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TESTING CARRIER MODELS OF COTRANSPORT USING THE BINDING KINETICS OF NON-TRANSPORTED COMPETITIVE INHIBITORS

R. JAMES TURNER * and M. SILVERMAN

Department of Medicine, University of Toronto, Medical Science Bldg. Rm. 7226, Toronto, Ontario M5S 1A8 (Canada)

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Summary

The kinetic equations representing the binding of a non-transported competitive inhibitor are derived from three variations of the carrier model of cotransport. These are (a) the model in which the binding sequence of activator and substrate is random (random bi-bi); (b) the model in which activator must bind before substrate (ordered bi-bi, activator essential), and (c) the model in which substrate must bind before activator (ordered bi-bi, activator non-essential). In general it is found that the kinetic equations for inhibitor binding are considerably simpler and easier to test than the corresponding transport equations. The effect of *trans*-inhibitor, transported substrate, activator concentration and membrane potential on inhibitor binding are examined in some detail. The use of these results to test and characterize the three transport models is emphasized. Applications to transport mechanisms which are not of the mobile carrier type are also discussed. A summary of relevant experimental data interpreted in terms of the theoretical models concludes the paper.

Introduction

It is now widely accepted that many organic solutes are transported across cell membranes against their electrochemical gradients by secondary active cotransport processes. The driving forces for these systems are provided by

* Present address: Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, 20205, U.S.A.

Abbreviations: NPG, *p*-nitrophenyl-α-D-glucopyranoside; TMG, methyl-1-thio-β-D-galactopyranoside.

the transmembrane electrochemical gradients for the cotransported species. These are typically monovalent cations such as Na^+ or H^+ which are actively pumped across the membrane at some other location distinct from the solute transport site. This coupling of organic solute transport to active ion transport is often referred to as the 'gradient hypothesis' (see Ref. 1 for a recent review).

Transport systems making use of this principle have now been described for a variety of cell types [1]. However, to date, relatively few kinetic studies designed to test the applicability of various theoretical models of the cotransport mechanism have been carried out. This is due at least in part to the complexity of the kinetic equations predicted by even the simplest models of coupled transport [2] and to the resulting difficulties involved in designing and interpreting experiments to distinguish between them.

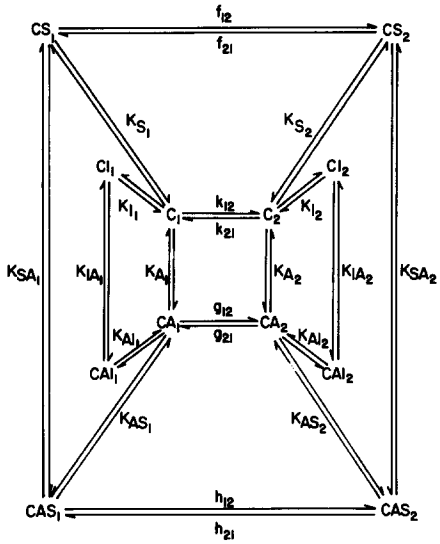
It is the purpose of the present paper to illustrate that the binding kinetics of non-transported competitive inhibitors can provide considerable information about cotransport processes and thus may be used to complement results obtained from transport studies. In particular we demonstrate that for models of the carrier type the kinetic equations representing the binding of such an inhibitor are considerably simpler than the corresponding equations for substrate transport. This is a direct result of the less complex interaction of the inhibitor with the carrier (binding for the inhibitor vs. binding plus translocation for the substrate). Although this simplification limits in some ways what can be learned about the system, the binding equations still retain many features which are characteristic of the assumptions of the transport model. By taking advantage of this fact we describe a number of qualitative and quantitative tests using inhibitor binding studies which may be employed to distinguish between and characterize several variations of the carrier model for cotransport.

We conclude with a discussion of the published experimental results on inhibitor binding to cotransport systems.

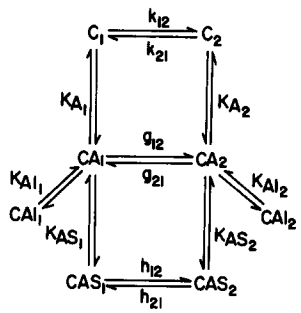
Theoretical derivations

Description of the models

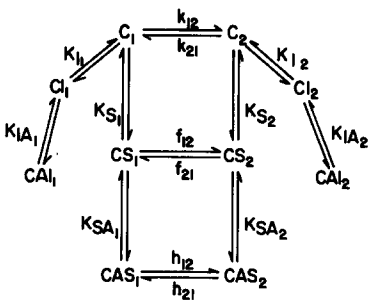
As a basis for the work presented in this paper we will first give the solution to the completely asymmetric 'general model' shown schematically in Fig. 1a. Here we refer to the two cotransported species as the substrate, S, and the activator, A, since such a distinction is commonly made in physiological systems. In the figure the 'free' carrier on side n of the membrane is represented by C_n, the carrier plus bound substrate by CS_n, the carrier plus bound activator by CA_n and the carrier plus bound activator and substrate by CAS_n. The external and internal faces of the membrane are labelled by $n = 1$ and $n = 2$, respectively. The rate constants for the translocation of the various free and loaded carrier species across the membrane are designated k_{12}, f_{12}, g_{12} and h_{12} for inward diffusion and k_{21}, f_{21}, g_{21}, and h_{21} for outward diffusion. The inhibitor, I, competes with the substrate for its binding site on the carrier. For completeness we have assumed that the inhibitor may bind to the carrier on both membrane faces, however, neither CI_n nor CAI_n are allowed to move across the membrane. The dissociation constants K_{A_n}, K_{AS_n}, K_{AI_n}, etc. which



(a) GENERAL MODEL



(b) AS MODEL



(c) SA MODEL

Fig. 1. Schematic representations of the three cotransport models analyzed in this paper (see text for details). The general model is analogous to a so-called 'random bi-bi' mechanism in enzyme kinetics [13] since the order of binding of substrate and activator is arbitrary. The AS and SA models are analogous to 'ordered bi-bi' enzyme mechanisms [13]. In the AS model the presence of activator is essential for substrate transport whereas in the general and SA models transport can occur in the presence or absence of activator.

characterize the various binding events at the two membrane faces are discussed below.

Two special cases of our general model will also be treated in detail in this paper. These are the 'AS model' shown in Fig. 1b in which the activator must bind to the carrier before the substrate (or inhibitor) and the 'SA model' show in Fig. 1c in which the substrate (or inhibitor) must bind before the activator.

Although to our knowledge no analysis of the effects and kinetics of inhibitor binding to cotransport models has been published previously, several authors have derived transport rate equations in the absence of inhibitors for models similar to those shown in Fig. 1 [3—8]. For example, Goldner et al.

