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Evidence for a Two-State Mobile Carrier Mechanism in Erythrocyte Choline Transport: Effects of Substrate Analogs on Inactivation of the Carrier by N-Ethylmaleimide

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Summary. Choline transport in erythrocytes is irreversibly inhibited by N-ethylmaleimide. The hypothesis that the carrier alternates between outward-facing and inward-facing forms and that only the latter reacts with the inhibitor (Martin, K. (1971) *J. Physiol. (London)* **213**:647–667; Edwards, P.A. (1973) *Biochim. Biophys. Acta* **311**:123–140) is here subjected to a quantitative test. In this test the effects of a series of substrate analogs upon rates of inactivation and rates of choline exit are compared. By hypothesis the effect of an analog in the external solution on the inactivation rate depends only on how it affects the proportion of the inward-facing carrier. Since ^{14}C-choline efflux is necessarily proportional to the concentration of free carrier in the inward-facing form, the analogs should have related effects on the two rates. In every case the observed effects were identical, whether the analogs accelerated transport or inhibited it. Analysis of the results demonstrates that (1) the transport mechanism depends on the operation of a mobile element; (2) distinguishable inward-facing and outward-facing conformations of the free carrier, carrier-substrate complex, and carrier-inhibitor complex exist, and only the inward-facing forms react at a significant rate with N-ethylmaleimide; (3) carrier mechanisms involving a single form of free carrier or a single form of carrier-substrate complex are ruled out; and (4) dissociation of the carrier-substrate complex is a rapid step with all substrate analogs.

Key words. Carrier, inactivation, transport mechanism, N-ethylmaleimide, choline transport, conformational change.

N-Ethylmaleimide (NEM), which reacts with an essential thiol group in the choline transport system of human erythrocytes, was used by Martin [11] and Edwards [6] as a probe of conformational states of the carrier. From the effects of two substrates and two reversible inhibitors on the rates of inactivation of the system by NEM it was concluded that two separate conformations of the carrier exist: an inward-facing and an outward-facing form. However, in a purely theoretical analysis of transport and its irreversible inhibition under steady-state conditions, Lieb and Stein [9, 10, 12] came to the conclusion that formulations of the classical carrier model having one or two forms of carrier-substrate complex (Figs. 1 and 2) cannot be distinguished. Moreover in order to establish the existence of separate forms of the free carrier, Lieb and Stein [10] concluded that the parameters of substrate transport and irreversible inhibition must be related in a definite way, but that insufficient data were available on the choline system to apply the proposed test.

That this theoretical analysis is not general is proven by the fact that in theory separate forms of complex with a nontransported substrate analog may be readily and unambiguously demonstrated, as we explain below. We also show that if the experimental parameters for transport and irreversible inhibition are correlated for a series of analogs rather than a single substrate, then it becomes possible to demonstrate two forms of complex with substrates in general.

We now report experiments required to test the hypothesis of Martin and Edwards. A series of substrate analogs has been used, ranging by degrees from a good substrate to a nontransported inhibitor [5]. The observations are clearly inconsistent with the one-complex formulation of the carrier model, but are in quantitative agreement with the usual

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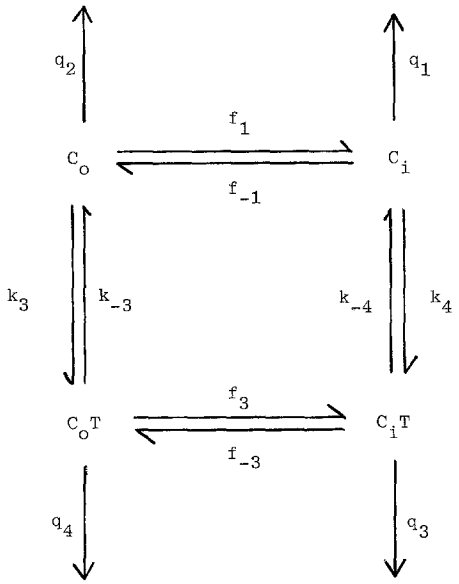


