J. Membrane Biol. 80, 71–80 (1984)

The Journal of
Membrane Biology

The Comparative Specificity of the Inner and Outer Substrate Transfer Sites in the Choline Carrier of Human Erythrocytes

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Summary. The substrate specificities on the inner and outer surfaces of the cell membrane have been compared by determining the relative affinities, inside and outside, of a series of choline analogs. The results of two different methods were in agreement: (1) the carrier distribution was determined in the presence of a saturating concentration of an equilibrated analog, using N-ethylmaleimide as a probe for the inward-facing carrier; (2) the degree of competition was measured between an equilibrated analog and choline in the external solution. The carrier sites are found to have markedly different specificities: the outer site is more closely complementary to the structure of choline than is the inner, and even a slight enlargement of either the trimethylammonium or hydroxyethyl group gives rise to preferential binding inside. It is also found that a nonpolar binding region, which is adjacent to the outer site, is absent from the inner site. As the transport mechanism involves the exposure of only one site at a time, first on one surface and then the other, it follows that an extensive reorganization of the structure of the substrate site may occur during the carrier-reorientation step, or alternatively that two distinct sites may be present, only one of which is exposed at a time.

Key Words carrier mechanism · substrate specificity · choline transport · conformational change · N-ethylmaleimide · choline analogs

Introduction

Choline is transported across the membrane of the red blood cell by a saturable carrier [1, 17]. Certain essential features of the carrier mechanism have already been revealed by experiments on the kinetics of transport of choline and choline analogs, and on the kinetics of irreversible inhibition by N-ethylmaleimide. One such feature is a substrate site which is alternately exposed on opposite sides of the membrane, as in the conventional carrier model in Fig. 1 [8, 12, 16]. Another is a conformational change in the carrier that occurs as the substrate is transferred through the membrane [8, 16]. The forces of attraction between the substrate and the carrier site may be expended, in part, to facilitate this conformational change, which occurs more readily in the carrier-substrate complex than in the free carrier [14, 17]. The bonding energy of the hydroxymethyl group of choline does not appear to be utilized in this way [7], and it is likely that interactions of the quaternary ammonium group are responsible [15].

The carrier is highly specific in forming a complex with substrate analogs, but it is even more specific in the transport step; the evidence [7] suggests that the substrate, bound at a surface site in the initial complex, is completely enclosed by the carrier as it moves across the membrane. The behavior could be explained if two half-sites, complementary to two faces of the substrate molecule, cooperate in the process of transport: the substrate may first be bound at a half-site exposed on one side of the membrane, and then, in the transfer step, a second half-site may move in to cover it.

In the simplest possible version of the conventional carrier model, the same substrate site, with the same geometry, becomes exposed first on one side of the membrane and then on the other. In a more complicated version, such as that suggested by the observations on the choline transport system, the substrate site could exhibit a different geometry when approached from the internal or external compartment, either because the structure is altered as the site shifts in orientation, or because two wholly different sites exist, which can become exposed on opposite sides of the membrane.

The problem which we now wish to address is how changes in the structure of the substrate site can be detected, should they occur. Perhaps the most obvious way is by comparing the affinity of substrate analogs on the inner and outer surfaces of the membrane, and this is the approach that we shall make.

The difficulty here is that the substrate dissocia-

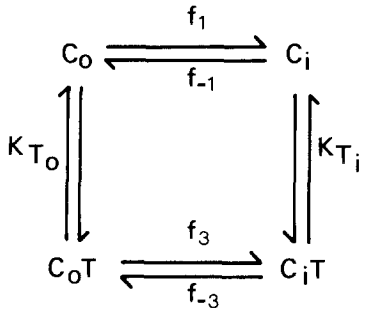

Fig. 1. The conventional carrier model, involving two main forms of the carrier, one outward-facing (C_o) and one inward-facing (C_i). The substrate T in the external or internal solution adds to these carrier forms, giving the complex C_oT or C_iT, respectively

tion constant is not directly measurable, either in transport, or in binding experiments [11]. The kinetic analysis of the conventional carrier model shows that the half-saturation constants found in transport assays are a function of the relative rates of movement of the free carrier and the carrier complex across the membrane, as well as of the actual affinity for the carrier site. The analysis also shows that the same difficulty arises when binding is measured directly, as in the technique of equilibrium dialysis; for even where the affinities at the inner and outer sites are unequal, binding assays would reveal simple saturation kinetics, and, with the substrate at equilibrium, the single half-saturation constant would be identical to the constant for equilibrium exchange. This is true not only of the intact carrier in the membrane, but of an isolated carrier in solution.

In this paper we present a method that avoids such difficulties by allowing the ratio of the dissociation constants on the two sides of the membrane to be measured, rather than the individual constants. The ratio is found from the carrier distribution in the presence of an equilibrated substrate analog. In the case of the choline transport system of erythrocytes, the carrier distribution can be estimated by means of the thiol reagent, N-ethylmaleimide.

The rationale of the method is as follows: At saturating concentrations of an analog which has come to equilibrium across the cell membrane, the distribution of the carrier between inward-facing and outward-facing forms depends on the ratio of the substrate dissociation constants at the inner and outer sites. If the analog binds most tightly to the outer carrier form, then the complex of this form will predominate; if it binds most tightly to the inner carrier form, that form will predominate; and if it binds with similar affinity to both, the two forms will be present at similar concentrations.

