Biochimica et Biophysica Acta, 1030 (1990) 24–31
Elsevier

BBAMEM 75026

A simple test for the sidedness of binding of transport inhibitors

Rosa Devés [1] and Richard M. Krupka [2]

[1] *Department of Physiology and Biophysics, Faculty of Medicine, University of Chile, Santiago (Chile) and* [2] *Research Centre, Agriculture Canada, London (Canada)*

(Received 1 March 1990)
(Revised manuscript 10 July 1990)

Key words: Transport inhibitor; Sidedness; Carrier model; Kinetics; Inhibition mechanism

A new method is described for determining the sidedness of action of nonpolar inhibitors that rapidly diffuse through the lipid bilayer and could therefore interact with the carrier on both sides of the membrane. Sidedness is deduced from the effect of the inhibitor on the flux ratio for the substrate (the ratio of the rates of exchange and net transport). The advantages of the method are that the experimental measurements are made after the inhibitor has equilibrated rather than in the brief period when it is present on only one side of the membrane, and that any reversible inhibitor can be tested, whether the inhibition mechanism is competitive, noncompetitive, uncompetitive, or mixed.

Introduction

The inhibition of membrane transport is complicated by factors that do not ordinarily enter into the inhibition of enzyme-catalyzed reactions, and therefore the kinetics of the two types of system have to be treated separately. The complications derive from the vectorial nature of transport and associated asymmetries in the carrier system [1]. Under non-equilibrium conditions, the substrate gradient and the resulting net flow of substrate from one side of the membrane to the other creates an element of spatial asymmetry; aside from this, asymmetry could pre-exist in the structure of the transmembrane carrier protein and in the mechanism of transport, or it could be imposed, by having the inhibitor on only one side of the membrane. Depending on the circumstances, an inhibitor might attack one asymmetrical carrier conformation, or a limited region of the transport protein accessible from only one compartment, or a step in translocation occurring at one surface of the membrane. As a result, the inward and outward transport of the substrate could be inhibited in different ways. Asymmetrically-acting inhibitors are of interest because they can be used to probe the transport mechanism, for example the involvement of separate inward-facing and outward-facing carrier conformations in translocation [2,3], or the function of defined regions of the carrier and individual amino acid side chains [4–7].

Correspondence: R.M. Krupka, Research Centre, Agriculture Canada, 1400 Western Road, London, Ontario, Canada, N6G 2V4.

The problem of asymmetrical inhibition is most easily grasped in terms of the carrier model. According to this model, the carrier exists in two main conformations, inward-facing, with the substrate site exposed inside the cell, and outward-facing, with the site exposed outside. An inhibitor may be able to add to one or both these forms, depending on its location inside or outside the cell and depending also on the location of inhibitor binding sites in relation to the substrate site, the plane of the membrane, and the carrier conformation. If, for whatever reason, the inhibitor binds to only one carrier form, then the inhibition is asymmetric, with predictable consequences for the distribution of the carrier between the two forms, and also for the kinetics of transport.

Another point needs to be made concerning the site of inhibition. Because carriers are integral membrane proteins, spanning the membrane and exposed on both sides, an inhibitor could conceivably add to one particular conformation, the outward-facing carrier for example, either from the same compartment as the substrate (outside) or from the opposite compartment (inside), depending on whether the inhibition site is located on the same surface of the membrane as the exposed substrate site, or on the opposite surface. The kinetics of inhibition can tell us which carrier form the inhibitor attacks – outward-facing or inward-facing – but not the location of the inhibitor binding site. Other information is required to decide this point. For the sake of brevity, one often speaks of binding 'outside' as if it meant binding to the outward-facing carrier, but the distinction should be kept in mind.

Under the conditions of an experiment, the possibility of an inhibitor attacking the transport system from both sides of the membrane may depend on its ability to diffuse across the lipid bilayer in a short period of time. Inhibitors that are highly polar molecules, unable to penetrate the cell membrane, are normally confined to one compartment and would approach the carrier from one side, while lipid-soluble molecules, which diffuse across the lipid bilayer, could approach the carrier from both sides. In either case, the problem would be to determine which carrier form the inhibitor acts upon and which step in translocation it blocks. In the following discussion we consider the general case of penetrating inhibitors, though the conclusions apply equally to non-penetrating inhibitors.