Fig. 1. The two-complex formulation of the classical carrier model. A carrier system is represented having two separate carrier-substrate complexes, one on the inner ($C_i T$) and one on the outer ($C_o T$) surface of the membrane. Each carrier species reacts with an irreversible inhibitor at a rate given by the constants q_1 to q_4. Rate constants for carrier reorientation are designated by the letter f, and those for complex formation by the letter k

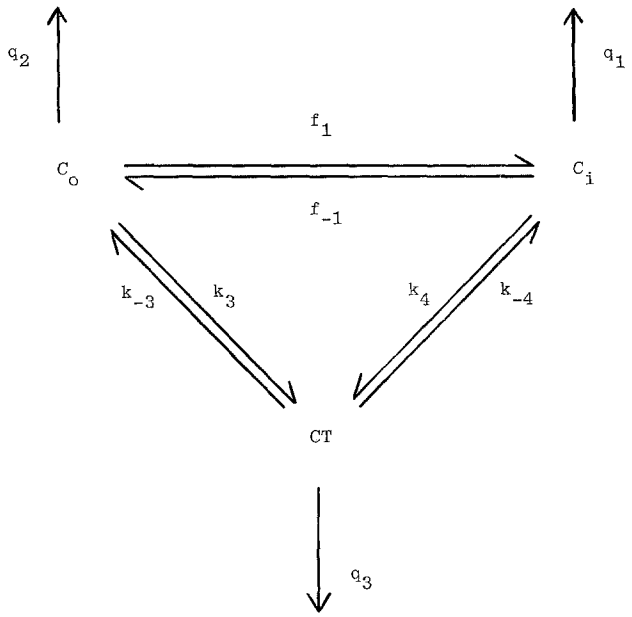


Fig. 2. The one-complex formulation of the carrier model. Whether the substrate adds to the inner or outer form of the free carrier, the same complex is formed. Each carrier species reacts with an irreversible inhibitor at a rate given by the constants q_1 to q_3

two-complex formulation. The results show that the free carrier and the substrate complex exist in two different conformational states, one inward-facing and the other outward-facing, and further that the rate-limiting step in transport involves the interconversion of these states, and not dissociation of the substrate from the complex with the carrier.

Testing the Hypothesis on N-Ethylmaleimide

According to the interpretation of Martin and Edwards only the inward-facing conformation of the carrier reacts with NEM. Briefly, their essential observations were as follows. The substrates choline and tetramethylammonium ion, when located in the extracellular medium, were found to accelerate the rate of inactivation by NEM, whereas protection was observed when cells were pre-loaded with these substrates. The opposite effects were observed with two nontransported substrate analogs: external decamethonium protected, and cells pre-loaded with metanephrine, which enters the cells by passive diffusion, were more susceptible to NEM. It was proposed that the substrate analogs exerted their effect by altering the carrier distribution between the two membrane faces. In general, any force shifting the carrier towards the inner surface should increase the inactivation rate, whereas any force drawing the carrier towards the outer surface should reduce it.

In devising a quantitative test of this hypothesis we note that the direction and strength of the pressure exerted by a substrate analog depends on its rate of transport, and hence a series of substrate analogs having different transport rates should permit us to gradually alter the carrier distribution. A kinetic analysis further demonstrates that if the hypothesis is correct, the effect of the analog on the rate of inactivation by NEM should bear a definite relationship to its maximum transport rate, and this relationship becomes the basis of a quantitative test of the hypothesis. The analysis cannot be applied to the data available because the two substrates used in the earlier studies are transported at the same rate while the inhibitors are not transported at all.

The relationship between the maximum transport rates of analogs and their effects on inactivation by NEM is most simply explained in the following way. When the cells are suspended in pure buffer the carrier exists as an equilibrium mixture of the outer and inner free carrier forms (C_0 and C_i, Fig. 1) and by hypothesis the measured inactivation rate (Q) is proportional to the concentration of the internal

form (C_i)[1]. Upon addition of a saturating concentration of a substrate analog to the external solution there is a redistribution of the carrier. In the new steady state all the carrier on the outside surface of the membrane will be present as the complex, and initially, while the internal substrate concentration is effectively zero, all the internal carrier will exist in the free form (assuming for the moment that the rate of substrate dissociation is rapid compared to the rate of transfer through the membrane). Since only the inner form can react with NEM, the inactivation rate under these conditions (Q_{To}) will again be proportional to the concentration of the free carrier on the inner surface. The ratio of inactivation rates in the presence and absence of external substrate may therefore be written as

$$\frac{Q_{To}}{Q}=\frac{[C_i]_{To\to\infty}}{[C_i]_{To=0}}. \tag{1}$$

Slowly transported substrates will tend to protect the system by holding the carrier in the external complex. Good substrates, for which movement of the complex is faster than return of the unloaded carrier, will drive the carrier into the internal face of the membrane, thereby quickening inactivation.