[3], Schultz and Curran [4] and Jacquez [5] have solved simplified versions of the transport scheme represented by Fig. 1a. However, in contrast to our completely asymmetric general model, Goldner et al. [3] assume complete symmetry, Schultz and Curran [4] assume symmetry of the diffusional rate constants (i.e. $k_{12} = k_{21}$, $g_{12} = g_{21}$, etc.) and Jacquez [5] assumes symmetry in the binding reactions at the two sides of the membrane (i.e. $K_{A_1} = K_{A_2}$, $K_{AS_1} = K_{AS_2}$, etc.) A related but more complex transport model has also been treated by Heinz et al. [6] and Geck and Heinz [7].

Kotyk and Janacek [2] and Kotyk [8] have derived the solution to a completely symmetric variation of Fig. 1a in which a distinction is made between two forms of the fully loaded carrier, one resulting from the reaction $CAS_n \rightleftharpoons CA_n + S_n$ and the other from $CSA_n \rightleftharpoons CS_n + A_n$. Several authors have also solved transport schemes similar to our AS and SA models in the absence of inhibitors. The most general treatments are due to Stein [9] and Hill [10].

In order to simplify their transport equations, some authors [3,8,11,12] have considered the possibility that the partially loaded forms of the carrier, CA_n and CS_n, may not be mobile, i.e., that $g_{12} = g_{21} = 0$ and $f_{12} = f_{21} = 0$. In order to retain the generality of our results, we have not made this assumption.

Finally, it should be stressed that we refer to the models shown in Fig. 1 as being of the 'carrier type' for historical reasons only. Such transport schemes are often discussed in terms of a 'mobile carrier' shuttling back and forth across the membrane, however, they are in fact considerably more general. These models assume only that the binding sites of the transporter are alternately exposed on one or the other side of the membrane and that these translocations are characterized by the rate constants k_{12}, k_{21}, g_{12}, g_{21}, etc. No further assumptions about the physical mechanism of the transport event are made. The mobile carrier represents only one possible physical interpretation of this scheme. Another will be discussed later in this paper.

Assumptions

We make the usual assumptions associated with the equilibrium carrier model [2,5,6], namely that:

1. The rate-limiting step in the transport (binding) process is the movement of the transporter binding site from one membrane surface to the other; thus, the transporters are in equilibrium with the ligands at the membrane faces.

2. The total number of transporters, free and loaded, is constant and equal to C_0.

3. There is no net movement of transporter binding sites from one face of the membrane to the other; in other words, a steady state exists at the time of measurement.

In addition we assume that:

4. Inhibitor, I, is not transported or so poorly transported that its interaction with the carrier may be essentially regarded as a binding event.

5. The transporter itself is not primary active.

Solution of the 'general model'

It can be seen from the schematic representation of the general model in Fig. 1a that the undirectional flux of substrate from side n to side m of the

membrane is given by $f_{nm}[CS_n] + h_{nm}[CAS_n]$ and that the total inhibitor bound to side n is given by $[CI_n] + [CAI_n]$. Thus a complete solution consists of deriving expressions for $[CS_n]$, $[CAS_n]$, $[CI_n]$ and $[CAI_n]$ in terms of the parameters of the model and the substrate and activator concentrations (as is commonly done in studies of transport models we use concentrations in place of chemical activities).

From assumption 1 above and Fig. 1a we have that the concentrations of carrier and ligand species at side n of the membrane are related by the dissociation constants defined by

$$K_{S_n} = \frac{[C_n][S_n]}{[CS_n]}, \qquad K_{SA_n} = \frac{[CS_n][A_n]}{[CAS_n]}, \qquad K_{AS_n} = \frac{[CA_n][S_n]}{[CAS_n]},$$

$$K_{I_n} = \frac{[C_n][I_n]}{[CI_n]}, \qquad K_{IA_n} = \frac{[CI_n][A_n]}{[CAI_n]}, \qquad K_{AI_n} = \frac{[CA_n][I_n]}{[CAI_n]},$$

$$K_{A_n} = \frac{[C_n][A_n]}{[CA_n]}$$

It follows directly from the above equations that

$$K_{S_n} K_{SA_n} = K_{A_n} K_{AS_n} \tag{1a}$$

$$K_{I_n} K_{IA_n} = K_{A_n} K_{AI_n} \tag{1b}$$

In order to keep our mathematical expressions as compact as possible we introduce the following 'relative concentrations'

$$s_n = \frac{[S_n]}{K_{S_n}}, \qquad s'_n = \frac{[S_n]}{K_{AS_n}}, \qquad a'_n = \frac{[A_n]}{K_{SA_n}};$$

$$i_n = \frac{[I_n]}{K_{I_n}}, \qquad i''_n = \frac{[I_n]}{K_{AI_n}}, \qquad a''_n = \frac{[A_n]}{K_{IA_n}}$$

$$a_n = \frac{[A_n]}{K_{A_n}}$$

Thus from Eqn. 1a and b we have that

$$s_n a'_n = s'_n a_n \tag{2a}$$

$$i_n a''_n = i''_n a_n \tag{2b}$$

Using assumptions 2—4 and some standard algebra we find that the solution to the general model for side 1 of the membrane can be expressed in the following simple form

$$\begin{aligned} [CS_1] &= s_1 Q_{12}, \qquad [CAS_1] = s_1 a'_1 Q_{12} \\ [CI_1] &= i_1 Q_{12}, \qquad [CAI_1] = i_1 a''_1 Q_{12} \end{aligned} \tag{3a}$$

The quantity Q_{12} which occurs in all of these expressions is given by

$$Q_{12} = \frac{C_0 F_{21}}{\alpha_1 F_{21} + \alpha_2 F_{12}} \tag{3b}$$

where

$$F_{nm} = k_{nm} + a_n g_{nm} + s_n f_{nm} + s_n a'_n h_{nm} \tag{4a}$$

and

$$\alpha_n = 1 + a_n + s_n + i_n + s_n a'_n + i_n a''_n \tag{4b}$$

In Eqn. 3a and b above as well as in all subsequent results given in this paper the corresponding solutions for side 2 of the membrane are simply obtained by exchanging the roles of the subscripts 1 and 2.

In the above derivations we have not yet made use of assumption 5, that the transporter is not primary active. Thus Eqns. 3 and 4 are also valid for the case of active transport. In the case of a secondary active cotransport mechanism the Second Law of Thermodynamics requires that the parameters of the model be related in such a way that when the electrochemical potential differences for both substrate and activator across the membrane are zero the net flux of each of these species is zero. Applying this condition in the presence of substrate only, activator only and substrate and activator together we find the following constraints on the parameters of the general model:

$$K_{S_2} f_{12} k_{21} e^{u_S} = K_{S_1} f_{21} k_{12} \tag{5a}$$

$$K_{A_2} g_{12} k_{21} e^{u_A} = K_{A_1} g_{21} k_{12} \tag{5b}$$

$$K_{SA_2} h_{12} f_{21} e^{u_A} = K_{SA_1} h_{21} f_{12} \tag{5c}$$

and

$$K_{AS_2} h_{12} g_{21} e^{u_S} = K_{AS_1} h_{21} g_{12} \tag{5d}$$

where

$$u_S = \frac{z_S F}{RT} \Delta\psi \tag{6a}$$

and

$$u_A = \frac{z_A F}{RT} \Delta\psi \tag{6b}$$

Here F, R and T have their usual thermodyamic definititions, z_S and z_A are the electrical charges on the substrate and activator, respectively, and $\Delta\psi = \psi_2 - \psi_1$ is the electrical potential difference (membrane potential) between side 2 and side 1 of the membrane. Only three of the above four constraints are in fact mathematically independent, since given any three the fourth can always be derived from them using Eqn. 1a.

