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Apparent Noncompetitive Inhibition of Choline Transport in Erythrocytes by Inhibitors Bound at the Substrate Site

R. Devés and R.M. Krupka

Department of Physiology and Biophysics, Faculty of Medicine, University of Chile, Santiago, Chile, and Research Institute, Agriculture Canada, London, Ontario, Canada N6A5B7

Summary. According to the conventional carrier model, an inhibitor bound at the substrate transfer site inhibits competitively when on the same side of the membrane as the substrate, but noncompetitively when on the opposite side. This prediction was tested with the nonpenetrating choline analog dimethyl-*n*-pentyl (2-hydroxyethyl) ammonium ion. In zero *trans* entry and infinite *trans* entry experiments, where the labeled substrate and the inhibitor occupy the same compartment, the inhibition was competitive, but in zero *trans* exit it was noncompetitive, in accord with the model. Similar behavior was seen with dimethyl-*n*-decyl (2-hydroxyethyl) ammonium ion. With this property of the choline transport system established, it becomes possible to estimate the relative affinity inside and outside of inhibitors present on both sides of the membrane. The tertiary amine, dibutylaminoethanol, which enters the cell by simple diffusion, is such an inhibitor. Here the inhibition kinetics were the reverse of those for nonpenetrating inhibitors; zero *trans* and infinite *trans* exit was inhibited competitively, and zero *trans* entry noncompetitively. It follows that dibutylaminoethanol binds predominantly to the inner carrier form.

Key Words choline transport · carrier model · inhibition mechanism · competitive · noncompetitive · erythrocyte

Introduction

In theory, the type of inhibition of transport produced by a reversible inhibitor which is bound at the substrate site, and which is present on the opposite side of the membrane from the transported substrate, depends on the carrier mechanism. Some models predict competitive inhibition [2, 5] while others, including any mechanism having the broad features of the conventional carrier[1], predict noncompetitive inhibition [3, 5]. The actual behavior has never been unambiguously demonstrated. As transport mechanisms are still in doubt, it is important to establish this property by experiment.

For an unequivocal test, an inhibitor with the following characteristics is required. First, it should be a close analog of the substrate in order to bind directly in the substrate transfer site, and this expected competition with the substrate should be demonstrated in experiments in which the substrate and inhibitor are initially present in the same compartment. Second, the analog must not undergo transport on the carrier, since *trans* substrates and *trans* inhibitors have different effects [3]. Finally, the inhibitor must bind to the carrier on only one side of the membrane.

Reversible inhibitors with just these properties are available [8]. An example is dimethyl-*n*-pentyl (2-hydroxyethyl) ammonium ion, which differs from choline only in the replacement of one N-methyl by an N-pentyl group. Because of this substituent it fails to undergo transport on the carrier, and being a quaternary ammonium ion is unable to penetrate the membrane by simple diffusion either. It therefore cannot enter the cell, and if it binds to the carrier can only do so on the external surface of the membrane. The pentyl analog strongly inhibits transport (with a half-saturation constant of 7 μM), and considering its structural similarity to choline it is likely to be bound at the same site. In support of this, a constant difference in affinity has been found between corresponding dimethyl and diethyl analogs of structure $(CH_3)_2R\overset{\oplus}{N}-C_2H_4OH$ and $(C_2H_5)_2R\overset{\oplus}{N}-C_2H_4OH$, where R is an alkyl group varying in length from ethyl to decyl, the ethyl derivatives being more weak-

[1] In mechanisms of this kind a substrate transfer site appears first on one side of the membrane and then the other, and in the course of transport the carrier cycles between inward-facing and outward-facing forms.