Now we can show that the rate of choline exit is also proportional to the concentration of C_i. In terms of the carrier scheme in Fig. 3, the rate of exit for labeled choline (S) is given by $f_{-2}[C_iS]-f_2[C_oS]$. By following the method of derivation given before [4], a completely general solution of this expression may be found without any assumptions as to the rate-limiting steps, in the case where the external concentration of the labeled substrate $[S_o]$ is zero, but where a second substrate, unlabeled, is present in the external solution at a given concentration $[T_o]$. The rate of exit of the labeled substrate S is found to be

$$f_{-2}[C_iS]-f_2[C_oS]=\frac{k_{-1}k_2f_{-2}[C_i][S_i]}{k_{-1}f_{-2}+k_{-1}k_{-2}+k_{-2}f_2}. \tag{2}$$

The ratio of exit rates in the presence and absence of external substrate must therefore be:

$$\frac{\tilde{v}^T}{\bar{v}}=\frac{[C_i]_{To\to\infty}}{[C_i]_{To=0}}. \tag{3}$$

[1] The symbols for the experimental inactivation rate constants in the presence or absence of substrate analogs are defined in the Appendix. In brief, Q is the rate of inactivation of the undisturbed transport system, and a subscript *To*, *Ti* or *To Ti* attached to this constant indicates that the substrate T is present at a saturating concentration either outside the cell (o), or inside (i), or both. The inactivation rate constants for individual carrier forms are q_1 to q_4, as shown in Fig. 1.

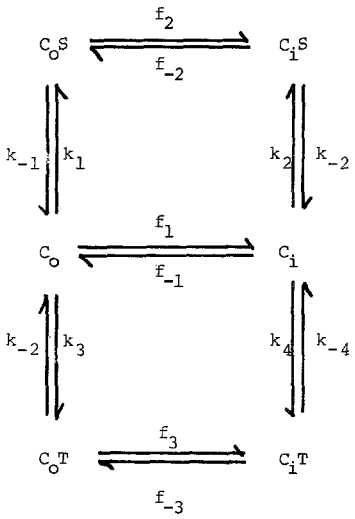


Fig. 3. The two-complex formulation of the carrier, showing two substrates, S and T

From Eqs. (1) and (3) it follows that:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{Si\to 0}=\frac{Q_{To}}{Q}. \tag{4}$$

Since the internal substrate concentration is zero in the determination of Q_{To} and Q, the necessary condition for this equality is indicated in Eq. (4), namely that the internal choline concentration in the exit experiment must be well below its half-saturating concentration, in order not to disturb the carrier distribution.

As stated above, the effect of an analog on the rate of inactivation is related to its maximum rate of transport, and this follows because the ratio of the parameters $(\tilde{v}^T/\bar{v})_{Si\to 0}$ for two different analogs is equal to the ratio of their maximum zero-trans entry rates [4]. Hence,

$$\frac{Q_{To}/Q)_1}{(Q_{To}/Q)_2}=\frac{(\bar{V}_{To})_1}{(\bar{V}_{To})_2} \tag{5}$$

where the subscripts 1 and 2 denote two different substrate analogs, and where $\bar{V}_{To}$ is the maximum rate of substrate entry.

In the above analysis we assumed that substrate dissociation is a rapid step compared to transfer of the substrate complex across the membrane $(k_{-3}\gg f_{-3},\ k_{-4}\gg f_3)$. Were it a slow process, the predictions would be different. With a transported substrate outside the cell the internal carrier would then exist partly or wholly as the substrate complex. If this complex as well as the free carrier reacts with NEM, as the evidence cited below shows, while exit of radioactive choline depends on the free carrier alone, then obviously the effects on inactivation and

transport rates would bear no simple relationship to one another. However, were identical effects actually observed in all cases, it would be possible to conclude that substrate dissociation is always a rapic process.