N-ethylmaleimide is an irreversible inhibitor of the choline transport system, and it was shown to react with the inward-facing form of the carrier (both the free carrier and the complex) but not, at a significant rate, with the outward-facing forms [8, 9, 12, 18]. The rate at which it reacts with the system, which is the rate of inactivation, therefore depends on the proportion of the carrier, under steady-state conditions, in the inner form.

Equilibration of the substrate analogs is ensured by making use of tertiary (rather than quaternary) amines, which rapidly penetrate the cell membrane in their un-ionized form [15]. At neutral pH the predominant form is a cation, which is capable of binding strongly to the choline site; thus dimethylaminoethanol has an apparent affinity nearly as high as choline's [15].

Kinetic Method

The Carrier Distribution is Directly Related to the Substrate Dissociation Constants on the Inner and Outer Surfaces of the Membrane

In the steady state, the fraction of the carrier forming a complex with the substrate T is given by the following general expressions, which were derived by applying the method of King and Altman [13] to the scheme in Fig. 2:

$$\frac{[C_oT]}{C_t} = \frac{f_{-1}k_3(f_{-3}+k_{-4})[T_o] + f_1f_{-3}k_4[T_i] + f_{-3}k_3k_4[T_o][T_i]}{\Sigma} \quad (1)$$

$$\frac{[C_iT]}{C_t} = \frac{f_1k_4(f_3+k_{-3})[T_i] + f_{-1}f_3k_3[T_o] + f_3k_3k_4[T_o][T_i]}{\Sigma} \quad (2)$$

where $[T_o]$ and $[T_i]$ are the external and internal concentrations of the substrate analog, and C_t is the total concentration of the carrier. The denominator of Eqs. (1) and (2) is given by:

$$\begin{aligned}\Sigma = {} & (f_1+f_{-1})(f_{-3}k_{-3}+k_{-3}k_{-4}+f_3k_{-4}) \\ & + \{f_3k_3(f_{-1}+k_{-4}) + f_{-1}k_3(f_{-3}+k_{-4})\}[T_o] \\ & + \{f_{-3}k_4(f_1+k_{-3}) + f_1k_4(f_3+k_{-3})\}[T_i] \\ & + k_3k_4(f_3+f_{-3})[T_o][T_i].\end{aligned}$$

The ratio of the concentrations of the external and internal complex, C_oT and C_iT, respectively, is found from the ratio of Eqs. (1) and (2). Under equilibrium conditions, where $[T_i] = \alpha[T_o]$, the expres-

sion reduces to a simple form. This happens because the ratio of substrate concentrations inside and outside, attained in the final steady state, bears a necessary relationship to the rate constants in the transport scheme in Figs. 1 and 2 (based on the principle of microscopic reversibility):

$$\alpha = \left(\frac{[T_i]}{[T_o]}\right)_{\text{final}} = \frac{f_{-1}f_3K_{T_i}}{f_1f_{-3}K_{T_o}} = \frac{f_{-1}f_3k_{-4}k_3}{f_1f_{-3}k_{-3}k_4}. \quad (3)$$

Substitution of Eq. (3) into the expression for the ratio of $[C_oT]/[C_iT]$ (from Eqs. (1) and (2)) yields:

$$\frac{[C_oT]}{[C_iT]} = \frac{f_{-1}K_{T_i}}{\alpha f_1K_{T_o}}. \quad (4)$$

Equation (4), being independent of f_3 and f_{-3} (the rate constants for transfer of the substrate across the membrane), holds for both transported and non-transported analogs. In the latter case, the equation is easily verified by substituting $f_3 = f_{-3} = 0$ into Eqs. (1) and (2).

The N-Ethylmaleimide Inactivation Rate is Directly Related to the Distribution of the Carrier in the Membrane

The rate of inactivation for the system equals the sum of the rates of inactivation of each carrier species (Fig. 2):

$$Q_{\text{app}} = \frac{q_1[C_i] + q_2[C_o] + q_3[C_iT] + q_4[C_oT]}{C_t} \quad (5)$$

where Q_{app} is the observed rate constant, and q_1 to q_4 are the rate constants for reaction of the individual carrier species with the inhibitor. When the substrate analog T is present at a saturating concentration, a negligible proportion of the carrier remains in the free form (C_o and C_i), and therefore

$$C_t = [C_oT] + [C_iT]. \quad (6)$$

Under these conditions the inactivation rate is given by:

$$Q_{T_oT_i} = \frac{q_3[C_iT] + q_4[C_oT]}{C_t}. \quad (7)$$

The inhibitor has been shown to react at a far faster rate with the inward-facing than with the outward-facing complex ($q_3 \gg q_4$), and consequently the inactivation rate, $Q_{T_oT_i}$, depends on the proportion of the two carrier forms, C_iT and C_oT.