The kinetics of substrate flux

Our main concern in this paper is with the kinetics of inhibitor binding, however, for completeness and later reference we include here the expressions for the substrate flux equations in the general model of Fig. 1a. As previously stated the unidirectional flux of substrate from side 1 to side 2 of the membrane, $J_S^{1\to 2}$, is given by $f_{12}[CS_1] + h_{12}[CAS_1]$. From Eqns. 3 and 4 it can be

shown that his quantity can be written in Michaelis-Menten form as

$$J_S^{1\to 2} = \frac{V_S^{1\to 2}[S_1]}{K_S^{1\to 2} + [S_1]} \tag{7}$$

where $V_S^{1\to 2}$, the maximum velocity of transport, and $K_S^{1\to 2}$, the 'Michaelis constant' for transport, are given by

$$V_S^{1\to 2} = \frac{C_0(f_{12} + a_1' h_{12})F_{21}}{(1 + a_1')F_{21} + \alpha_2(f_{12} + a_1' h_{12})} \tag{8a}$$

and

$$K_S^{1\to 2} = K_{S_1} \frac{(1 + a_1 + i_1 + a_1'' i_1)F_{21} + \alpha_2(k_{12} + a_1 g_{12})}{(1 + a_1')F_{21} + \alpha_2(f_{12} + a_1' h_{12})} \tag{8b}$$

The quantities F_{12}, F_{21} and α_2 are as previously defined in Eqn 4a and b.

A detailed study of the substrate flux equations arising from the models treated here is planned for a future publication.

The kinetics of inhibitor binding

The total inhibitor bound to side 1 of the membrane is made up of two components, $[CAI_1]$ and $[CI_1]$. These correspond to inhibitor binding to two subpopulations of carriers, one to which activator is bound and the other to which it is not bound. Using Eqns. 3 and 4 we find that each of these components can be expressed in Michaelis-Menten form as

$$[CI_1] = \frac{C_0}{1 + a_1''} \cdot \frac{[I_1]}{K_{d_1}^{app} + [I_1]} \tag{9a}$$

and

$$[CAI_1] = \frac{C_0 a_1''}{1 + a_1''} \cdot \frac{[I_1]}{K_{d_1}^{app} + [I_1]} \tag{9b}$$

where

$$K_{d_1}^{app} = K_{I_1} \frac{\alpha_2 F_{12} + (1 + a_1 + s_1' + a_1' s_1)F_{21}}{F_{21}(1 + a_1'')} \tag{10}$$

As one would expect the number of binding sites associated with $[CI_1]$, namely $C_0/(1 + a_1'')$, decreases with increasing activator concentration while the number of sites associated with $[CAI_1]$, $C_0 a_1''/(1 + a_1'')$, increases. The quantity $K_{d_1}^{app}$ is discussed below.

In experimental binding studies it is the sum of these two components of inhibitor binding which is commonly measured. From Eqn. 9a and b we have that $[CI_1] + [CAI_1]$, the total inhibitor bound to side 1 of the membrane is given by the simple expression

$$\frac{C_0[I_1]}{K_{d_1}^{app} + [I_1]} \tag{11}$$

Thus the total number of inhibitor binding sites is simply equal to C_0, the total number of transporters, and the apparent dissociation constant for inhibitor binding, $K_{d_1}^{app}$, is given by Eqn. 10. It should be stressed that $K_{d_1}^{app}$ is a complex quantity which incorporates both the binding constants K_{I_1} and K_{AI_1} of the inhibitor to the carrier as well as a number of other model parameters. In this respect it is more properly interpreted in Eqns. 9 and 11 as a half-saturation constant rather than a true binding constant.

The mathematical simplicity of inhibitor binding over substrate flux can be readily appreciated by comparing the binding Eqns. 10 and 11 to the corresponding flux Eqns. 7 and 8. In the case of inhibitor binding any variation in $[A_1]$, $[A_2]$, $[S_1]$, $[S_2]$ or $[I_2]$ must be expressed simply as a change in apparent affinity since the total number of binding sites, C_0, is a constant. By contrast in the case of substrate flux both $V_S^{1\to 2}$ and $K_S^{1\to 2}$ can change simultaneously with variations in $[A_1]$, $[A_2]$, $[S_2]$ or $[I_2]$ ($K_S^{1\to 2}$ is also a function of $[I_1]$).

Solutions to the AS and SA models

The AS and SA models may each be regarded as limiting cases of the general model in which the binding of the activator and substrate (or inhibitor) to the carrier must occur in a specific order. These requirements may be expressed as a set of mathematical conditions which when applied to Eqns. 3 and 4 reduce them to the solutions of the appropriate model. These conditions are given below:

To obtain the solutions of the AS model from those of the general model let

$f_{nm} \to 0$

$K_{S_n} \to \infty$ and $K_{SA_n} \to 0$ such that $K_{S_n}K_{SA_n} \to K_{A_n}K_{AS_n}$

$K_{I_n} \to \infty$ and $K_{SI_n} \to 0$ such that $K_{I_n}K_{IA_n} \to K_{A_n}K_{AI_n}$

To obtain the solutions of SA model from those of the general model let

$g_{nm} \to 0$

$K_{A_n} \to \infty$ and $K_{AS_n} \to 0$ such that $K_{A_n}K_{AS_n} \to K_{S_n}K_{SA_n}$

$K_{A_n} \to \infty$ and $K_{AI_n} \to 0$ such that $K_{A_n}K_{AI_n} \to K_{I_n}K_{IA_n}$

The expressions for $V_S^{1\to 2}$, $K_S^{1\to 2}$ and $K_{d_1}^{app}$ in each of these simpler models can likewise be obtained by carefully applying the above conditions to Eqns. 8 and 10. Note that in the case of the AS Model as $[A_1] \to 0$, $K_{d_1}^{app} \to \infty$, corresponding to the fact that in the absence of activator there can be no inhibitor binding in this model.

The explicit expressions for $K_{d_1}^{app}$ in the general, AS and SA models in the absence of substrate are given in Table I for future reference. We use the obvious notational convention $K_{d_1}^{app}([S_1] = [S_2] = 0)$ to refer to these quantities.