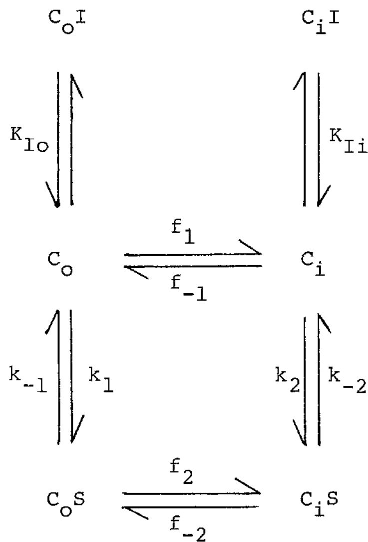

Fig. 1. Transport scheme for the classical carrier model in which the substrate site in the carrier faces either inward (C_i) or outward (C_o). A substrate or inhibitor in the internal solution forms a complex with C_i, while a substrate or inhibitor in the external solution forms a complex with C_o

ly bound by a factor of 6.5 [8]. All members of the series therefore add to a site, or sites, having the same ability to discriminate between dimethyl and diethyl derivatives. The smallest pair are transported substrates, which directly implicates the substrate transfer site. The largest pairs are nontransported inhibitors, and in between the smaller member of the pair undergoes transport and the larger does not. Hence the constant affinity difference indicates that all the analogs are bound at the transfer site.

The observation reported previously [8], that dimethylpentyl (2-hydroxyethyl) ammonium ion competitively inhibits the zero *trans* entry of choline, is in agreement with binding at the substrate site. However, a kinetic analysis of noncompetitive inhibition [13] shows that zero *trans* experiments may not give unequivocal evidence of a competitive mechanism, unlike experiments on infinite *trans* flux or equilibrium exchange. The reason, in essence, is as follows. If in zero *trans* entry experiments the substrate complex crosses the membrane much faster than the free carrier returns ($f_2 \gg f_{-1}$; *see* Fig. 1), then in the steady state the carrier will accumulate in the inward-facing form out of reach of an inhibitor confined to the external solution. As a result, increasing concentrations of the substrate should overcome the effects of even a noncompetitive inhibitor, making the behavior competitive. This should not happen in infinite *trans* or equilibrium exchange experiments, where the carrier returns as the substrate complex rather than in the free form.

In the choline system[2] the ratio f_2/f_{-1} is at least 4, and as a result a noncompetitive inhibitor could give rise to partly competitive kinetics in zero *trans* experiments [13]. Additional evidence for a competitive mechanism has been obtained in an infinite *trans* entry experiment. Following this demonstration, it is also shown that in zero *trans* exit experiments, where the substrate and inhibitor are on opposite sides of the membrane, the inhibition is noncompetitive.

Because the observations are in accord with the conventional carrier model, a definite interpretation can be given to similar experiments in which inhibitors are present on both sides of the membrane. As shown earlier, the relative affinities for inner and outer carrier forms may be estimated in zero *trans* entry and zero *trans* exit experiments [3, 4], and the method is now applied to a tertiary analog of choline, dibutylaminoethanol. At pH 6.8, where transport is studied, the amine exists almost entirely as a cation capable of binding at the negatively charged substrate transfer site [10]; this species is in equilibrium with the unprotonated amine, which is neutral and passively diffuses across the cell membrane.

A kinetic analysis of competitive inhibition is given in the Appendix, and it is shown how the experimental behavior – competitive, noncompetitive, partially noncompetitive or partially uncompetitive – depends on where the inhibitor binds in relation to the substrate, and also on the transport properties of the substrate, in particular the relative mobility of the free and loaded carrier.

Materials and Methods

Materials

The synthesis of dimethyl-*n*-pentyl (2-hydroxyethyl) ammonium iodide and dimethyl-*n*-decyl (2-hydroxyethyl) ammonium bromide was described previously [8]. 2-Di-*n*-butylaminoethanol was of commercial reagent grade and was redistilled at reduced pressure before use.