One way of proceeding, the first to be worked out, is to place the inhibitor in either the inner or outer compartment, and to determine its effect before it has had time to equilibrate across the membrane. The difficulty is that diffusion rates for the inhibitor must be known, and that the rates of substrate transport must be measured in times far shorter than those required for equilibration of the inhibitor across the membrane. The advantage is that both competitive and noncompetitive inhibitors can be tested. This method, useful where equilibration is not too rapid, was employed by Baker and Widdas [8] in studies of the inhibition of glucose transport in erythrocytes by the amphipathic substrate analog, ethylidene glucose, which slowly enters the cell by simple diffusion through the lipid bilayer, independent of the carrier. Later, the method was applied by Barnett and co-workers [9] to other amphipathic glucose analogs. The analogs, which are bound at the substrate site in competition with glucose but are not transported, added preferentially on either the inner or outer surface of the membrane, depending on their structure. It followed that the configuration of the substrate site exposed inside and outside differs, an important finding for an understanding of the carrier mechanism. The same approach was recently taken in studies of the inhibition of anion exchange in red cells by phloretin [10], though here the technical difficulties are more severe, owing to rapid diffusion across the cell membrane of the highly nonpolar inhibitor molecule.

Another approach, developed for the case of cytochalasin B inhibition of glucose transport [11–13], has the advantage of depending on rate measurements made after the inhibitor has equilibrated rather than before. The limitation of the method is that it does not apply to noncompetitive inhibitors. Here, the sidedness of action of a competitive inhibitor is deduced from its differing effects on substrate entry and substrate exit. A substrate in one compartment, binding to the same carrier site in the same carrier form as the inhibitor, can overcome the inhibition, while a substrate in the opposite compartment, binding to the opposite carrier form (to which we suppose the inhibitor does not bind), does not overcome the inhibition. In an exit experiment, for example, where the substrate is inside the cell, the behavior will be competitive if the inhibitor binds exclusively to the inward-facing carrier; but in an entry experiment, with the substrate outside, the same competitive inhibitor will inhibit noncompetitively. Equilibrated cytochalasin B was shown, by means of such experiments, to add in competition with the substrate to the inward-facing glucose carrier; it did not add to the outward-facing form. Again, the findings demonstrate the existence of structural asymmetry in the transport protein with respect to the plane of the membrane.

A somewhat different, but related, method was used in studies of the inhibition of choline transport in red cells by permeant substrate analogs. These analogs, which rapidly equilibrate across the lipid bilayer by simple diffusion, are bound at the carrier site but are not transported. The inhibition of exit of labeled choline was measured with cells suspended in a medium containing either pure buffer or a solution of unlabeled choline at a high concentration. As the inhibition by an analog bound to the outward-facing carrier is diminished through competition with unlabeled choline in the external compartment, while the inhibition by an analog bound to the inward-facing carrier is not, the sidedness of inhibitor binding could be deduced [14].

An entirely different approach, which does not depend on displacement of the inhibitor by the competing substrate and which therefore applies to both competitive and noncompetitive inhibitors, was introduced in studies of the same choline analogs [14]. Here, the effect of an inhibitor on the partition of the carrier between inward-facing and outward-facing forms was assayed with a chemical reagent, *N*-ethylmaleimide, that only reacts with the inward-facing form of the carrier. An inhibitor preferentially bound to one carrier form shifts the partition to favor that form, whether it binds at the substrate site or not. The results of the two methods, one dependent and one not dependent on competition between the substrate and inhibitor, were in agreement. As in the glucose system, striking differences were revealed in the specificity of the inner and outer transport sites.

Among these approaches to the problem of an equilibrated inhibitor, only the last applies to both competitive and noncompetitive mechanisms, though here a special probe is needed to measure the partition of the carrier. We now describe a more convenient method, which involves the carrier partition and is therefore applicable to inhibitors of all types, but which requires the measurement of transport rates only, specifically, flux ratios for the substrate in the presence and absence of an equilibrated inhibitor. The method depends on the interrelationship between inhibition, substrate flux, and the carrier partition, in particular on the fact that an

asymmetrically bound inhibitor alters the carrier partition, that the substrate can affect the inhibition by altering the carrier partition, and that the flux ratio is responsive to the carrier partition. As the following kinetic analysis of these relationships shows, flux ratios provide a simple measure of the sidedness of inhibitor attack. Aside from its general applicability, the new method has several advantages over that involving inhibition patterns in entry and exit experiments: (1) Flux may be determined in either entry or exit whereas both measurements were required before. (2) The radioactive substrate is used at a single low concentration rather than over a range of concentrations. (3) Conclusions may be drawn from only a few rate determinations.