Note also that only the thermodynamic constraints given by Eqn. 5b and d apply to the AS model while only those given by Eqn. 5a and c apply to the SA model.

TABLE I

EXPRESSIONS FOR $K_{d_1}^{app}([S_1] = [S_2] = 0)$, THE APPARENT DISSOCIATION CONSTANT OF INHIBITOR BINDING IN THE ABSENCE OF TRANSPORTED SUBSTRATE

Model	$K_{d_1}^{app}([S_1] = [S_2] = 0)$
General	$K_{I_1} \dfrac{(1 + a_2 + i_2 + i_2 a_2'')(k_{12} + a_1 g_{12}) + (1 + a_1)(k_{21} + a_2 g_{21})}{(k_{21} + a_2 g_{21})(1 + a_1'')}$
AS	$K_{AI_1} \dfrac{(1 + a_2 + i_2'' a_2)(k_{12} + a_1 g_{12}) + (1 + a_1)(k_{21} + a_2 g_{21})}{(k_{21} + a_2 g_{21}) a_1}$
SA	$K_{I_1} \dfrac{k_{12}(1 + i_2 + i_2 a_2'') + k_{21}}{k_{21}(1 + a_1'')}$

Testing inhibitor binding models

Using the preceding theoretical formulation it is possible to design experiments to test the applicability of the various models of inhibitor binding shown in Fig. 1 to a given experimental system. In this section we present a number of the more interesting predictions of the models. We begin by describing a qualitative test for investigating the order of binding of the inhibitor and activator and continue with more quantitative theoretical results.

A simple test for distinguishing between the AS model and the general or SA models

In principle a system to which the AS model applies can be readily distinguished from one to which the general or SA model applies by comparing the characteristics of inhibitor binding in the presence and absence of activator. In the absence of activator the AS model predicts no binding to the transporter whatsoever whereas the general and SA models predict residual binding in the form CI_n. The problem is of course that in real systems there is always a degree of unspecific binding which is not associated with the transporter and thus tends to confuse the issue. Nevertheless, it should be possible to distinguish between unspecifically bound inhibitor and inhibitor bound as CI_n by observing the effect of transported substrate (S) on binding in the absence of activator. No effect of substrate would suggest that activator is essential for inhibitor binding and thus that the AS model is relevant to the system whereas competitive inhibition of binding by substrate would suggest that either the general or SA models may be more appropriate. More quantitative tests which may also be used to distinguish between these models are discussed later in this section.

Inhibition of inhibitor binding by trans-inhibitor

Referring back to Eqn. 10 we see that $[I_2]$, the concentration of inhibitor on side 2 of the membrane, appears only in the numerator of $K_{d_1}^{app}$ through the parameter α_2. Rearranging Eqn. 10 we find that it can be rewritten in the form

$$K_{d_1}^{app} = K_{d_1}^{app}([I_2] = 0)\left[1 + \frac{[I_2]}{K_{d_2}^{app}([I_1] = 0)}\right] \tag{12}$$

were $K_{d_1}^{app}([I_2] = 0)$ and $K_{d_2}^{app}([I_1] = 0)$ are the expressions for $K_{d_1}^{app}$ and $K_{d_2}^{app}$ when $[I_2] = 0$ and $[I_1] = 0$, respectively. Eqn. 12 has the usual form associated with competitive inhibition of enzyme reactions [13], namely,

$$K_m\left[1 + \frac{[inh]}{K_{inh}}\right] \tag{13}$$

where K_m is the 'Michaelis constant' in the absence of inhibitor, [inh] is the inhibitor concentration and K_{inh} is the so-called 'inhibitor constant'.

There are two important points about Eqn. 12 we wish to stress. First, it is a completely general result which holds for all models treated here in the presence or absence of substrate and/or activator. Secondly, although it is pleasing that the effect of $[I_2]$ on $K_{d_1}^{app}$ can be written in this simple form, it is by no means obvious that this should be the case. Indeed, since inhibitor molecules on opposite sides of the membrane do not share the same compartment they cannot compete with one another for binding sites in the conventional sense. Furthermore examination of Eqn. 8a and b shows that in the case of substrate flux both $V_S^{1\to 2}$ and $K_S^{1\to 2}$ are functions of $[I_2]$ and thus the effect on $J_S^{1\to 2}$ of inhibitor on side 2 of the membrane is non-competitive.

Inhibition of inhibitor binding by substrate

In analogy to the above treatment for $[I_2]$ we find that $[S_1]$ also occurs only in the numerator of $K_{d_1}^{app}$ and that Eqn. 10 can be rewritten as

$$K_{d_1}^{app} = K_{d_1}^{app}([S_1] = 0)\left[1 + \frac{[S_1]}{K_S^{1\to 2}([I_1] = 0)}\right] \tag{14}$$

Here $K_{d_1}^{app}([S_1] = 0)$ is the expression for $K_{d_1}^{app}$ when $[S_1] = 0$ and $K_S^{1\to 2}([I_1] = 0)$ is the Michaelis transport constant for unidirectional substrate flux from side 1 to side 2 when $[I_1] = 0$ (cf. Eqn. 8b).

The presence of substrate on side 2 of the membrane also leads to competitive inhibition of inhibitor binding on side 1. However, because $[S_2]$ appears in both the numerator and denominator of $K_{d_1}^{app}$, the form of this inhibition is in general considerably more complex than Eqn. 13. An exception to this situation occurs when the substrate and activator are each in thermodynamic equilibrium across the membrane. This is a generalization of the usual equilibrium exchange (e.e.) conditions for facilitated transport and is characterized by $[S_1] = [S_2]\, e^{u_S}$ and $[A_1] = [A_2]\, e^{u_A}$ where u_S and u_A are defined in Eqn. 6a and b. Under these conditions it can be shown using Eqn. 5a—d that $k_{21}F_{12} = k_{12}F_{21}$ and this relation may be used to prove that

$$K_{d_1}^{app}(e.e.) = K_{d_1}^{app}(e.e.; [S_1] = [S_2] = 0)\left[1 + \frac{[S_1]}{K_S^{1\to 2}(e.e.; [I_1] = 0)}\right] \tag{15}$$

where

$$K_S^{1\to 2}(e.e.; [I_1] = 0) = K_{S_1}\frac{f_{21}}{k_{21}}\left[\frac{k_{21}(1 + a_1) + k_{12}(1 + a_2 + i_2 + i_2 a_2'')}{f_{21}(1 + a_1') + f_{12}(1 + a_2')}\right]$$

It can be shown from Eqns. 3—5 that $K_S^{1\to 2}(e.e.;[I_1] = 0)$ defined above is the

Michaelis transport constant for substrate flux from side 1 to side 2 of the membrane measured under equilibrium exchange conditions with $[I_1] = 0$. Like Eqn. 12, Eqns. 14 and 15 are completely general results which hold for all models treated here.