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[2] f_2/f_{-1} may be estimated from the ratio of the maximum rates of equilibrium exchange ($\bar{V}_S$) and zero *trans* entry ($\bar{V}_{So}$), which Martin [14] reported to be approximately 5. In terms of individual constants in the transport scheme in Fig. 1, this ratio is given by [7]:

$$\frac{\bar{V}_S}{\bar{V}_{So}} = \frac{1+f_2/f_{-1}}{1+f_2/f_{-2}} \cong 5.$$

Measurement of Transport Rates

The method of preparing red cells from outdated human blood and the details of the procedures used in determining rates of exit and entry of [methyl ^{14}C] choline were reported earlier [6, 8]. All rates were measured at 37 °C in a solution of 154 mM NaCl and 5 mM sodium phosphate buffer, pH 6.8.

Infinite trans Entry Experiments. Washed cells were initially incubated in a shaking water bath with 250 µM choline chloride, in solutions containing 0.1 % glucose and 0.02 % chloramphenicol, for a period of 18.5 hr at 37 °C. During this period, the cells become loaded with a saturating concentration of choline (the half-saturation constant inside, $\bar{K}_{Si}$, is approximately 40 µM). Plating on agar gel revealed no bacterial contamination. The cells were harvested and washed as described before [6]. Rates of uptake of radioactive choline were determined by withdrawing 0.5 ml samples at intervals from a suspension of 0.7 ml cells in 2 ml of salt-buffer solution containing various concentrations of ^{14}C-choline and a reversible inhibitor. The uptake rates were found to be linear, as in the previously described zero *trans* uptake experiments [8].

Zero trans Entry Experiments. Cells washed free of endogenous choline were placed in solutions containing varying concentrations of radioactive choline, with or without an inhibitory choline analog, and were sampled at intervals for 20 min. Uptake rates were linear over this period. In the case of dibutylaminoethanol, the cells were pre-incubated with the tertiary amine for approximately 1 hr before choline transport rates were determined, in order to allow it to come to equilibrium across the membrane.

Exit Experiments. Cells were incubated at 37 °C for a period of 18 hr in radioactive choline at various concentrations, and then were washed four times in ice-cold buffer. The radioactivity in the packed cells was determined by lysing the cells in a solution of trichloroacetic acid, as described before [6], and the internal choline concentration was calculated from the known specific activity. In experiments with dibutylaminoethanol, the amine was added to the cell suspension following the incubation period at 37 °C, and was included in the cold buffer used to wash the cells; the tertiary amine rapidly penetrates the cell, independent of the carrier, and attains equilibrium during this period. In the rate assay at 37 °C, samples were removed periodically over a period of 10 min and the radioactivity in the suspending medium was determined, according to procedures described before.

Permeability of 2-Di-n-Butylaminoethanol. The permeability of the cell membrane to 2-di-*n*-butylaminoethanol was determined by the light-scattering method [15], which depends on the rapid osmotic shrinking and swelling of the cells in response to the intracellular solute concentration.

Choline Metabolism During Prolonged Loading Periods

In exit and in infinite *trans* entry experiments, choline is preincubated with cells for 18 hr, and we may ask whether it is metabolized during this time. Askari [1] detected no metabolism of radioactive choline after a 2-hr period, and assays have now been run after 20 hr of loading. Cells (50 % hematocrit) were incubated at 37 °C in salt-buffer solution containing 60 µM radioactive choline, 0.1 % glucose, and 0.02 % chloramphenicol; they were harvested and washed as described above, and the radioactivity in the packed cells was determined in two ways. In one, trichloroacetic acid was used, as in determinations of the initial internal choline concentration in exit experiments. Cells (50 µl) were suspended in 0.4 ml of salt solution (0.9 % NaCl), and 0.5 ml of 5 % trichloroacetic acid was added; after sedimentation the radioactivity in the supernatant solution was determined by scintillation counting in Aquasol. In the second method, 50 µl of cells were suspended in 0.1 ml of salt solution, and 0.54 ml ethanol was added. The cells were sedimented, and the radioactivity in the supernatant was determined. The recovery of radioactivity in the two methods was similar (96.9 ± 3.3 % and 95.1 ± 3.3 %, respectively).