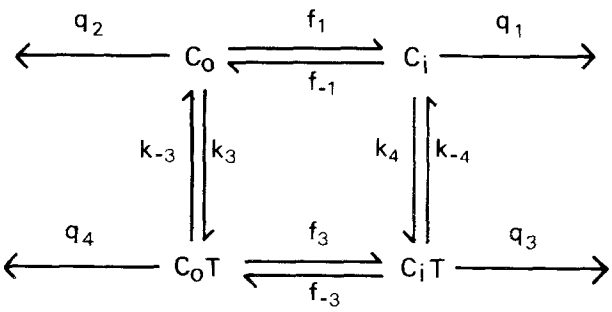

Fig. 2. Kinetic scheme for the reaction of the carrier with an irreversible inhibitor; the rate constants governing reaction with the four carrier species are q_1, q_2, q_3 and q_4

Therefore the Rate of Carrier Inactivation in the Presence of a Saturating Concentration of an Equilibrated Analog Depends on the Relative Affinity of the Analog for the Inward-Facing and Outward-Facing Forms of the Carrier

From Eqs. (4), (6) and (7), the inactivation rate is found to be

$$Q_{T_oT_i} = \frac{\left(q_3 + \frac{f_{-1}K_{T_i}q_4}{\alpha f_1K_{T_o}}\right)}{\left(1 + \frac{f_{-1}K_{T_i}}{\alpha f_1K_{T_o}}\right)}. \quad (8)$$

Rearranging Eq. (8) gives

$$\frac{K_{T_i}}{K_{T_0}} = \frac{\alpha f_1}{f_{-1}}\left(\frac{q_3 - Q_{T_oT_i}}{Q_{T_oT_i} - q_4}\right). \quad (9)$$

In the choline transport system, α may be taken as unity [19], and the f_1/f_{-1} ratio also has a value near unity [14]. The rate constant for inactivation of C_oT, q_4, was estimated previously [8], and q_3, for inactivation of C_iT, is determined in the experimental section that follows. Hence, from Eq. (9), the relative substrate affinities inside and outside may be calculated from the rate of inactivation of transport by N-ethylmaleimide in the presence of an equilibrated substrate analog.

Experimental Methods

Inactivation by N-Ethylmaleimide

The procedure for measuring inactivation rates has been described before [8]. In the present study, cells were equilibrated with a choline analog for 30 min, and then were treated with 1 mM N-ethylmaleimide for varying lengths of time (usually up to 6 min) in a solution of 154 mM NaCl and 5 mM sodium phosphate

Table 1. Rates of inactivation of the choline transport system by 1 mM N-ethylmaleimide in the presence of an equilibrated choline analog (pH 6.8, 37°C)

Analog	I_{50}[a] (mM)	Concentration[b] (mM)	Relative inactivation rate[c] $Q_{T_oT_i}/q_1$	Estimated relative affinity[d] K_{T_i}/K_{T_o}
$(CH_3)_2\overset{+}{N}H$ 	1.76	10.0–19.4	0.37 ± 0.014(2)	5.0
$(CH_3)_2\overset{+}{N}HC_2H_4C$ 	0.75	9.1	0.39 ± 0.02(2)	4.7
$(CH_3)_2\overset{+}{N}H—CH_2$ 	1.35	10.3–18.0	0.46 ± 0.04(4)	3.7
$(CH_3)_3\overset{+}{N}CH_2CH_2OH$[e]	0.006	0.05	0.69 ± 0.05(4)	2.0
$(CH_3)_2\overset{+}{N}HCH_2CH_2OH$[e]	0.019	0.009–0.05	0.69 ± 0.06(4)	2.0
$(CH_3)_2\overset{+}{N}HCH_2CH_2CH_2Cl$	6.8	92	1.44 ± 0.11(2)	0.42
$(CH_3)_2\overset{+}{N}HCH_2CH_2CH_2OH$	0.37	5.3	1.45 ± 0.07(2)	0.41
$(C_2H_5)_2\overset{+}{N}HCH_2CH_2OH$	0.15	2.0	1.51 ± 0.08(3)	0.35
$(C_4H_9)_2\overset{+}{N}HCH_2CH_2OH$[e]	1.59	Saturating	1.71 ± 0.17(5)	0.24
$(C_3H_7)_2\overset{+}{N}HCH_2CH_2OH$	0.66	10	2.03 ± 0.13(5)	0.00

[a] The average value of the concentration of an equilibrated analog required to give 50% inhibition of choline exit (Table 2), calculated from Eq. (10). With a transported analog, the kinetic analysis of the conventional carrier model shows that I_{50} can be higher than the true constant.

[b] Where experiments to determine the inactivation rate were run at more than one concentration of the analog, the highest and lowest concentrations are listed.

[c] The figure in parentheses is the number of experiments done to determine the relative inactivation rate: averages and standard deviations are given.

[d] The relative affinities were calculated from Eq. (11), with $q_4/q_1 = 0.039$, $q_3/q_1 = 2.0$, and $\alpha = f_1/f_{-1} = 1.0$ (*see* text).

[e] From reference [9].

buffer, pH 6.8, 37°C; the reaction was stopped by adding mercaptoethanol (10.6 mM). The transport activity of treated cells was determined from the rate of uptake of ^{14}C-choline [7], and the rates of inactivation were calculated from plots of the logarithm of the transport rate against the time of treatment [8].