Dependence of inhibitor binding on activator concentration

In this section we examine the effects of activator concentration on inhibitor binding. In contrast to the above derivations where we presented general results applicable to all three models here we will discuss the general, AS and SA models separately and indicate how the predictions of these models differ and how the models themselves may be characterized using inhibitor binding studies.

For simplicity we assume that $[S_1] = [S_2] = 0$, i.e. that no substrate is present on either side of the membrane. The expressions for $K_{d_1}^{app}([S_1] = [S_2] = 0)$ have already been given in Table I. However, these formulae are still too complex to test easily in a real system. Therefore we will also set $i_2 = i_2'' = 0$. This condition is satisfied if $[I_2] = 0$ or if K_{I_2} and K_{AI_2} are very large. Arranging for $[I_2] = 0$ should not be difficult experimentally since most competitive inhibitors tend to be relatively large impermeant molecules and thus can be excluded from intracellular or intravesicular spaces. For some competitive inhibitors the latter situation (K_{I_2} and $K_{AI_2} \to \infty$) may also apply, i.e. the inhibitor may bind significantly to the transporter at only one side of the membrane. It is well known for example that many inhibitors of sugar and anion transport in the red blood cell are much more effective at one membrane face than the other [14,15].

Finally we restrict ourselves to the following activator conditions:

(i) $[A_2] = 0$, or 'zero *trans*'.
(ii) $[A_1] = [A_2]e^{u_A}$, or 'equilibrium exchange'.
(iii) $[A_2] \to \infty$, or 'infinite *trans*'.
(iv) $[A_1] \to \infty$, or 'infinite *cis*'.
(v) $[A_1] = 0$, or 'zero *cis*'.

The expressions for $K_{d_1}^{app}([S_1] = [S_2] = 0;\ i_2 = i_2'' = 0)$ under conditions i–v have been derived from Table I and listed in Table II. A discussion for each model follows.

SA model. The SA model shows the simplest dependence of inhibitor binding on activator concentration. It can be seen directly from Table I that when $i_2 = 0$ $K_{d_1}^{app}([S_1] = [S_2] = 0)$ for this model is completely independent of $[A_2]$. Thus in Table II the predictions for the zero *trans*, equilibrium exchange and infinite *trans* experiments in the SA model are identical. In these three cases a plot of $1/K_{d_1}^{app}([S_1] = [S_2] = 0;\ i_2 = 0)$ vs. $[A_1]$ should give the same straight line. In the infinite *cis* procedure we find that $K_{d_1}^{app} \to 0$ as $[A_1] \to \infty$, indicating that high $[A_1]$ results in complete saturation of all binding sites. For the zero *cis* procedure $K_{d_1}^{app}$ reduces to the equation for the binding of a competitive inhibitor to the usual (single substrate) asymmetric carrier model. Note that even in this case the apparent binding constant is not equal to the dissociation constant for the inhibitor-carrier complex since it still retains a dependence on the rate constants k_{12} and k_{21}.

AS model. It can be seen from Table II that the AS model predicts a linear

TABLE II

EXPRESSIONS FOR $K_{d_1}^{app}([S_1] = [S_2] = 0; i_2 = i_2'' = 0)$ UNDER VARIOUS ACTIVATOR CONDITIONS

For activator conditions see text.

Activator condition	Model		
	SA	AS	General
(i) Zero *trans*	$\frac{K_{I_1}K_{IA_1}}{K_{IA_1}+[A_1]}\left(\frac{k_{12}}{k_{21}}+1\right)$	$K_{AI_1}\left[\frac{K_{A_1}}{[A_1]}\left(\frac{k_{12}}{k_{21}}+1\right)+\frac{g_{12}}{k_{21}}+1\right]$	$\frac{K_{I_1}K_{IA_1}}{K_{IA_1}+[A_1]}\left[\frac{[A_1]}{K_{A_1}}\left(\frac{g_{12}}{k_{21}}+1\right)+\frac{k_{12}}{k_{21}}+1\right]$
(ii) Equilibrium exchange	$\frac{K_{I_1}K_{IA_1}}{K_{IA_1}+[A_1]}\left(\frac{k_{12}}{k_{21}}+1\right)$	$K_{AI_1}\left[\frac{K_{A_1}}{[A_1]}\left(\frac{k_{12}}{k_{21}}+1\right)+\frac{g_{12}}{g_{21}}+1\right]$	$\frac{K_{I_1}K_{IA_1}}{K_{IA_1}+[A_1]}\left[\frac{[A_1]}{K_{A_1}}\left(\frac{g_{12}}{g_{21}}+1\right)+\frac{k_{12}}{k_{21}}+1\right]$
(iii) Infinite *trans*	$\frac{K_{I_1}K_{IA_1}}{K_{IA_1}+[A_1]}\left(\frac{k_{12}}{k_{21}}+1\right)$	$K_{AI_1}\left[\frac{K_{A_1}}{[A_1]}\left(\frac{k_{12}}{g_{21}}+1\right)+\frac{g_{12}}{g_{21}}+1\right]$	$\frac{K_{I_1}K_{IA_1}}{K_{IA_1}+[A_1]}\left[\frac{[A_1]}{K_{A_1}}\left(\frac{g_{12}}{g_{21}}+1\right)+\frac{k_{12}}{g_{21}}+1\right]$
(iv) Infinite *cis*	$\to 0$	$K_{AI_1}\left[\frac{K_{A_2}+[A_2]}{\frac{k_{21}}{g_{12}}K_{A_2}+\frac{g_{21}}{g_{12}}[A_2]}+1\right]$	$K_{AI_1}\left[\frac{K_{A_2}+[A_2]}{\frac{k_{21}}{g_{12}}K_{A_2}+\frac{g_{21}}{g_{12}}[A_2]}+1\right]$
(v) Zero *cis*	$K_{I_1}\left(\frac{k_{12}}{k_{21}}+1\right)$	$\to\infty$	$K_{I_1}\left[\frac{(K_{A_2}+[A_2])}{\frac{k_{21}}{k_{12}}K_{A_2}+\frac{g_{21}}{k_{12}}[A_2]}+1\right]$

relationship between $K_{d_1}^{app}([S_1] = [S_2] = 0;\ i_2'' = 0)$ and $1/[A_1]$ for the zero *trans*, equilibrium exchange and infinite *trans* procedures. In contrast to the SA model each of these lines will be different. Examination of the table also shows that the slopes of the equilibrium exchange and zero *trans* plots are identical as are the intercepts on the $K_{d_1}^{app}$ axis for the equilibrium exchange and infinite *trans* plots. These predictions may be used as additional tests of the model.