The ethanol extract was chromatographed on thin-layer cellulose (Avasil) plates in the upper layer of butanol/acetic acid/water, 4:1:5, and moved as a single sharp peak which co-chromatographed with ^{14}C-choline (*Rf* = 0.36). The radioactivity in the peak and adjoining regions was determined by scraping the cellulose powder off the plate and counting in Aquasol. At least 93 % of the radioactivity was recovered in the choline band.

Results

Di-n-Butylaminoethanol Permeability

Cells were preincubated with dibutylaminoethanol in salt-buffer solution, pH 6.8, for a period of 1 hr, to give a final concentration of 81.6 mM. The rate of exit was followed at 37 °C, after injection of 0.4 ml of a 20 % cell suspension into 65 ml of saline solution (Fig. 2). The escape of dibutylaminoethanol from the cells is independent of the choline carrier, and occurs by passive diffusion of the uncharged tertiary amine. Equilibrium is seen to be approached closely in 2 to 3 min.

Inhibition of Choline Transport by Dimethyl-n-Pentyl (2-hydroxyethyl) Ammonium Iodide, Dimethyl-n-Decyl (2-Hydroxyethyl) Ammonium Bromide, and 2-Di-n-Butylaminoethanol

In an infinite *trans* entry experiment, the inhibition pattern produced by dimethy-*n*-pentyl-(2-hydroxyethyl) ammonium ion is purely competitive (Fig. 3), demonstrating that the inhibition mechanism is competitive. The inhibition by each of the three inhibitors in zero *trans* entry and zero *trans* exit experiments is shown in Figs. 4–6. The two quaternary analogs, which can only bind on the external surface of the membrane, inhibit choline entry competitively

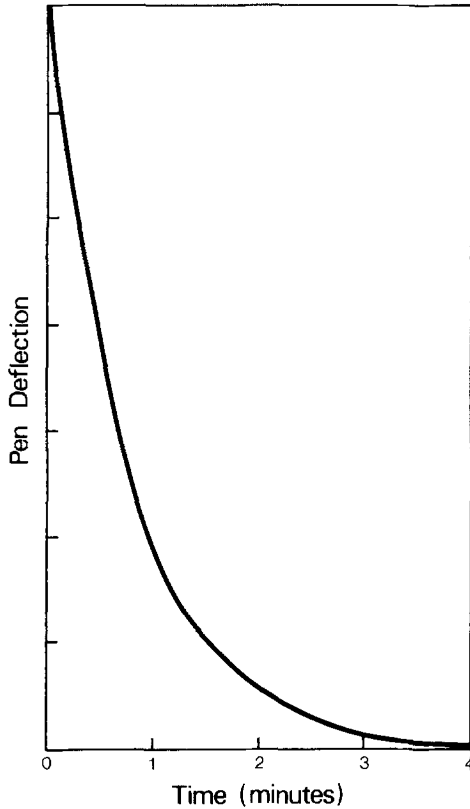

Fig. 2. Recorder tracing of exit of 2-di-*n*- butylaminoethanol (initial intracellular concentration 81.6 mM) into saline solution, pH 6.8, at 37 °C, as determined by the light-scattering method. The pen deflection, in arbitrary units, falls to zero when the amine reaches an equilibrium concentration (0.46 mM)

