface, being linear for some models and related to the square of the concentration in others. These tests distinguish all but two of the models, namely II and V. It is to be noted that the simultaneous existence of internal and external binding sites should become obvious in inhibitor studies, but may be masked in experiments with substrates. The latter, however, reveal properties of the system not disclosed by inhibitors.

(2) Unlabeled trans substrates

Tests with a trans substrate are summarized in Table III. In three of the models (I, II and IV) inhibition is inevitable. In Model V inhibition is possible, but depending on rate constants acceleration may occur. In model III either acceleration or the absence of any effect is expected of a good substrate, though inhibition is seen if loaded carrier re-orientates more slowly than free carrier ($f_1 > f_2$).

In many respects the behavior of Models II and V is similar, but the occurrence of acceleration in V but not II would distinguish between them. Further, a trans substrate in Model II would reduce the rate to zero. In Model V on the other hand retardation may be only partial.

The inhibition produced by trans substrate may be competitive or non-competitive in different models and this too serves to distinguish among them (second column). The characteristics of acceleration in Models III and V may also differ (third column). In Model V acceleration is purely 'non-competitive', in the sense that at varying concentrations of the labeled substrate whose movement is followed the fractional increase in rate due to trans substrate is a constant. In Model III the behavior is governed by Eqn. 11. Where T and S are identical substrates, $f_3 = f_2$ and $K_{To} = K_{So}$. Clearly, acceleration occurs when $f_2 > f_1$ (i.e. loaded carried re-orientates faster than free carrier). The form that

TABLE III

EFFECTS OF TRANS SUBSTRATE (UNLABELED T_0) ON SUBSTRATE EFFLUX (LABELED S_i)

Model	Type of effect	Characteristic pattern [a]		$\frac{V_{ex}}{V_{in}} + \frac{V_{ex}}{V_{out}}$ [b]	Dependence of equilibrium exchange rates on [S] [c]
		Inhibtion	Acceleration		
I	Inhibition	competitive	—	1	hyperbolic
II	Inhibition	non-competitive	—	0	substrate inhibition
III	Can accelerate	competitive	uncompetitive	$1 + \left(\frac{1/f_1 + 1/f_{-1}}{1/f_2 + 1/f_{-2}}\right)$	hyperbolic
IV	Inhibition	competitive	—	1	hyperbolic
V	Can accelerate	non-competitive	non-competitive	$f_{-3}\left(\frac{1}{f_1} + \frac{1}{f_{-1}}\right)$	sigmoid, hyperbolic, or substrate inhibition

[a] The patterns seen with a trans substrate and a trans inhibitor (Table II) are necessarily related since both depend on f_{-1}/f_{-2}.

[b] V_{ex}, V_{in} and V_{out} are maximum rates of exchange, influx and efflux, respectively. Equations for zero trans influx were omitted in the text, for the sake of brevity. They are easily found by inspection of equations for zero trans efflux and the corresponding transport schemes, since influx and efflux expressions mirror one another, with interchange of entry and exit rate constants and concentrations.

[c] $[S] = [S_i] = [T_0]$.

[d] Hyperbolic if $f_{-1} = f_{-3}$, in which case a trans substrate has no effect on the rate (second column). Sigmoid if $f_{-3} > f_{-1}$, when a trans substrate accelerates efflux. Substrate inhbition if $f_{-1} > f_{-3}$.

acceleration takes depends on the relative values of f_{-1} and f_{-2}, as inspection of the rate equation shows.

The expression $(V_{ex}/V_{in}) + (V_{ex}/V_{out})$, i.e. the sum of the ratio of exchange and influx rates plus the ratio of exchange and efflux rates (fourth column), is also informative. In Models I and IV the value is unity, in Model II it is zero, in model III it is greater than unity, and in Model V it may have any value depending on the relative rate constants in the scheme. Only model V can give a value between 0 and 1.

As shown in the last column of Table III, reciprocal plots for equilibrium exchange are non-linear in Model II and may be non-linear in Model V.

(3) Obligatory exchange

Many of the transport systems of the mitochondrial inner membrane function exclusively under conditions of exchange. Only Models III and V can account for this behavior. In Model III obligatory exchange results if free carrier cannot undergo re-orientation ($f_1 = f_{-1} = 0$), and in Model V if substrate movement occurs only in the ternary complex of carrier with two substrate molecules ($f_1 = f_{-1} = f_2 = f_{-2} = 0$).

(4) Limitations of pore models

After all relevant tests have been applied, ambiguity should remain in only one special case: Schemes II and V are indistinguishable if exchange in the latter system does not occur at saturating substrate concentrations, owing to a failure of substrate interchange in the CS_2 complex ($f_{-3} = 0$). However any pore

mechanism such as Scheme II suffers from a weakness which no semi-permeable membrane could afford except possibly in the case of small ions such as Na^+ and K^+. The difficulty is that all molecules smaller than the substrate should be able to navigate the channel (baring electrostatic repulsion). Since transport systems exist for compounds as large as disaccharides, nucleosides, uridine diphosphate glucose, arginine and phenylalanine, and ATP and ADP, high selectivity for smaller molecules would be difficult to account for [12]. In any case specificity is not easily explained, since binding sites in a pore would retard molecules of high affinity rather than the facilitate their movement. Binding sites would accord better with the specificity of inhibition by non-transported substrate analogs. Substrate specificity is more likely to be explained by a sieving action, as suggested by Keynes [4], resulting from exclusion on the basis of either size or electrostatic charge.

In our analysis we have postulated that a simple pore could not exhibit accelerated exchange (Table II), but it is alleged that unstirred layers of sufficient depth at the surface of the membrane could produce this behavior [13]. A labeled substrate, once having traversed the pore, would remain for a time in the unstirred layer and could therefore return; an unlabeled trans substrate would compete in this back reaction and increase the net flux of label. However a layer of the required dimensions is found to be a practical impossibility [14—17]. Even without this objection the hypothesis is questionable, for it depends on substrate molecules passing simultaneously in opposite directions within the rate-limiting segment of the pore. If this is not possible a trans substrate at high concentration eliminates flux altogether (Eqns. 3 and 7).

(5) Active transport

In our earlier analysis [1] of reversible inhibition for a mechanism of the classical carrier type (III), the characteristic kinetic patterns produced by an inhibitor were shown to be identical whether transport was facilitated (non-active) or concentrative (active). Similarly the patterns found here for other models should apply to active as well as facilitated transport systems.

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