Kinetic theory

The flux ratio (for exit) is the ratio of the unidirectional efflux of a low concentration of labeled substrate, either into a solution containing a high concentration of unlabeled substrate (an infinite-trans experiment) or into pure buffer (a zero-trans experiment). These two fluxes depend on different steps in the transport cycle and may therefore differ in magnitude. In a simple carrier mechanism (Fig. 1), transport involves the following steps: substrate binding, translocation of the complex across the membrane, substrate dissociation, and return of the carrier to its initial position. The measured rate of transport is seen to depend on the rate of return of the carrier, which differs in the two experiments: in an infinite-trans experiment the carrier returns as the complex with unlabeled substrate, but in a zero-trans experiment as the free carrier.

In the absence of an inhibitor the flux ratio for exit is

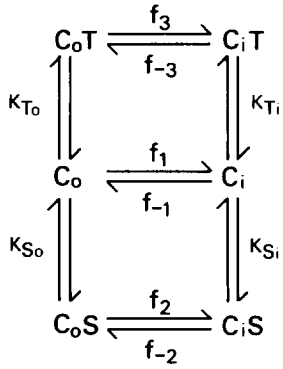


Fig. 1. Kinetic scheme for the simple carrier model, involving two substrates, S and T. The free carrier exists in two forms, outward-facing (C_o) and inward-facing (C_i). Substrates in the external solution form a complex with C_o (C_oS and C_oT), substrates in the internal solution with C_i. The substrate dissociation constants for S and T are K_{So} and K_{To} on the outer face of the membrane and K_{Si} and K_{Ti} on the inner face, respectively. The rate constants for carrier reorientation are f_1, f_{-1}, f_2, f_{-2}, f_3 and f_{-3}.

given by the following expression [15] based on the carrier model (Fig. 1):

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_i \to o} = \frac{(1+(f_{-1}/f_1))}{(1+(f_{-1}/f_3))} \tag{1}$$

From Eqn. 1 it can be seen that where the external substrate T is rapidly transported, with the carrier complex more mobile than the free carrier ($f_3 \gg f_{-1}$), the flux ratio approaches $(1 + (f_{-1}/f_1))$, which is equal to 2 if the carrier is equally distributed between its two forms ($[C_o]/[C_i] = f_{-1}/f_1 \approx 1$). Where the external substrate is slowly transported and the mobility of the complex is low ($f_3 \ll f_{-1}$), the flux ratio falls below unity (Eqn. 1). In an intermediate case, where the mobilities of the free carrier and the complex are identical ($f_3 = f_1$), the flux ratio equals unity. The flux ratio is seen to depend on the substrate in the external solution, which may be the unlabeled form of the internal substrate, or another substrate.

From the effect of an inhibitor on the flux ratio, it is possible to decide whether the site of action isi in the outward-facing or inward-facing carrier, or both. The predicted flux ratios can be found from equations for rates in the presence and absence of an inhibitor; the derivation is outlined in the Appendix. In the general case, where the inhibitor can add to both the free carrier and the carrier-substrate complex, the flux ratio for exit, $(\tilde{v}^T/\bar{v})^I_{S_i \to o}$, is given by

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)^I_{S_i \to o} = \left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_i \to o} \left\{\frac{1+([I_o]/\bar{K}_{Io})+([I_i]/\bar{K}_{Ii})}{1+([I_o]/\bar{K}_{ToIo})+([I_i]/\bar{K}^T_{Ii})}\right\} \tag{2}$$

where $[I_o]$ and $[I_i]$ are the external and internal inhibitor concentrations. The inhibitor half-saturation constants are

$$\bar{K}_{Io} = K_{Io}(1+(f_1/f_{-1})) \tag{3}$$

$$\bar{K}_{Ii} = K_{Ii}(1+(f_{-1}/f_1)) \tag{4}$$

$$\bar{K}_{ToIo} = K_{ToIo}(1+(f_3/f_{-1})) \tag{5}$$

$$\tilde{K}^T_{Ii} = K_{Ii}(1+(f_{-1}/f_3)) \tag{6}$$

K_{Io} and K_{Ii} are the inhibitor dissociation constants for the external and internal free carrier, respectively, and K_{ToIo} the inhibitor dissociation constant for the external carrier-substrate complex (Fig. 2). With a noncompetitive inhibitor, which does not affect substrate binding, $K_{ToIo} = K_{Io}$; with a competitive inhibitor, which does, K_{ToIo} is extremely large, and the term $[I_o]/\bar{K}_{ToIo}$ drops out of Eqn. 2. Thus, the form of the equations, and the predicted effect of an inhibitor on the flux ratio, depends on the mechanism of inhibition.