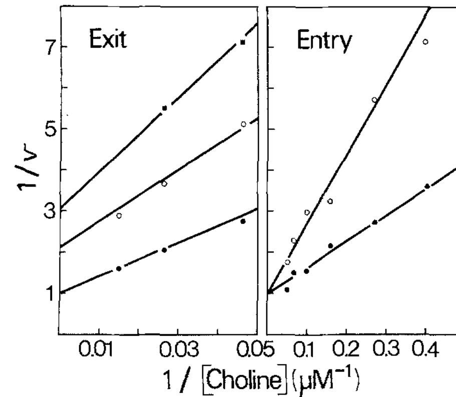

Fig. 4. Inhibition of choline transport by dimethyl-*n*-pentyl (2-hydroxyethyl) ammonium iodide in zero *trans* experiments. *Left:* rates of choline exit, at varying choline concentrations, into pure saline solution (lower line) or into a solution containing 3.9 or 7.8 μM inhibitor (middle and upper lines, respectively). *Right:* rates of choline entry in the absence of inhibitor (lower line) or in the presence of 12.9 μM inhibitor (entry data taken from Ref. [8]). Rates were determined at 37 °C in solutions of 154 mM NaCl and 5 mM sodium phosphate buffer, pH 6.8. The rates are expressed in relative units, with the maximum velocity in the absence of inhibitor set at unity. The maximum rate of entry is approximately 0.14 μmol min^{-1} per liter of packed cells, and the maximum rate of exit approximately 0.23 μmol min^{-1} per liter of packed cells. From a least-squares analysis, $\bar{K}_{I_o}$, measured in entry where the inhibition is competitive, is 8.2±1.6 μM. The theoretical lines shown in the exit experiment were calculated from the constants: $\bar{K}_{I_o}=7$ μM; $\bar{K}_{I_o}/\bar{K}^S_{I_o}=2.1$; $\bar{K}_{S_i}=40$ μM

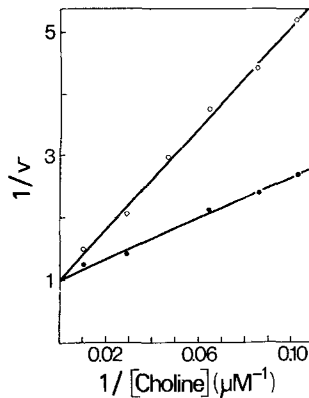

Fig. 3. Inhibition of infinite *trans* entry by dimethyl-*n*-pentyl (2-hydroxyethyl) ammonium bromide (8.04 μM). Cells were pre-incubated with 250 μM cold choline, and rates of uptake of varying concentrations of external ^{14}C-choline were determined. The upper and lower lines represent the reciprocal of the relative rates in the presence or absence of the inhibitor, respectively

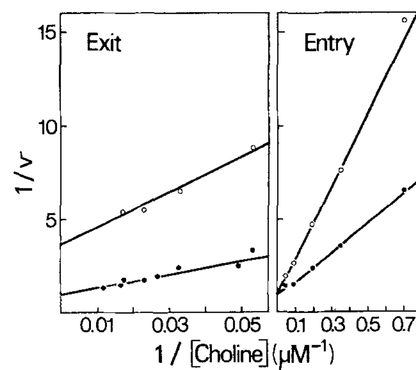

Fig. 5. Inhibition of choline transport by dimethyl-*n*-decyl (2-hydroxyethyl) ammonium bromide in zero *trans* experiments. *Left:* rates of choline exit into pure saline solution (below) or a solution of 0.29 μM inhibitor (above). *Right:* rates of choline entry in pure saline solution (below) or with 0.41 μM inhibitor (above). Units of rate and experimental conditions as in Fig. 2. From a least-squares analysis, $\bar{K}_{I_o}$, the competitive inhibition constant measured in entry, is 0.23±0.02 μM. The constants determined in exit are $\bar{K}_{I_o}=0.19\pm0.057$, and $\bar{K}^S_{I_o}=0.10\pm0.024$ μM

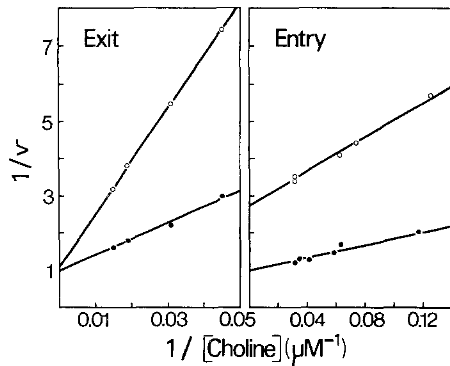

Fig. 6. Inhibition of choline transport by 2-di-*n*-butylaminoethanol in zero *trans* experiments. *Left:* rates of choline exit into pure saline solution (below) or a solution of 2.4 mM inhibitor (above). Right: rates of choline entry in pure saline solution (below) or a solution of 2.4 mM inhibitor (above). The cells were equilibrated with the inhibitor before choline transport rates were measured. Units of rate and experimental conditions as in Fig. 2. From a least-squares analysis the competitive inhibition constant as measured in exit is 1.13 ± 0.18 mM