Permeability of Tertiary Amines

The permeability of the red cell membrane was tested by the light-scattering method [20], which depends on the rapid osmotic shrinking and swelling of the cells in response to the intracellular solute concentration. Red cells were incubated for an hour or more with an amine at a concentration of approximately 80 mM, after which the rate of exit was monitored. Dimethylaminoethanol and dibutylaminoethanol, results for which were presented earlier [10, 15], came to equilibrium within approximately 4 min. Rapid permeation is a general property of the tertiary amines used in this study, and equilibration times under 4 min were also observed with the following compounds: dimethylaminopropanol, dimethylaminopropylchloride, dimethylbenzylamine, dimethylcyclohexylamine, dimethylpropiophenone, diethylaminoethanol, di-isopropylaminoethanol.

Transport Assays

The inhibition of ^{14}C-choline exit by the analogs was determined as follows [5]: Cells (45% suspension) were equilibrated overnight with ^{14}C-choline; the final internal concentration, approximately 5 μM, was low compared to the internal half-saturation constant, approximately 35 μM [10]. Equilibrated cells were washed in ice-cold buffer and packed by centrifugation, and aliquots of the packed cells were added (10% hematocrit) to solutions containing a choline analog, either in the presence or absence of unlabeled choline at a saturating concentration (550 μM). The pH was 6.8, the temperature 37°C. Samples were withdrawn at intervals quenched in decamethonium bromide, and rapidly centrifuged in the cold; the radioactivity in the supernatant solution was determined by scintillation counting. Plots of radioactivity versus time were approximately linear, as expected if the amines rapidly come to equilibrium across the cell membrane (*see* Fig. 3); a gradual increase in their concentration inside the cell would have caused the plots to be nonlinear. Control rates were determined in the absence of the analog.

The concentrations of the analogs required for 50% inhibition of transport were calculated from exit rates in the presence and absence of the inhibitor (in experiments lacking external choline). The formula used to calculate this concentration (I_{50}) was

$$I_{50} = [I] \Big/ \left(\frac{v_o}{v} - 1\right) \tag{10}$$

where v and v_o are choline exit rates in the presence and absence of the inhibitor, respectively.

To confirm that saturating concentrations of the analogs were used in the experiments on N-ethylmaleimide (Table 1), the inhibition of choline transport was determined at the same high concentrations. In cells preincubated with a low concentration of

radioactive choline (5.5 μM), the percentage inhibition of exit was within 3% of that predicted from the I_{50} values found with lower concentrations of the analogs, as described above. In every case, the inhibition was 90% or greater; the fraction of the carrier in the form of an inhibitor complex is roughly equal to the fractional inhibition.

Results

Inactivation Rates in the Presence of Equilibrated Substrate Analogs

The results for choline and nine analogs are listed in Table 1. As a control in each experiment, the rate of inactivation was determined with a saturating concentration of choline in the external solution, where virtually all the carrier is in the form of C_i, and the observed inactivation rate is therefore equal to q_1 [9][1]. For this reason, the Table reports the ratio of inactivation rates in the presence of an equilibrated substrate analog, and in the presence of saturating choline outside. The relative affinities were calculated from Eq. (11), found by dividing the numerator and denominator of Eq. (9) by q_1:

$$\frac{K_{T_i}}{K_{T_o}} = \frac{\alpha f_1}{f_{-1}} \left(\frac{\frac{q_3}{q_1} - \frac{Q_{T_oT_i}}{q_1}}{\frac{Q_{T_oT_i}}{q_1} - \frac{q_4}{q_1}} \right). \tag{11}$$

The ratio of the pseudo-first-order rate constants for reaction of C_oT and C_i, q_4/q_1, was previously shown to be 0.041 ± 0.016 [8]. The ratio of the rate constants for reaction of C_iT and C_i, q_3/q_1, was found to be greater than unity [9], and its precise value has now been estimated from the inactivation rate in the presence of dipropylaminoethanol, which is shown below to bind exclusively to the inner carrier form; q_3/q_1, taken as the average of five different determinations on different samples of cells, is equal to 2.00 ± 0.08. As noted in the preceding section, f_1/f_{-1} and α are both close to unity.

[1] The carrier-choline complex was shown to move inward through the membrane, and to dissociate inside, at a far faster rate than the free carrier returns to the outer surface; as a result, the carrier accumulates as the free form on the inside (C_i), and in the steady state virtually all the carrier (C_t) is present in this form [14]. Further, the rate of entry of choline into the cell is slow ($\bar{V}_{s_o}$ is approximately 0.2 μmol per liter of packed cells per min [7]) relative to the time of treatment with *N*-ethylmaleimide (up to 6 min), and during the experiment the internal choline concentration remains very low relative to the internal half-saturation constant, which is approximately 35 μM.