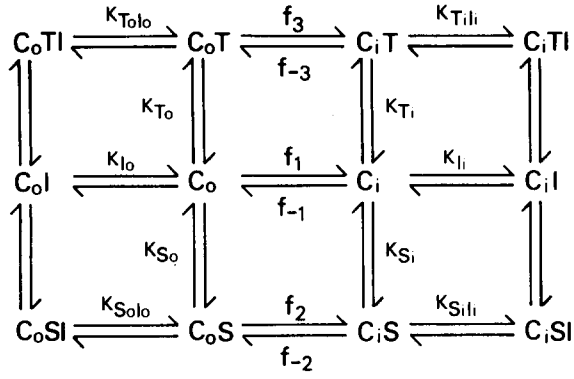


Fig. 2. Transport scheme for two substrates, S and T, in the presence of a noncompetitive inhibitor, I. In purely competitive inhibition the ternary complexes C_oSI, C_iSI, etc. are not formed (K_{TiIi}, K_{SoIo}, and K_{SiIi} approach infinity).

Flux ratios in the presence of an inhibitor

(a) Noncompetitive inhibitors

(i) The inhibitor binds to the external carrier form only ($K_{Ii} \to \infty$). The flux ratio approaches a limit as the inhibitor concentration is raised. This limit is found by substitution of Eqns. 1, 3 and 5 into Eqns. 2:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}^{I\to\infty} = f_3/f_1 \tag{7}$$

(ii) The inhibitor binds to the internal carrier form only (K_{Io} and $K_{ToIo} \to \infty$). The limiting value of the flux ratio at increasing inhibitor concentrations is found by substitution of Eqns. 1, 3 and 4 into Eqn. 2:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}^{I\to\infty} = 1 \tag{8}$$

(iii) The inhibitor binds equally to the two carrier forms ($K_{Io} = K_{Ii} = K_{ToIo}$). The bracketed expression in inhibitor concentration in Eqn. 2 is now equal to unity at all inhibitor concentrations. It follows that the inhibitor does not change the flux ratio.

(iv) The inhibitor binds predominantly but not exclusively to one carrier form. If the ratio of affinities on the two sides is $K_{Ii}/K_{Io} = \beta$, the limiting value of the flux ratio reduces to:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}^{I\to\infty} = \frac{1+\beta(f_{-1}/f_1)}{1+\beta(f_{-1}/f_3)} \tag{9}$$

(b) Competitive inhibitors

With a competitive inhibitor, as noted above, $K_{ToIo} \to \infty$, and the term $[I_o]/\overline{K}_{ToIo}$ in Eqn. 2 disappears.

(i) The inhibitor binds to the external carrier form only ($K_{Ii} \to \infty$). The flux ratio is found to depend on the inhibitor concentration:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}^{I} = \left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}\{1+[I_o]/\overline{K}_{Io}\} \tag{10}$$

Therefore the flux ratio rises without limit as the inhibitor concentration increases:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}^{I\to\infty} = \infty \tag{11}$$

(ii) The inhibitor binds to the internal carrier form only ($K_{Io} \to \infty$). The behaviour is the same as with a noncompetitive inhibitor, with the flux ratio approaching unity (see Eqn. 8).

TABLE I

The sidedness of inhibitor binding in relation to effects on the flux ratio for exit [a]

In the presence of an inhibitor, there is either an increase (↑) a decrease (↓), no change (–), or little change (≈), in the flux ratio. The limiting value of the flux ratio at high inhibitor concentrations is given. Predictions are shown for noncompetitive, competitive, and uncompetitive inhibitors bound to either the outward-facing carrier, the inward-facing carrier, or both. The behaviour depends on the control flux ratio, which may be greater than, less than, or equal to, unity.

Control flux ratio (F)	Effect of an inhibitor on the flux ratio	Noncompetitive inhibitor			Competitive inhibitor			Uncompetitive inhibitor		
		out	in	both	out	in	both	out	in	both
$F>1$	Direction of change	↑	↓	–	↑	↓	≈ [b]	↓	↓	↓
	Limiting value	>1	1		∞	1	>1	0	1	0
$F=1$	Direction of change	–	–	–	↑	–	↑	↓	–	↓
	Limiting value				∞		>1	0	1	0
$F<1$	Direction of change	↓	↑	–	↑	↑	↑	↓	↑	↓
	Limiting value	<1	1		∞	1	>1	0	1	0

[a] By interchanging the 'in' and 'out' columns, the table may be used to interpret experiments on the flux ratio for entry.