Table. The inhibition of infinite *trans* exit of ^{14}C-choline by dibutylaminoethanol[a]

Internal concentration of ^{14}C-choline (μM)	Rate of exit (μmol min^{-1} per liter of packed cells)		$\frac{v}{v_I}-1$
	Control	+ Dibutylaminoethanol (3.70 mM)	
3.4	0.096 ± 0.0032	0.0244 ± 0.0058	3.09 ± 0.16
102.7	1.70 ± 0.050	0.73 ± 0.019	1.34 ± 0.09

[a] Cells were preincubated with either a high or a low concentration of ^{14}C-choline, and rates of exit were measured into solutions containing 220 μM cold choline, either in the presence or absence of 3.7 mM dibutylaminoethanol, pH 6.8. In a noncompetitive mechanism the inhibition should be independent of the internal substrate concentration, while in a competitive mechanism the relative inhibition is given by

$$\left(\frac{v}{v_I}-1\right)=\frac{[I_i]/\tilde{K}^S_{Ii}}{1+[S_i]/\tilde{K}^S_{Si}}$$

where v_I and v are rates in the presence or absence of the inhibitor. The results show that choline reduces the inhibition and that the mechanism is therefore competitive. Standard deviations of rates were determined in a least-squares analysis.

and choline exit noncompetitively (Figs. 4 and 5). The tertiary analog, present on both sides of the membrane, gives rise to the opposite behavior: noncompetitive in entry and competitive in exit (Fig. 6). The mechanism of inhibition by dibutylaminothanol is competitive, as demonstrated by the results of an infinite *trans* exit experiment given in the Table.

Discussion

Nonpenetrating Choline Analogs

According to evidence cited in the Introduction, the quaternary analogs of choline, dimethylpentyl-(2-hydroxyethyl) ammonium ion and dimethyldecyl-(2-hydroxyethyl) ammonium ion, are bound at the substrate site but do not enter the cell, either on the carrier or by simple diffusion. An infinite *trans* entry experiment (Fig. 3) shows that the inhibition mechanism is competitive. For inhibitors with these properties, the conventional carrier model makes definite predictions about the behavior in zero *trans* entry and zero *trans* exit experiments. In zero *trans* entry the inhibition should be purely competitive, as found; but in zero *trans* exit the character of the inhibition depends on the flux ratio for the substrate, which is the ratio of exchange to zero *trans* flux, $(\tilde{v}^S/\bar{v})_{S_o \to 0}$. If this ratio is much less than unity, the inhibition should be competitive; if equal to unity, purely noncompetitive; and if greater than unity, partially uncompetitive. More exactly, the ratio of the substrate-dependent and substrate independent inhibition constants should equal the flux ratio:

$$\bar{K}_{Io}/\tilde{K}^S_{Io}=(\tilde{v}^S/\bar{v})_{S_o \to 0} \qquad \text{(Eqs. A5 and A6).}$$

The results on dimethyl-*n*-decylaminoethanol (Fig. 5) agree: $\bar{K}_{Io}$ and $\tilde{K}^S_{Io}$ calculated in the exit experiment are 0.19 ± 0.057 μM and 0.10 ± 0.024 μM, respectively, with a ratio of 1.90 ± 0.56; and $(\tilde{v}^S/\bar{v})_{S_o \to 0}$ is 2.07 ± 0.05 [11]. The results on dimethylpentylaminoethanol (Fig. 4) are less precise but are consistent with the theoretical lines drawn in the plot, which were calculated from $\bar{K}_{Io}=7$ μM and $\bar{K}_{Io}/\tilde{K}^S_{Io}=2.1$. Values of $\bar{K}_{Io}$ found in entry experiments are 7.3 ± 1.2 μM [8] and 8.2 ± 1.6 μM (Fig. 4).