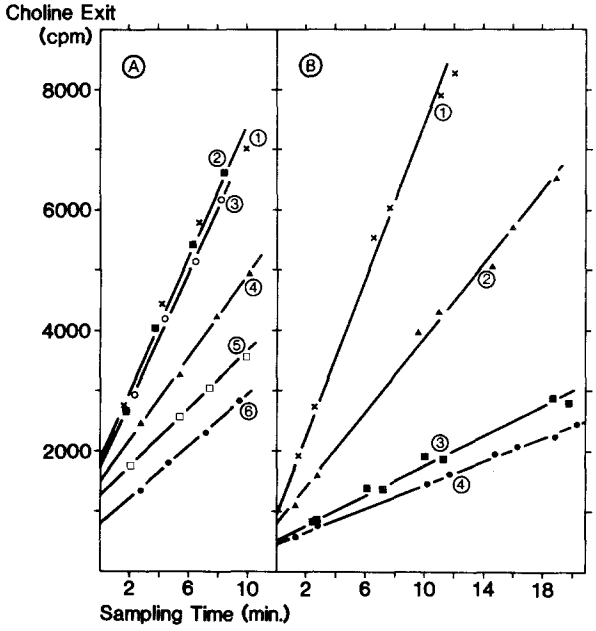

Fig. 3. The exit ^{14}C-choline in the presence or absence of an equilibrated inhibitor, with or without choline (550 μM) in the external medium (pH 6.8, 37°C). (*A*) Inhibition by 3 μM dimethyl-*n*-decyl(2-hydroxyethyl)ammonium bromide (*I*), or 39 μM dimethyl-*n*-dodecylamine (*II*): (1) no inhibitor, + external choline; (2) *I*, + external choline; (3) *II*, + external choline; (4) Control; (5) *II*; (6) *I*. (B) Inhibition by 1.50 mM dipropylaminoethanol (*III*): (1) no inhibitor, + external choline; (2) Control; (3) *III*, + external choline; (4) *III*. In *A*, the concentration of ^{14}C-choline in the cell water was 3.8 μM; in *B*, 5.5 μM. The plot shows the actual counts recorded by scintillation counting; a change of 1000 cpm is equivalent to a change in the internal choline concentration of 0.118 μM

The Determination of q_3/q_1

Of all the substrate analogs tested, dipropylaminoethanol is the most effective in increasing the rate of inactivation by N-ethylmaleimide (Table 1). The rate is somewhat faster than with dibutylaminoethanol, which was shown, in experiments on the kinetics of choline exit and entry, to be bound predominantly to the inner carrier form [10], and it follows that dipropylaminoethanol may be exclusively bound inside.

This hypothesis was tested in the following way: the inactivation rate was determined, first in the presence of equilibrated dipropylaminoethanol, and then in the presence of equilibrated dipropylaminoethanol together with saturating external choline. If the inhibitor adds partly to the outer carrier form, external choline should both displace it, and cause the carrier to accumulate in the internal form where it can complex with the analog inside the cell. From the kinetics of the carrier model, it may be shown that all the carrier would then be in

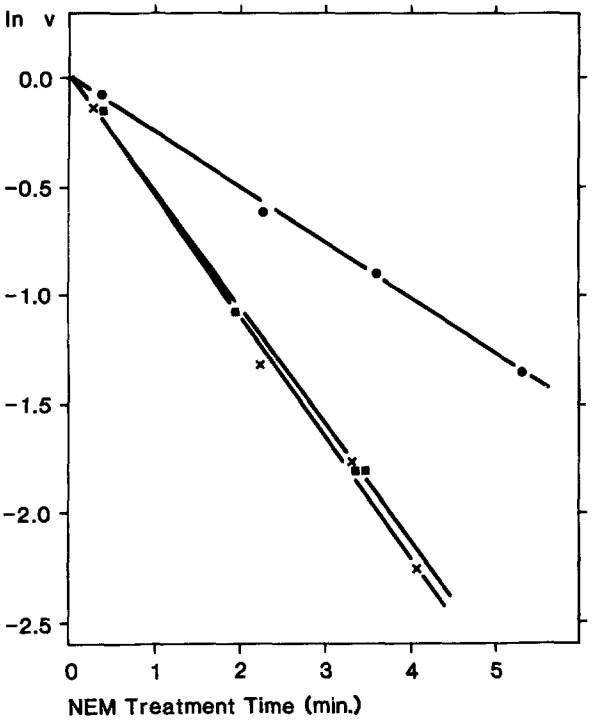

Fig. 4. The effect of external choline on the N-ethylmaleimide inactivation rate in the presence of dipropylaminoethanol. The logarithm of the transport activity is plotted as a function of the time of treatment with 1 mM N-ethylmaleimide at pH 6.8, 37°C, under three different conditions: ●, with 0.26 mM choline in the external medium; ×, in 5.5 mM dipropylaminoethanol; ■, in 5.5 mM dipropylaminoethanol together with 0.26 mM choline. The pseudo-first-order inactivation rate constants calculated from the slopes of the lines are, respectively: 0.254 ± 0.009 min^{-1}, 0.551 ± 0.027 min^{-1} and 0.531 ± 0.019 min^{-1}

the form of the internal complex[1] (*see* Appendix Eq. (A9)), and the inactivation rate would therefore equal q_3, the inhibition constant for C_iT.

Eq. (A9) applies to a substrate analog which is either not transported at all, or transported slowly compared with choline. From observations on the substrate specificity of the choline system [7], dipropylaminoethanol is not expected to be transported at a significant rate: even slight enlargements of the substituents on the nitrogen atom were found to impede transport. For example, with the introduction of a single propyl group, or of two ethyl groups, the transport rate is reduced, and the rate of movement of the carrier complex with the analog relative to that with choline is estimated to be 1/8 or less. With three ethyl substituents, or two ethyl and one propyl, the transport rate falls to nearly zero. The closely related tertiary amine, dibutylaminoethanol, does not undergo transport on the carrier [10].

The experimental result, given in Fig. 4, shows that dipropylaminoethanol is exclusively bound to the inner carrier form (since external choline does not increase the inactivation rate). The q_3/q_1 ratio is calculated to be 2.13 ± 0.15.