[b] The control flux ratio is equal to $(1=(f_{-1}/f_1))/(1+(f_{-1}/f_3))$ and the limiting value of the flux ratio in the presence of inhibitor is $1+(f_{-1}/f_1)$ (see Eqns. 1 and 13). When the control flux ratio is greater than unity, f_3 must be greater than f_{-1}, and therefore the control flux ratio and that in the presence of the inhibitor have similar values.

(iii) The inhibitor is bound equally to the two carrier forms ($K_{Io} = K_{Ii}$). The flux ratio approaches a limit given by:

$$\left(\frac{\vec{v}^T}{\vec{v}}\right)_{S_{i\to o}}^{I\to\infty} = 1 + (f_{-1}/f_1) \tag{12}$$

(iv) The inhibitor is bound predominantly but not exclusively to one carrier form. With a ratio of affinities of $K_{Ii}/K_{Io} = \beta$, the limiting value of the flux ratio is:

$$\left(\frac{\vec{v}^T}{\vec{v}}\right)_{S_{i\to o}}^{I\to\infty} = 1 + \beta(f_{-1}/f_1) \tag{13}$$

The results of the analysis are summarized in Table I, which serves as a guide in interpreting the experimental results. For the sake of completeness, uncompetitive inhibition is included, though it is rarely encountered; the case of an uncompetitive inhibitor, which adds to the carrier-substrate complex but not the free carrier, is treated in the Appendix. The following discussion deals mainly with competitive and noncompetitive inhibition.

How inhibitors alter the flux ratio

The flux ratio (the ratio of infinite-trans to zero-trans flux) changes when the inhibitor has different effects on transport into a medium containing, or not containing, unlabeled substrate at a high concentration. A substrate in the external medium could alter the degree of inhibition by shifting the distribution of the carrier between inner and outer forms, which may have a different sensitivity to the inhibitor. In addition, the substrate could weaken the inhibition by displacing a competitive inhibitor from its binding site, or could strengthen the inhibition by enabling an uncompetitive inhibitor to add to the carrier.

With regard to the carrier partition, substrates can be divided into three groups, depending on the flux ratio in their presence:

(a) Substrates with a flux ratio greater than one. Here the carrier-substrate complex is more mobile than the free carrier ($f_3 > f_1$; Eqn. 1), and substrate in the external solution therefore increases the steady-state concentration of the inward-facing carrier and decreases that of the outward-facing carrier (see Fig. 1). Hence, an inhibitor that binds to the outward-facing carrier is less effective in the presence of the substrate, less of the carrier being available to form the complex. Infinite-trans flux is then less inhibited than zero-trans flux, and the flux ratio increases. On the other hand, an inhibitor that binds to the inward-facing carrier, being more effective in the presence of the substrate, causes the flux ratio to decrease. An inhibitor bound equally to the two carrier forms affects the two rates equally and leaves the ratio unchanged. While these effects do not depend on whether the inhibition is competitive, noncompetitive, or uncompetitive, any direct interaction between the substrate and inhibitor would by itself shift the flux ratio. Thus a substrate could diminish competitive inhibition, and increase the flux ratio, in two ways: by directly displacing the inhibitor from the binding site, and by shifting the carrier partition toward the opposite compartment.

(b) Substrates with a flux ratio equal to one. Because the free carrier moves at the same rate as the complex (Eqn. 1), the substrate has no effect on the carrier distribution. Here, a noncompetitive inhibitor would leave the flux ratio unchanged. A competitive inhibitor in the same compartment as the substrate would alter the flux ratio, but a competitive inhibitor in the opposite compartment would not.

(c) Substrates with a flux ratio smaller than one. Inward movement of the carrier-substrate complex (f_3) is slower here than that of the free carrier (f_1), and the carrier shifts outward. The effect of the substrate now depends on the inhibition mechanism. If it is noncompetitive, the outward shift of the carrier reinforces the effect of an externally bound inhibitor, but protects against an internally bound inhibitor, and has no effect on a symmetrically bound inhibitor. If the mechanism is competitive, the substrate protects against an externally bound inhibitor, by displacing it from the external site, and also protects against an internally bound inhibitor, by drawing the carrier to the outside.

The choice of substrate in the trans compartment

It can be seen from Table I that with competitive and noncompetitive inhibitors the most convenient experimental design involves the use in the external medium of a good substrate, that is, a substrate with a flux ratio greater than unity. With such a substrate the flux ratio increases if the inhibitor binds 'outside' (strictly speaking, to the outward-facing carrier), decreases if it binds 'inside' (that is, to the inward-facing carrier), and is unchanged if it binds equally to both. Because the predictions are simple, the sidedness of inhibition can be deduced from measurements at a single inhibitor concentration.