Two entirely different models are in qualitative agreement with the observed inhibition patterns [5], but are ruled out by other evidence. One of these is a simple pore able to accommodate two or more substrate molecules in the section that limits diffusion: the inhibition of entry should be competitive, and the inhibition of exit purely noncompetitive. The other model

is a mobile carrier with binding sites simultaneously exposed to both compartments. Entry, again, is blocked competitively; on the other hand the inhibition of exit can range from partially uncompetitive to mixed competitive and noncompetitive, but bears no predictable relationship to the flux ratio for the substrate. In these models, unlike the conventional carrier, a ternary complex is formed with two inhibitor molecules, one from each compartment [5]. A test for the ternary complex has been made, with the result that both models are excluded [12]. Moreover, mechanisms based on a simple pore do not explain accelerated exchange.

Two other mechanisms, which predict competitive inhibition of both entry and exit, fail to account for the observed inhibition kinetics. These are (a) a simple pore in which only one substrate molecule can occupy the section limiting the rate of substrate diffusion across the membrane; and (b) a dual-site carrier with sites simultaneously exposed in both compartments, where addition of the substrate or inhibitor to one induces a conformational change that occludes the other.

Binding of an Equilibrated Inhibitor

Dibutylaminoethanol inhibits choline exit competitively, and choline entry noncompetitively (Fig. 6), and it acts by a competitive mechanism (Table). A kinetic analysis of the conventional carrier model supplies an obvious interpretation, which runs as follows. First, a competing substrate cannot produce noncompetitive inhibition, regardless of its location relative to the substrate, whereas a nontransported substrate analog can; therefore dibutylaminoethanol is an inhibitor rather than a substrate. Next, an inhibitor that binds on both sides of the membrane would be noncompetitive in both entry and exit; therefore dibutylaminoethanol, though present both within the cell and in the suspending medium, does not add to both the inner and outer carrier forms. Finally, the inhibition patterns in entry and exit are the reverse of those seen with nonpenetrating inhibitors, which bind exclusively on the outer surface of the membrane, and therefore dibutylaminoethanol must add predominantly on the inner surface [4, 5].

This preference for the inner site could be related to the bulkiness of the substituted ammonium group, assuming that the inner and outer sites differ in shape or in openness to approaching cations. Certainly asymmetrical binding is not characteristic of tertiary amines, for the smaller dimethylaminoethanol, the tertiary analog of choline, appears to bind about equally on both sides, like choline [9].

Though there is no doubt that dibutylaminoethanol binds asymmetrically, any quantitative interpretation of the observations in Fig. 6 is uncertain. If the inhibitor failed entirely to add to the outer carrier form, the ratio of the substrate-dependent and substrate-independent inhibition constants, $\bar{K}_{Ii}/\tilde{K}^S_{Ii}$, would equal the substrate flux ratio for exit $(\tilde{v}^S/\bar{v})_{Si\to 0}$ [7], paralleling the case of nonpenetrating inhibitors. The flux ratio lies between 1.5 and 2.5 in different cell samples, with a usual value of 1.9 [7], whereas the calculated inhibition constants are roughly equal, 1.29 ± 0.36 and 1.40 ± 0.27 mM, respectively. On the other hand, if there is even a small component of binding to the outer carrier form, which seems likely, the predicted ratio of the inhibition constants would fall. Whether this entirely accounts for the results remains undecided.

Appendix

Inhibition Patterns and Substrate Transport

With a competitive inhibitor across the membrane from the substrate, the observed pattern of inhibition should depend on the rate-limiting steps in transport; these steps are revealed by the *trans* effect of the substrate, specifically by an experimental parameter called the flux ratio. The behavior may be explained in the following way.