The dependence of $K_{d_1}^{app}$ on $[A_2]$ found for the infinite *cis* procedure in this model is more difficult to test than those discussed above since a linear relationship between simple functions of $K_{d_1}^{app}$ and $[A_2]$ is not predicted. In the zero *cis* situation the apparent binding constant becomes infinitely large since no binding is possible in the AS model under these circumstances.

General model. None of the predictions listed in Table II for the general model can be rearranged to give linear relationships between simple functions of $K_{d_1}^{app}([S_1] = [S_2] = 0;\ i_2 = i_2'' = 0)$ and activator concentration. Thus in order to determine whether a given set of experimental measurements of $K_{d_1}^{app}$ vs. activator concentration are consistant with this model non-linear data-fitting procedures must be employed. Use may also be made of the fact that when activator concentrations are very large (i.e., much greater than K_{IA_n}) or very small (i.e. much less than K_{A_n}) the predictions of the general model reduce to those of the AS and SA models, respectively.

The above results illustrate that the general, AS and SA models predict distinctly different qualitative and quantitative relationships between the apparent affinity of inhibitor binding and the concentration of activator on the two sides of the membrane. It should therefore be possible to distinguish between the models on the basis of these types of measurements and to go on to evaluate a number of the kinetic parameters associated with the one which is appropriate to the experimental system under study.

As we discuss in the following section, inhibitor binding can also be affected by membrane potential. Thus in studies such as those described above it is important to ensure that the membrane potential is kept constant (ideally equal to zero) as the activator concentrations are varied.

Dependence of inhibitor binding on membrane potential

Simplifying assumptions and mathematical derivations. Analysing the effect of an applied electric field on a membrane transport model can be very complex since in general all of the parameters of a given model may exhibit some electrical dependence. Thus considerable information about the nature of the transporter and its interaction with the field are required for a complete treatment. Such a detailed analysis is beyond the scope of the present article. Accordingly, in this section we make several assumptions about the electrical dependence of the parameters of our models in order to illustrate how inhibitor binding can be affected by a transmembrane electrical potential difference, $\Delta\psi = \psi_2 - \psi_1$ (membrane potential).

For carrier-type models such as those shown in Fig. 1 it is obvious that the rate constants k_{12}, k_{21}, g_{12}, etc. can be sensitive functions of the membrane potential since these parameters often characterize processes involving the net transfer of electrical charges across the membrane. On the other hand, any

effect of membrane potential on the binding constants K_{S_1}, K_{S_2}, K_{A_1}, etc. would presumably have to occur through some structural modification of the binding site due to the electric field and might be expected to be of secondary importance. Therefore as a first approximation we will assume here that only the rate constants of carrier models are functions of $\Delta\psi$.

Using this simplification Eqn. 5a—d, the constraints due to the second law of thermodynamics, can be expressed in a more convenient form for our purposes as follows. Let k_{12}^0, k_{21}^0, g_{12}^0, etc be the values of k_{12}, k_{21}, g_{12}, etc. when $\Delta\psi = 0$. Rearranging Eqn. 5a—d and using the fact that the binding constants K_{S_1}, K_{S_1}, K_{A_1}, etc. are independent of $\Delta\psi$ we find that

$$\begin{aligned}
\frac{f_{12}k_{21}}{f_{21}k_{12}} &= \frac{f_{12}^0 k_{21}^0}{f_{21}^0 k_{12}^0}\, e^{-u_S} \\
\frac{g_{12}k_{21}}{g_{21}k_{12}} &= \frac{g_{12}^0 k_{21}^0}{g_{21}^0 k_{12}^0}\, e^{-u_A} \\
\frac{h_{12}f_{21}}{h_{21}f_{12}} &= \frac{h_{12}^0 f_{21}^0}{h_{21}^0 f_{12}^0}\, e^{-u_A} \\
\frac{h_{12}g_{21}}{h_{21}g_{12}} &= \frac{h_{12}^0 g_{21}^0}{h_{21}^0 g_{12}^0}\, e^{-u_S}
\end{aligned} \tag{16}$$

where u_A and u_S were previously defined in Eqn. 6a and b. On the right-hand side of Eqn. 16 only u_A and u_S are functions of $\Delta\psi$.

In order to obtain explicit expressions for the dependence of the rate constants on $\Delta\psi$ we still require information about the shape of the activation energy barrier and the variation of the electrical potential across the membrane [16]. At the present time this type of information is not available. Let us therefore simply assume that the rate constants for the free carrier are given by

$$k_{12} = k_{12}^0\, e^{-\beta\eta u}, \qquad k_{21} = k_{21}^0\, e^{\beta(1-\eta)u} \tag{17a}$$

where $u = F\Delta\psi/RT$ and $1 \geqslant \eta \geqslant 0$. Eqn. 17a have a simple interpretation in terms of a mobile carrier. Here β is the charge on the free carrier and $-\eta\Delta\psi$ is the electrical potential difference between side 1 of the membrane and the point at which the transition state of the carrier from form C_1 to C_2 occurs (e.g. if the transition state occurs at the mid-point of the electrical potential drop then $\eta = 0.5$). A more general interpretation of the parameter β is given later in this paper.

In analogy with Eqn. 17a we write the remaining rate constants as

$$g_{12} = g_{12}^0\, e^{-\gamma\eta u}, \qquad g_{21} = g_{21}^0\, e^{\gamma(1-\eta)u} \tag{17b}$$

$$f_{12} = f_{12}^0\, e^{-\delta\eta u}, \qquad f_{21} = f_{21}^0\, e^{\delta(1-\eta)u} \tag{17c}$$

$$h_{12} = h_{12}^0\, e^{-\epsilon\eta u}, \qquad h_{21} = h_{21}^0\, e^{\epsilon(1-\eta)u} \tag{17d}$$

Here we have assumed that the transition state for all carrier species occurs at the same point in the potential drop.

Substituting Eqn. 17a—d in Eqn. 16 we find that γ, δ and ϵ can be expressed

in terms of the single parameter β as follows

$$\gamma = \beta + z_A$$
$$\delta = \beta + z_S \tag{18}$$
$$\epsilon = \beta + z_S + z_A$$

In terms of the mobile carrier these equations represent the law of conservation of electrical charge.

The explicit functional dependence of $K_{d_1}^{app}$ on membrane potential can now be derived using Eqns. 17a—d and 18. To illustrate the discussion which follows we will refer to the expressions for $K_{d_1}^{app}([S_1] = [S_2] = 0;\ i_2 = i_2'' = 0)$ already presented in Table II. The membrane potential dependence of these formulas under the assumptions we have made above can be easily obtained using the following substitutions

$$\frac{k_{12}}{k_{21}} = \frac{k_{12}^0}{k_{21}^0} e^{-\beta u}, \qquad \frac{g_{12}}{k_{21}} = \frac{g_{12}^0}{k_{21}^0} e^{-(\beta+\eta z_A)u}$$
$$\frac{g_{12}}{g_{21}} = \frac{g_{12}^0}{g_{21}^0} e^{-(\beta+z_A)u}, \qquad \frac{k_{12}}{g_{21}} = \frac{k_{12}^0}{g_{21}^0} e^{-[\beta+z_A(1-\eta)]u} \tag{19}$$

For sufficiently small membrane potentials we can use the approximation $e^{-x} \approx 1 - x$ (good to approx. 5% accuracy for $x < 0.3$) to simplify Eqn. 19. Under these conditions all of the $K_{d_1}^{app}$ given in Table II will be approximately linear functions of $\Delta\psi$. Note, however, that at room temperature $u = F\Delta\psi/RT = 38.9\ \Delta\psi$. Thus for typical intracellular membrane potentials in the order of 50—80 mV this approximation will not usually be valid.