If the flux ratio for the substrate is equal to unity, the method works with competitive but not noncompetitive inhibitors. With competitive inhibitors, exclusive binding to the inner carrier form can be distinguished at a single inhibitor concentration from binding outside (Table I). Exclusive binding outside is distinguished from binding on both sides at varying inhibitor concentrations. If the inhibitor only binds outside the flux ratio increases linearly with its concentration (Eqn. 11), whereas if it binds on both sides the flux ratio rises to a limit. This limit depends on the equilibrium distribution of the free carrier between outward-facing and inward-facing forms, $[C_o]/[C_i]$; with the proportions equal, the limiting value is 2 (Eqn. 12).

If the flux ratio for the substrate is less than unity, a single inhibitor concentration suffices with a noncompetitive inhibitor. With a competitive inhibitor, sidedness is revealed by the concentration dependence of the flux ratio: exclusive binding outside gives rise to a linear relationship between the flux ratio and the inhibitor concentration, exclusive binding inside to a limiting value of unity, and binding on both sides to a limiting value greater than unity.

Flux ratios for entry

The above analysis applies specifically to exit experiments, but the conclusions can easily be adapted to flux ratios for entry. The same table (Table I) may be consulted in interpreting exit experiments, provided the column marked 'In' and 'Out' are interchanged.

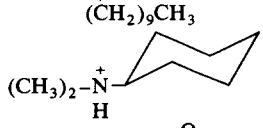
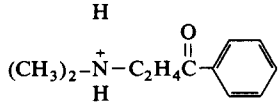
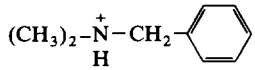
Application of the method

The method of flux ratios is applied in the following paper to a series of noncompetitive inhibitors of erythrocyte choline transport. Here we apply the method to competitive inhibitors of the same system. Flux ratios for choline exit in the presence of various substrate analogs, calculated from previously published data [14], are listed in Table II. The analogs are tertiary amines which, in their unprotonated, uncharged form, rapidly migrate across the cell membrane and are therefore present both outside and inside the cell. In the protonated, cationic form the amines add to the carrier site in competition with the substrate, but being larger than choline, do not undergo transport. The reference point for an analog bound only to an outward-facing carrier site is set by the quaternary ammonium compound, dimethyl-*n*-decyl(2-hydroxyethyl)ammonium bromide, which cannot diffuse across the cell membrane and is therefore restricted to the external solution. One of the tertiary amines, *N,N*-dipropylaminoethanol, shown in other experiments to bind almost exclusively to inward-facing carrier sites, serves as a reference point for internal binding. As expected, the flux ratio is much increased in the presence of the quaternary ammonium ion and much decreased in the presence of dipropylaminoethanol. Among the tertiary amines as a whole, one group increases the flux ratio, indicating a prefer-

TABLE II

Changes in the flux ratio for choline transport in the presence of equilibrated inhibitors

The inhibitors are tertiary amines, analogs of choline that compete for the substrate site. The reference for solely external binding is the non-permeant quaternary ammonium compound, dimethyl-*n*-decyl-2-hydroxyethylammonium ion. In the experiments [14], rates of exit of [^{14}C]choline (4.9 μM) from human erythrocytes were measured in the presence and absence of an inhibitor, with or without unlabeled choline (550 μM) in the external medium; the medium was isotonic NaCl buffered with 5 mM sodium phosphate (pH 6.8).

Inhibitor	Inhibitor concentration (mM)		%Inhibition (zero-trans)	Flux ratio	
	external	internal		control	+ inhibitor
$(CH_3)_2-\overset{+}{N}(-(CH_2)_9CH_3)-C_2H_4OH$	0.033	0	44	1.92	3.39
	1.46	1.46	48	1.92	2.98
	0.97	0.97	55	1.94	3.15
	1.22	1.22	47	1.95	2.71
$(CH_3)_2-\overset{+}{N}H-(CH_2)_{11}CH_3$	0.039	0.039	39	1.93	2.91
$(CH_3)_2-\overset{+}{N}H-C_3H_6Cl$	13.1	13.1	61	1.96	1.66
$(CH_3)_2-\overset{+}{N}H-C_3H_6OH$	0.44	0.44	48	1.87	1.00
$(C_2H_5)_2-\overset{+}{N}H-C_2H_4OH$	0.23	0.23	53	1.87	1.17
$(C_3H_7)_2-\overset{+}{N}H-C_2H_4OH$	1.50	1.50	70	1.90	1.16

ence for the outer carrier site, and the other group decreases the flux ratio, indicating a preference for the inner site. These conclusions agree with other observations on the system [14].