Competition between Equilibrated Analogs and External Choline in Transport Assays

To confirm these estimates of the sidedness of binding, an entirely different test was applied, one dependent on the ability of external choline to compete with an externally bound analog, and its inability to compete with an internally bound analog [10]. In the assay, the inhibition of choline exit by a substrate analog is measured, either in the presence of a saturating concentration of unlabeled choline in the external solution, or in the absence of external choline. If the analog is predominantly bound to the outer carrier form, external choline should relieve the reversible inhibition; and if it is predominantly bound to the inner form, external choline should fail to relieve the inhibition.

As a check on the validity of the test, a nonpenetrating analog was included, dimethyl-*n*-decyl(2-hydroxyethyl) ammonium bromide. Being a quaternary ammonium compound, this analog does not diffuse through the cell membrane, and is therefore exclusively bound to the outward-facing carrier [7]. The related tertiary amine, dimethyl-*n*-dodecylamine was also included, though it was not tested with N-ethylmaleimide because it causes hemolysis at higher concentrations. As examples of the method, experiments with these analogs, as well as dipropylaminoethanol, are shown in Fig. 3.

The results, given in Table 2, support the conclusions reached with N-ethylmaleimide: external choline reduces the inhibition by analogs adding mainly on the outer surface of the membrane, but not the inhibition by analogs adding mainly inside.

While these general conclusions are obvious from an inspection of Table 2, a quantitative interpretation of the data would require that the following points be considered. First, the kinetic analysis of transport predicts that the inhibition by an analog bound exclusively inside should become more severe in the presence of choline. The reason is that external choline causes the carrier distribution to shift inward, making the carrier more available to the inhibitor[1]. In terms of a kinetic analysis [6], the inhibitor constant falls from $K_{I_i}(1 + f_{-1}/f_1)$ in the absence of external choline, to $K_{I_i}(1 + f_{-1}/f_2)$ in its presence; because $f_1 \simeq f_{-1}$, and because, for choline, $f_2 \gg f_{-1}$ [14], the latter inhibition constant is about half as large as the former. The inhibition by dipropylaminoethanol does, indeed, increase in the presence of external choline (Table 2), which gives added support to the conclusions.

Table 2. Competition between equilibrated analogs and choline (unlabeled) in the external solution[a]

Analog	Concentration	Choline exit rates (Relative values)					
		Control			Excess unlabeled choline outside		
		−Analog	+Analog	%Inhibition	−Analog	+Analog	%Inhibition
Analogs bound outside preferentially:							
$(CH_3)_2\overset{+}{N}H$ 	1.46	100	57	43	177	151	15
	1.46	100	52	48	192	155	19
$(CH_3)_2\overset{+}{N}HC_2H_4C$(=O) 	0.97	100	42	58	195	141	28
	0.97	100	45	55	194	142	27
$(CH_3)_2\overset{+}{N}HCH_2$ 	1.22	100	52	48	178	138	22
	1.22	100	53	47	195	144	26
$(CH_3)_2\overset{+}{N}H—(CH_2)_{11}—CH_3$	0.039	100	61	39	193	178	7
	0.039	100	64	36	170	161	5
$(CH_3)_2\overset{+}{N}—CH_2CH_2OH$[b]	0.003	100	56	44	192	190	1
$(CH_2)_9—CH_3$	0.003	100	61	39	170	168	1
Analogs bound inside preferentially:							
$(CH_3)_2\overset{+}{N}HCH_2CH_2CH_2Cl$	12.8	100	29	71	173	45	74
	13.1	100	39	61	196	65	67
$(CH_3)_2\overset{+}{N}HCH_2CH_2CH_2OH$	0.44	100	52	48	187	52	72
	0.44	100	36	64	174	35	80
$(C_2H_5)_2\overset{+}{N}HCH_2CH_2OH$	0.46	100	17	83	177	19	89
	0.23	100	47	53	187	55	70
$(C_4H_9)_2\overset{+}{N}HCH_2CH_2OH$	1.40	100	50	50	178	83	53
	1.39	100	56	44	177	85	52
$(C_3H_7)_2\overset{+}{N}HCH_2CH_2OH$	1.50	100	31	69	200	39	81
	1.50	100	30	70	190	35	81

[a] Cells were equilibrated with a low concentration (4.9 μM) of ^{14}C-choline, and the rates of exit were then measured in the presence and absence of an equilibrated choline analog, or of unlabeled choline (550 μM) in the external solution.
[b] Dimethyldecyl(2-hydroxyethyl)ammonium bromide, a quaternary analog which does not enter the cell, is included in order to demonstrate the effect of an inhibitor which is exclusively bound on the outer surface.

Another point to be taken into account is that different behavior is expected with transported and nontransported analogs. From the kinetic analysis [6], the effect of external choline depends on the magnitude of the analog's transport constants, f_3 and f_{-3} (*see* Fig. 4): if f_3 is as large as the corresponding constant for choline (i.e. if the analog is as rapidly transported inward), the analog bound outside would accelerate the exit of labeled choline, just as external choline does (*see* Table 2); consequently, the addition of choline to the external solution would have little effect on the inhibition by an equilibrated substrate. This case would not be mistaken for the case of an analog bound exclusively inside, where, as we have seen, the inhibition increases in the presence of external choline. It follows that the ability of external choline to reduce the inhibition by certain analogs provides evidence that they are bound predominantly outside, and also that they do not undergo transport.