The parameter β. In order to discuss the effects of membrane potential further it is useful to assign some reasonable values to the parameter β. If we invoke the mobile carrier hypothesis then as already discussed β is simply the charge on the unloaded carrier, C_n. By the same token γ, δ and ϵ are the charges on CA_n, CS_n and CAS_n, respectively (cf. Eqn. 18). As an illustration in Table III we have collected the predictions of the various models regarding the behavior of the $K_{d_1}^{app}([S_1] = [S_2] = 0;\ i_2 = i_2'' = 0)$ as functions of $z_A u$ for two popular variations of the mobile carrier model, namely, $\beta = 0$ or unloaded carrier uncharged, and $\beta = -z_A$ or carrier-activator complex uncharged. The physical basis of the effects documented in Table III can be easily understood by considering the behavior of charged mobile carriers moving to and fro across the membrane in response to the applied electric field. (For example, when $\beta = -z_A$ free carriers will be attracted to side 2 by positive $\Delta\psi$, thus inhibitor binding on side 1 will decrease and K_d^{app} will increase.)

As mentioned earlier, however, the kinetic models we are dealing with here are not limited to the mobile carrier type. Thus a more general interpretation of the parameter β would be useful. Fig. 2 represents an alternate transport mechanism which is also consistent with the kinetic schemes shown in Fig. 1. Here we envision the transporter as being made up of two (or more) protein subunits. The activator and substrate binding sites can be alternately exposed to one or the other side of the membrane through an appropriate conformational rearrangement of the subunits with respect to one another [17,28].

TABLE III

BEHAVIOR OF $K_{d_1}^{app}$ ($[S_1] = [S_2] = 0$; $i_2 = i_2'' = 0$) AS A FUNCTION OF INCREASING $z_A u$

Activator condition	$\beta = 0$		$\beta = -z_A$	
	SA model	General and AS models	SA model	General and AS models
(i) Zero *trans*	no effect	$0 < \eta \leqslant 1$ decreases $\eta = 0$ no effect	increases	increases
(ii) Equilibrium exchange	no effect	decreases	increases	increases
(iii) Infinite *trans*	no effect	decreases	increases	$0 < \eta \leqslant 1$ increases $\eta = 0$ no effect
(iv) Infinite *cis*	—	decreases	—	$0 \leqslant \eta < 1$ increases $\eta = 1$ no effect
(v) Zero *cis*	no effect	$0 \leqslant \eta < 1$ decreases $\eta = 1$ no effect	increases	increases

This mechanism applies equally well to passive and active transport processes.

It is obvious that the effect of membrane potential on the translocation rate constants of the transporter shown in Fig. 2 is not as easily interpreted as in the case of the mobile carrier. Nevertheless, in general terms one might imagine that the reorientation of the unloaded carrier from one side of the membrane to the other, $C_1 \leftrightharpoons C_2$, could result in a net movement of electrical charge and thus to a membrane potential dependence. This might occur through the physical displacement of localized bound charges or possibly through a change in an electrical dipole moment associated with the transporter. Regardless of these details, if the mathematical representation of the dependence of the reorientation events on $\Delta\psi$ can be written in the form of Eqn. 17a the parameter β may be interpreted as the 'effective charge' of the unloaded carrier (i.e. the charge that would be associated with an 'electrically equivalent' mobile carrier).

It is clear from the above discussion that for non-mobile carrier transport mechanisms there is no reason to expect β to assume only integer values.

General remarks concerning the predictions of the models. The combined results of Table II plus Eqn. 19 may be used to test the predictions of the general, AS and SA models regarding the effects of membrane potential on inhibitor binding. In this respect we note that all of the $K_{d_1}^{app}$ listed in the table for the SA model are linear functions of $e^{-\beta u}$. In contrast, for the AS and general models a linear dependence on an exponential function of u occurs only for certain special values of β, e.g. in the zero *trans* and equilibrium exchange experiments when $\beta = 0$ or in the equilibrium exchange and infinite *trans* experiments when $\beta = -z_A$. In circumstances when β does not assume such convenient values (which may be the rule rather than the exception) these expressions will be considerably more difficult to test in a quantitative way owing to their complex dependence on $\Delta\psi$.

It can also be seen from Eqn. 19 that all of the $K_{d_1}^{app}$ listed in Table II for the SA model are independent of η, the parameter which characterizes the position of the transition state relative to the electrical potential drop (cf. Eqn. 17a—d).