Appendix

I. Flux ratios in the presence of competitive and noncompetitive inhibitors

The effect of an inhibitor on zero-trans and infinite-trans efflux is found by substitution into the general rate equation for transport of substrate S in the presence of a reversible inhibitor I and a second substrate T (Fig. 2), which was derived earlier [15].

(1) Zero-trans exit

With a low initial concentration of labeled substrate inside the cell ($[S_i]/\bar{K}_{Si} \ll 1$), the rate of exit is:

$$\bar{v}_{S_{i\to o}} = \frac{\bar{V}_{Si}[S_i]/\bar{K}_{Si}}{1+([I_o]/\bar{K}_{Io})+([I_i]/\bar{K}_{Ii})} \tag{A-1}$$

(2) Infinite-trans exit

The labeled substrate, present at a low concentration ($[S_i]/\tilde{K}^T_{Si} \ll 1$), exits into a medium containing a saturating concentration of unlabeled substrate T:

$$\tilde{v}^T_{S_{i\to o}} = \frac{\tilde{V}^T_{Si}[S_i]/\tilde{K}^T_{Si}}{1+([I_o]/\bar{K}_{ToIo})+([I_i]/\tilde{K}^T_{Ii})} \tag{A-2}$$

All the constants in Eqns. A-1 and A-2 are experimental parameters: V is a maximum transport rate and K a half saturation constant. The type of experiment is indicated by subscripts and superscripts attached to each constant. Subscripts designate the substrate or inhibitor involved (S, T or I) and also its location either inside or outside the cell (i or o). Superscripts specify the condition of the experiment, either zero-trans (' '), with no substrate in the opposite compartment, or infinite-trans (' '), with a saturating concentration of substrate T in the opposite compartment. For example, $\bar{V}_{Si}$ is the maximum rate of exit of S in a zero-trans experiment, and $\tilde{V}^T_{Si}$ is the maximum rate of exit of S in an infinite-trans experiment. Expressions for the inhibitor half-saturation constants, in terms of rate constants for carrier reorientation (f_1, f_{-1}, f_2, etc. in Fig. 2), were given above (Eqns. 3–6), and expressions for the other parameters in Ref. 15.

The dependence of the flux ratio on the inhibitor concentration is found by dividing Eqn. A-2 by Eqn. A-1:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}} = \frac{\tilde{V}^T_{Si}\bar{K}_{Si}}{\bar{V}_{Si}\tilde{K}^T_{Si}}\left\{\frac{1+([I_o]/\bar{K}_{Io})+([I_i]/\bar{K}_{Ii})}{1+([I_o]/\bar{K}_{ToIo})+([I_i]/\tilde{K}^T_{Ii})}\right\} \tag{A-3}$$

The expression $\tilde{V}^T_{Si}\bar{K}_{Si}/(\bar{V}_{Si}\tilde{K}^T_{Si})$ was previously shown to be equal to the control flux ratio, $(\tilde{v}^T/\bar{v})_{S_{i\to o}}$; hence Eqn. A-3 is equivalent to Eqn. 2 above.

II. Flux ratios in the presence of uncompetitive inhibitors

An uncompetitive inhibitor is one that binds to the carrier-substrate complex but not the free carrier: in terms of the scheme in Fig. 2 the constants for addition of inhibitor to the free carrier (K_{Io} and K_{Ii}) approach infinity. In order to treat this case, the rate equations have to be rewritten in a more general form, for it is no longer permissible to assume that terms in $[I_i]\cdot[S_i]$ are negligible.

(1) Zero-trans exit

$$\bar{v} = \{\bar{V}_{Si}[S_i]/\bar{K}_{Si}\} \times\{1+([I_o]/\bar{K}_{Io})+([I_i]/\bar{K}_{Ii})+ ([S_i]/\bar{K}_{Si})[1+([I_o]/\tilde{K}^S_{Io})+([I_i]/\bar{K}_{SiIi})]\}^{-1} \tag{A-4}$$