Discussion

Several observations combine to show that the estimates of relative internal and external affinity are essentially correct. First, the estimates from the two tests (Tables 1 and 2) agree. Second, each test is clearly valid. The evidence that N-ethylmaleimide reacts specifically with inward-facing forms of the carrier, and therefore reports on the carrier distribution, may be summarized as follows. To begin with, two reference points are available: an inhibitor bound outside and an inhibitor bound inside.

The quaternary analog diethyl-*n*-decyl (2-hydroxyethyl) ammonium ion, which is exclusively bound outside (it does not enter the cell [7]), gives strong protection against N-ethylmaleimide [5]. Dibutylaminoethanol, which is predominantly bound inside (as shown by the kinetics of its inhibition of choline transport [16]) accelerates the inactivation rate by N-ethylmaleimide. More decisive evidence that N-ethylmaleimide reacts with the inward-facing forms of the carrier, and not with the outward-facing forms, was provided by the observed quantitative relationship, which is predicted by the carrier model, between the maximum transport rates for a series of substrate analogs and their effects on the rates of N-ethylmaleimide reaction [8]. The relationship cannot be explained by a bilateral carrier model (one with substrate sites exposed on both faces of the membrane simultaneously), or by reaction of N-ethylmaleimide with both carrier conformations [8, 12]. It has also been shown that thiol reagents unable to penetrate the cell membrane fail to inhibit choline transport [18].

The trends seen with the substrate analogs are best viewed in relation to choline: choline is bound to the outer site approximately twice as strongly as to the inner, and dimethylaminoethanol, the tertiary counterpart of choline, behaves in the same way (Table 1). Any slight enlargement of the dimethylaminoethanol structure, either by substitution on the nitrogen atom, or by lengthening of the hydroxyethyl chain, is found to shift the affinity in favor of the inner site. Thus, in the series of aminoethanol derivatives, the diethyl analog has three times higher affinity for the inner site than for the outer, and within the limits of observation the dipropyl analog is exclusively bound at the inner site; the still larger dibutylaminoethanol has four times higher affinity for the inner site than for the outer. Extension of the other end of the choline molecule, the hydroxyethyl group, also shifts the balance of affinity in favor of the inner site, as in the case of dimethylaminopropanol and dimethylaminopropylchloride. This evidence makes it clear that the geometry at the inner and outer transport sites is different.

It should be noted that, despite these differences, both sites are highly specific for choline. Judging by the I_{50} values in Table 1, diethylaminoethanol is bound 8 times less strongly than dimethylaminoethanol, dipropylaminoethanol 35 times less strongly, and dimethylaminopropanol 20 times less strongly. This degree of discrimination necessarily reflects the less specific of the two sites, i.e. the inner, and the corresponding factors for the outer site would be considerably greater. It was previously shown that the specificity of the carrier for choline is far higher than the specificity of the enzyme acetylcholinesterase for its substrate, acetylcholine [15].

An extended nonpolar binding region was shown in previous studies to be closely associated with the outer site [7], accounting for the preferential binding there of analogs with a single bulky substituent, as in dimethylcyclohexylamine, dimethylbenzylamine, dimethylaminopropiophenone, and dimethyl-*n*-dodecylamine. Presumably, this region does not exist in the inner carrier form, or is comparatively small in extent. The effect of the external nonpolar region is most marked with dimethyl-*n*-dodecylamine (Table 2), which is expected if the nonpolar region, as was previously shown, has an especially high affinity for long alkyl chains and is separated from the anionic center of the substrate site by the length of three methylene groups [7].

To sum up, the transport sites exposed in the inward- and outward-facing forms of the carrier differ strikingly. First, the outer site is more exactly complementary to choline than is the inner—at both the trimethylammonium and the hydroxymethyl sub-sites. Second, the outer site is adjacent to an extended nonpolar binding region, which is much reduced at the inner site. Third, the inward-facing carrier contains a reactive thiol group, which in the outward-facing form is masked (this being the basis of the N-ethylmaleimide test for the carrier distribution).

Binding asymmetry has also been observed in the glucose carrier of human erythrocytes [2–4]. The observation that derivatives of glucose substituted at C-4 and C-6 were preferentially bound outside, while a derivative substituted at C-1 was preferentially bound inside, was explained by Barnett et al. [4] on the basis of a single substrate site, part of which binds the substrate on one surface, and part on the other: at the outer site C-1 participates in binding, and C-4 and C-6 are in contact with the solvent, while at the inner site the reverse is true. Doubt was thrown on this hypothesis by the later report of Baker et al. [2], that two other analogs substiuted at the C-1 end of the glucose molecule are more firmly bound outside than inside. In the case of the choline transport system, a hypothesis involving a single unchanged substrate site is clearly unsatisfactory, because modifications of any part of the substrate structure give rise to preferential binding inside.

In the choline transport system, therefore, an extensive reorganization of the carrier structure, including the structure of the substrate site, probably occurs during the carrier reorientation step—the step in which the substrate site is removed from contact with the solution on one side of the mem-

brane to make contact with the solution on the other side. Indeed, the striking differences in the interactions of substrate analogs inside and outside may suggest that different regions of the carrier are involved in forming the two sites.