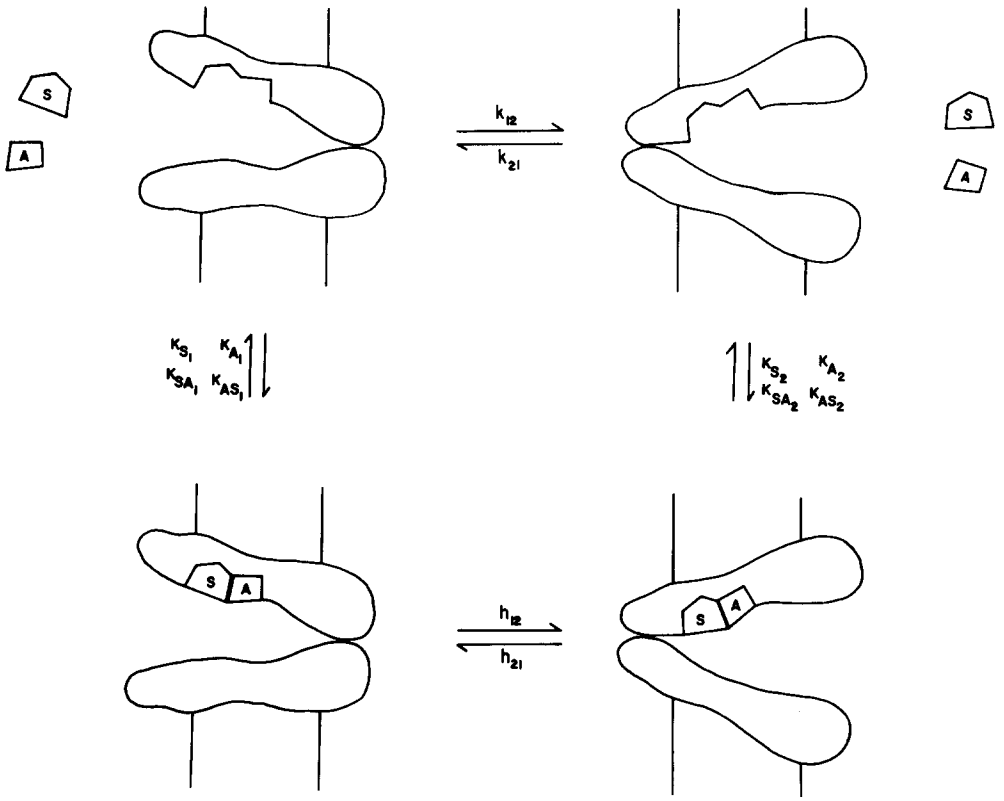


Fig. 2. A hypothetical transport mechanism (see text) which is consistent with the kinetic schemes of Fig. 1. The unloaded carrier species C_1 and C_2 are shown at the upper left and right, respectively, while the fully loaded forms CAS_1 and CAS_2 are at the lower left and right. For simplicity the partially loaded carrier species (CA_1, CS_1, etc.) have not been illustrated. The significance of the relevant rate constants and binding constants from Fig. 1 are indicated. The reaction $C_n \leftrightharpoons CAS_n$ may proceed through CA_n or CS_n or both, depending on the choice of model from Fig. 1.

For the AS and general models only the equilibrium exchange $K_{d_1}^{app}$ is independent of η.

Several preliminary applications of the results of this section are given in the discussion of experimental data which follows.

Experimental studies of inhibitor binding

To our knowledge the binding kinetics of only two non-transported competitive inhibitors of cotransport systems have been studied experimentally in any detail. These are phlorizin, the competitive inhibitor of the sodium-dependent renal and intestinal brush border membrane D-glucose transporters, and *p*-nitrophenyl-α-D-glucopyranoside (NPG), the competitive inhibitor of the sodium-dependent methyl-1-thio-β-D-galactopyranoside (TMG) transporter in *Salmonella typhimurium*.

The kinetics of phlorizin binding to renal and intestinal brush border mem-

branes has been studied by a number of authors [18,27]. There is now a wealth of evidence indicating that the high-affinity, sodium-dependent component of binding seen in these experiments is intimately associated with the sodium-dependent D-glucose transporter in the brush border membrane. D-Glucose is consistently found to be a competitive inhibitor of this binding site [18—20, 22—24] with an inhibitor constant, K_{inh} (see Eqn. 13) in good agreement with the Michaelis transport constant measured for glucose in the same system [23,24]. Frasch et al. [18], Glossmann and Neville [20], Chesney et al. [21] and ourselves [27] have also shown that the apparent dissociation constant for the high-affinity component of phlorizin binding decreases with increasing sodium (activator) concentration.

Aronson [25] has shown that more phlorizin binding to brush border membrane vesicles is seen under zero *trans* sodium conditions than under equilibrium exchange conditions at the same sodium concentration. From Table II we see that this observation cannot be accounted for by the SA model where inhibitor binding on one side of the membrane is independent of the activator concentration on the other. This observation is, however, consistent with either the general or AS models where the presence of activator on the *trans* side of the membrane can in effect act to recruit binding sites to that side.

Toggenburger et al. [23], Aronson [25] and ourselves [26,27] have shown that under zero *trans* sodium conditions phlorizin binding to brush border membrane vesicles is enhanced by more negative intravesicular electrical potentials. Our preliminary work [27] indicates that this is primarily due to a change in the apparent dissociation constant as predicted by the models discussed in this paper. From Table II and Eqn. 19 we find that these results can only be explained if $\beta < 0$, i.e. if the free glucose carrier has a negative effective charge.

In contrast to the above zero *trans* results we have found (Turner, R.J. and Silverman, M., unpublished observation) that under equilibrium exchange sodium conditions ([Na] = 100 mM), negative-inside, valinomycin induced, potassium diffusion potentials do not significantly enhance phlorizin binding to brush border membrane vesicles. This difference between the zero *trans* and equilibrium exchange phlorizin binding experiments is again inconsistant with the SA model which predicts identical results for both procedures. This observation in fact implies that the membrane potential-dependent terms of $K_{d_1}^{app}$ (e.e) for phlorizin binding to the glucose transporter must cancel one another out at this sodium concentration. The mathematical implications of this condition are very complex; however, since our experimentally induced membrane potentials were probably small let us assume that $u = (F\Delta\psi/RT) << 1$. Under these conditions it is possible to prove for both the general and AS model that

$$\beta \approx \frac{-z_A}{q+1} = -\frac{1}{q+1}$$

where

$$q = \frac{K_{A_1}}{[A_1]} \frac{g_{21}^0}{g_{12}^0} \frac{k_{12}^0}{k_{21}^0} = \frac{K_{A_2}}{[A_1]}$$

The second equality in the expression for q was found using Eqn. 5b. K_{A_2} is not yet known but it is safe to assume that it is approx. 10—100 mM, thus $-0.5 \gtrsim \beta \gtrsim -0.9$ (recall that $[A_1] = [Na] = 100$ mM). It is obvious that numerous assumptions and approximations have gone into this calculation but it is nevertheless an interesting result, particularly since it suggests that the glucose transporter may have a non-integral effective charge and thus could not be a mobile carrier.

Tokuda and Kaback [29] have studied the binding of NPG to membrane vesicles isolated from *S. typhimurium*. As in the case of phlorizin binding discussed above they report that TMG is a competitive inhibitor of NPG binding with an inhibitor constant in good accord with its Michaelis transport constant. In agreement with our theoretical models they find that the apparent dissociation constant for NPG binding decreases with increasing sodium concentration with little or no effect on the number of binding sites and that negative-inside membrane potentials enhance NPG binding again through an effect on apparent dissociation constant only. In contradiction to our models, however, they report that when binding under zero *trans* conditions is compared to binding at sodium equilibrium a change in the number of binding sites and no change in the apparent dissociation constant is seen. In this experiment the sodium gradient was dissipated by the addition of the ionophore monensin which catalyzes H^+/Na^+ exchange. Thus this discrepancy with the models could be due to the presence of the ionophore or the pH gradient it produces. Further studies of this system should prove interesting.

Concluding remarks

We have demonstrated how inhibitor binding studies may be used to distinguish between and characterize several variations of the carrier model of cotransport. In general the theoretical results we have derived are considerably simpler and easier to test than the corresponding transport equations. Although binding studies on only two non-transported competitive inhibitors of cotransport systems (phlorizin and NPG) have been reported in the literature there is every reason to expect that others will be discovered and/or synthesized in the future. We hope that the present paper will provide a theoretical framework for more quantitative investigations of the binding properties of these ligands.

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