All the inhibition constants in Eqn. A-4 are already defined except $\bar{K}_{SiIi}$, which is given by

$$\bar{K}_{SiIi} = K_{SiIi}(1+(f_{-2}/f_1)) \tag{A-5}$$

(2) Infinite-trans exit

$$\tilde{v}^T = \{\tilde{V}^T_{Si}[S_i]/\tilde{K}^T_{Si}\} \times\{1+([I_o]/\bar{K}_{ToIo})+([I_i]/\tilde{K}^T_{Ii})+ ([S_i]/\tilde{K}^T_{Si})[1+([I_o]/\tilde{K}^S_{ToIo})+([I_i]/\tilde{K}^T_{SiIi})]\}^{-1} \tag{A-6}$$

where

$$\tilde{K}^T_{SiIi} = K_{SiIi}(1+(f_{-2}/f_3)) \tag{A-7}$$

$\bar{K}_{Io}$, $\bar{K}_{Ii}$, $\tilde{K}^S_{Io}$, and $\tilde{K}^T_{Ii}$, the experimental half-saturation constants dependent on K_{Ii} and K_{Io}, which govern addition of the inhibitor to the free carrier, approach infinity in the case of a purely uncompetitive inhibitor, by definition, and therefore drop out of the rate equations. The flux ratio (Eqn. A-6 divided by Eqn. A-4) now reduces to:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)^{I}_{S_{i\to o}} = \left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}\{1+([S_i]/\bar{K}_{Si})[1+([I_i]/\bar{K}_{SiIi})]\} \times\{1+([I_o]/\bar{K}_{ToIo})+([S_i]/\tilde{K}^T_{Si}) \times[1+([I_o]/\tilde{K}^S_{ToIo})+([I_i]/\tilde{K}^T_{SiIi})]\}^{-1} \tag{A-8}$$

(a) The inhibitor binds to the external carrier form only ($K_{SiIi} \to \infty$):

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)^{I\to\infty}_{S_{i\to o}} = \left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_{i\to o}}\cdot\frac{\bar{K}_{ToIo}}{[I_o]} \tag{A-9}$$

The flux ratio is seen to fall to zero at high inhibitor concentrations.

(b) The inhibitor binds to the internal carrier form only ($K_{ToIo} \to \infty$). At high inhibitor concentrations the flux ratio approaches a limit:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_i \to o}^{I \to \infty} = \left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_i \to o} \left\{\frac{\tilde{K}_{Si}^T \cdot \tilde{K}_{SiIi}^T}{\bar{K}_{Si} \cdot \bar{K}_{SiIi}}\right\} \quad \text{(A-10)}$$

Eqn. A-10 is equal to unity, as may be shown by substitution of the half-saturation constants for the inhibitor (Eqns. A-5 and A-7) and for the substrate:

$$\bar{K}_{Si} = K_{Si}(f_1 + f_{-1})/(f_1 + f_{-2}) \quad \text{(A-11)}$$

$$\tilde{K}_{Si}^T = K_{Si}(f_{-1} + f_3)/(f_{-2} + f_3) \quad \text{(A-12)}$$

(c) The inhibitor binds equally to the two carrier forms ($K_{SiIi} = K_{ToIo}$). Because $[S_i]/\bar{K}_{Si} \ll 1$, Eqn. A-8 reduces to Eqn. A-9 and the result is the same as with an inhibitor bound exclusively outside.

Acknowledgements

This work was supported in part by Grants from the University of Chile (B2674) and FONDECYT-Chile (156-88).

References

1 Widdas, W.F. (1980) Curr. Top. Membr. Transp. 14, 165–223.
2 Edwards, P.A. (1973) Biochim. Biophys. Acta 311, 123–140.
3 Krupka, R.M. (1985) J. Membr. Biol. 84, 35–43.
4 Wang, J.-F., Falke, J.J. and Chan, S.I. (1986) Proc. Natl. Acad. Sci. USA 83, 3277–3281.
5 Rees, W.D., Gliemann, J. and Holman, G.D. (1987) Biochem. J. 241, 857–862.
6 Gibbs, A.F., Chapman, D. and Baldwin, S.A. (1988) Biochim. J. 256, 421–427.
7 May, J.M. (1989) J. Membr. Biol. 108, 227–233.
8 Baker, G.F. and Widdas, W.F. (1973) J. Physiol. (London) 231, 143–165.
9 Barnett, J.E.G., Holman, G.D., Chalkley, R.A. and Munday, K.A. (1975) Biochem. J. 145, 417–429.
10 Frohlich, O. and Mayer, S. (1989) Biophys. J. 55, 145a.
11 Basketter, D.A. and Widdas, W.F. (1977) J. Physiol. (London) 265, 39P–40P.
12 Basketter, D.A. and Widdas, W.F. (1978) J. Physiol. (London) 278, 389–401.
13 Devés, R. and Krupka, R.M. (1978) Biochim. Biophys. Acta 510, 339–348.
14 Devés, R. and Krupka, R.M. (1984) J. Membr. Biol. 80, 71–80.
15 Krupka, R.M. and Devés, R. (1983) Int. Rev. Cytol. 84, 303–352.