We thank Miss M. Latoszek for technical assistance. This work was supported in part by Grant B1540-8325 of the Departamento de Desarrollo de la Investigacion, Universidad de Chile.

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Received 22 September 1983; revised 12 December 1983

Appendix

The Rate Constant for Inactivation of the Internal Complex (q_3)

In the presence of a saturating concentration of a nontransported analog at equilibrium across the cell membrane, the carrier is entirely in the form of the internal complex once choline has been added to the external solution at a sufficiently high concentration. The proof of this statement follows:

With S representing choline, and T the substrate analog, as in Fig. 5, the inactivation rate Q_{app} is equal to the sum of the rates for each carrier species:

$$Q_{app} = \frac{q_1[C_i] + q_2[C_o] + q_3[C_iT] + q_4[C_oT] + q_5[C_iS] + q_6[C_oS]}{C_t} \quad \text{(A1)}$$

$$C_t = [C_i] + [C_o] + [C_iT] + [C_oT] + [C_iS] + [C_oS]. \quad \text{(A2)}$$

If dissociation of the complex is a rapid step (previously shown to be true of the choline transport system [6]), $[CS_o] = [C_o][S_o]/K_{S_o}$, $[CS_i] = [C_i][S_i]/K_{S_i}$, etc., and Eq. (A1) can be written as

$$Q_{app} = \frac{[C_o]\left(q_2 + q_4\frac{[T_o]}{K_{T_o}} + q_6\frac{[S_o]}{K_{S_o}}\right) + [C_i]\left(q_1 + q_3\frac{[T_i]}{K_{T_i}} + q_5\frac{[S_i]}{K_{S_i}}\right)}{[C_o]\left(1 + \frac{[T_o]}{K_{T_o}} + \frac{[S_o]}{K_{T_o}}\right) + [C_i]\left(1 + \frac{[T_i]}{K_{S_i}} + \frac{[S_i]}{K_{S_i}}\right)}. \quad \text{(A3)}$$

If $[S_i] = 0$, and $[S_o]/K_{S_o} \gg [T_o]/K_{T_o}$, Eq. (A3) may be written as

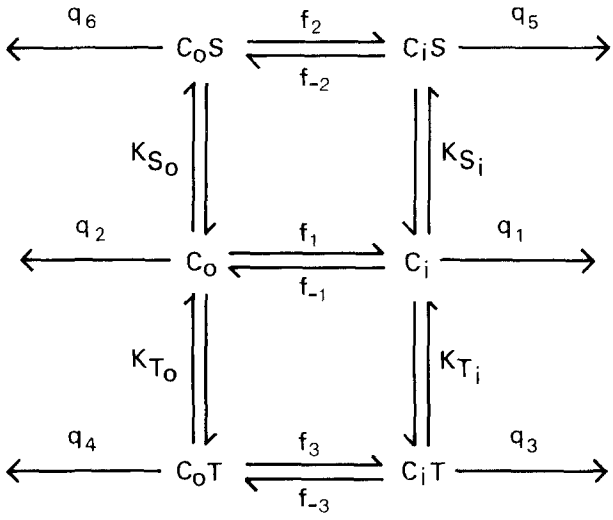

Fig. 5. Kinetic scheme for the reaction of the carrier with an irreversible inhibitor in the presence of two substrates, S and T; the rate constants for inactivation are $q_1 - q_6$

$$Q_{app} = \frac{q_6 \frac{[S_o]}{K_{S_o}} + \frac{[C_i]}{[C_o]}\left(q_1 + q_3 \frac{[T_i]}{K_{T_i}}\right)}{\frac{[S_o]}{K_{T_o}} + \frac{[C_i]}{[C_o]}\left(1 + \frac{[T_i]}{K_{T_i}}\right)}. \tag{A4}$$

The ratio $[C_i]/[C_o]$ is found from the steady-state relationship, which expresses the fact that the outward movement of the carrier balances the inward movement:

$$f_1[C_o] + f_2[C_oS] + f_3[C_oT] = f_{-1}[C_i] + f_{-2}[C_iS] + f_{-3}[C_iT] \tag{A5}$$

hence,

$$\frac{[C_i]}{[C_o]} = \frac{f_1 + f_2 \frac{[S_o]}{K_{S_o}} + f_3 \frac{[T_o]}{K_{T_o}}}{f_{-1} + f_{-2} \frac{[S_i]}{K_{S_i}} + f_{-3} \frac{[T_i]}{K_{T_i}}}. \tag{A6}$$

Equation (A6) may be simplified in the present case, where $[S_i] = 0$, $[S_o]/K_{S_o} \gg f_1/f_2$, and $f_3, f_{-3} \ll f_2$ (since T is not transported):

$$\frac{[C_i]}{[C_o]} = \frac{f_2[S_o]}{f_{-1}K_{S_o}}. \tag{A7}$$

Substitution of (A7) into (A4) yields

$$Q_{app} = \frac{q_6 + \frac{f_2}{f_{-1}}\left\{q_1 + q_3 \frac{[T_i]}{K_{T_i}}\right\}}{1 + \frac{f_2}{f_{-1}}\left\{1 + \frac{[T_i]}{K_{T_i}}\right\}}. \tag{A8}$$

At saturating concentrations of T_i, Eq. (A8) reduces to

$$Q_{app} = q_3. \tag{A9}$$