The rate of substrate exit into a solution containing a nonpenetrating inhibitor, but no substrate, is given by [7]:

$$v = \frac{\bar{V}_{Si}[Si]/\bar{K}_{Si}}{1 + \frac{[Io]}{\bar{K}_{Io}} + \frac{[Si]}{\bar{K}_{Si}}\left(1 + \frac{[Io]}{\tilde{K}^S_{Io}}\right)}. \quad \text{(A1)}$$

$\bar{V}_{Si}$ is the maximum rate of exit and $\bar{K}_{Si}$ is the substrate half-saturation constant. For convenience, Eq. (A1) may be rewritten in reciprocal form:

$$\frac{1}{v} = \frac{1}{\bar{V}_{Si}}\left[1 + \frac{[Io]}{\tilde{K}^S_{Io}} + \frac{\bar{K}_{Si}}{[Si]}\left(1 + \frac{[Io]}{\bar{K}_{Io}}\right)\right]. \quad \text{(A2)}$$

$\bar{K}_{Io}$ is seen to be the half-saturation constant for an external inhibitor determined from the substrate-dependent component of the inhibition (the slope in reciprocal plots), and $\tilde{K}^S_{Io}$ the constant determined from the substrate-independent component (the intercept). The inhibition is described as competitive if the inhibitor increases the slope but not the intercept ($\tilde{K}^S_{Io} \gg \bar{K}_{Io}$); as noncompetitive if it increases the slope and intercept by the same factor ($\tilde{K}^S_{Io} = \bar{K}_{Io}$); and as partially uncompetitive if it increases the intercept more than the slope ($\tilde{K}^S_{Io} < \bar{K}_{Io}$). The observed behavior thus reflects the relative values of $\bar{K}_{Io}$ and $\tilde{K}^S_{Io}$,

which in turn are functions of the rate constants for substrate transport:

$$\bar{K}_{Io} = K_{Io}(1 + f_1/f_{-1}), \tag{A3}$$

$$\tilde{K}_{Io}^{S} = K_{Io}\left[1 + \frac{f_1}{f_{-2}} + \frac{f_1}{k_{-1}}\left(1 + \frac{f_2}{f_{-2}}\right)\right]. \tag{A4}$$

An analysis of the conventional carrier model shows the ratio of the inhibition constants to be equal to the flux ratio for substrate entry:

$$\frac{\bar{K}_{Io}}{\tilde{K}_{Io}^{S}} = (\tilde{v}^S/\bar{v})_{So\to 0} = \frac{(1 + f_1/f_{-1})}{1 + \frac{f_1}{f_{-2}} + \frac{f_1}{k_{-1}}\left(1 + \frac{f_2}{f_{-2}}\right)}. \tag{A5}$$

The flux ratio, $(\tilde{v}^S/\bar{v})_{So\to 0}$, is a measure of the effect of unlabeled substrate inside the cell on the entry of labeled substrate S present outside at a low concentration $([S_o]/\bar{K}_{So} \ll 1)$: $\tilde{v}^S$ and $\bar{v}$ are entry rates measured with a saturating concentration of substrate inside, or in the absence of internal substrate, respectively.

It follows from Eqs. (A3)-(A5) that the inhibition type and the flux ratio have exactly the same dependence on the rate-limiting steps in substrate transport. As seen in Fig. 1, flux may be limited by reorientation of the free carrier (f_1 and f_{-1}), reorientation of the carrier-substrate complex (f_2 and f_{-2}), or dissociation of the substrate from the complex (k_{-1} and k_{-2}). If f_{-1} is roughly equal to f_{-2} or k_{-1} (not necessarily both), the flux ratio is not far from unity and the inhibition is noncompetitive. If f_{-1} is much larger than f_{-2} or k_{-1} (again not necessarily both), the ratio is small and the inhibition is competitive. If both f_{-2} and k_{-1} are much greater than f_{-1}, the ratio is greater than unity and the behavior is partially uncompetitive.

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