Materials and Methods

Washed red cells (5–10% hematocrit) were incubated for 12 hr or more at 37 °C, with one change of solution, to free them of endogenous choline. The isotonic saline medium contained 0.1% glucose and 0.02% chloramphenicol. The cells were then washed several times in ice-cold buffer and packed by centrifugation.

I. The Rate of Inactivation by N-Ethylmaleimide

A. Treatment of cells

Cells (2.5% suspension) were treated with 1 mM NEM in the presence of a choline analog at a saturating concentration outside the cells or in its absence. The suspending medium, held at 37 °C, contained 154 mM NaCl and 5 mM sodium phosphate buffer at pH 6.8. Solutions of NEM and the analog were added to the cell suspension at zero time, and samples (40 ml) were removed at intervals and placed in 50-ml tubes containing mercaptoethanol (final concentration, 10.6 mM). The tubes were immediately spun in a clinical centrifuge for 3 min, after which the supernatant was aspirated and the cells washed 3 times in saline solution. The treated cells were then transferred to conical tubes, packed by centrifugation (10 min) and assayed for choline uptake.

B. Uptake Assay

The procedure for determining uptake rates was described before [5]. The concentration of [methyl ^{14}C]choline (30 or 53 mCi/mmol) was 3.0 μM.

C. Determination of Inactivation Rates

Plots were constructed of the natural logarithm of the rate of choline entry, as determined in the uptake assay, against the time of treatment with NEM. The pseudo-first-order inactivation-rate constant was obtained from the slope of the line, by a least-squares analysis. Results under four different conditions are shown in Fig. 4. In every case a linear relationship is observed.

II. The Rate of Choline Exit

The rate of exit of ^{14}C-choline was determined in the presence of a substrate analog at a saturating concentration outside the cell or in its absence, as previously described [3]. The internal concentration of ^{14}C-choline was approximately 2 μM (compared to an internal half-saturation constant of 30 μM).

III. Materials

The synthesis of the choline analogs was described earlier [5]. Red cells were prepared from outdated blood from a blood bank [3].

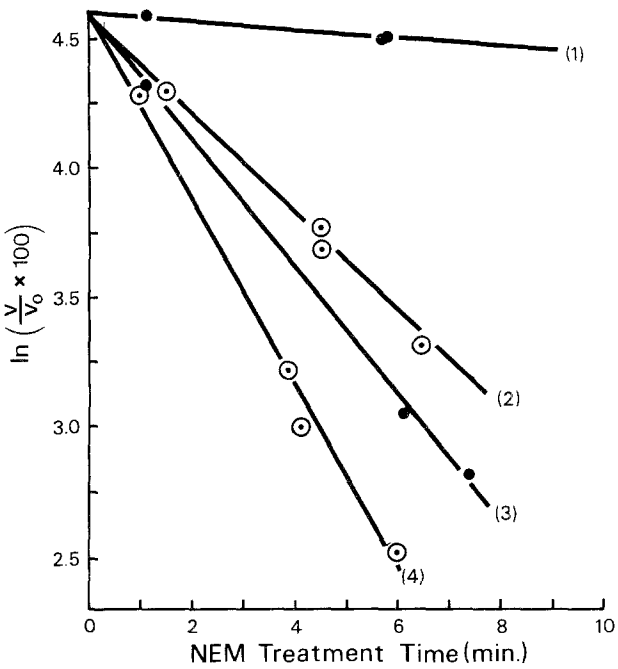


Fig. 4. The inhibition of choline transport activity as a function of time of treatment with 1 mM N-ethylmaleimide (NEM), pH 6.8, 37 °C, in the presence of different choline analogs. The analogs, their concentrations, and the pseudo-first order inactivation rate constants (k) calculated from the slopes of the lines, are as follows: *1*, dimethyl-*n*-decyl(2-hydroxyethyl) ammonium bromide (18 μM), $0.0194 \pm 0.0039\ \text{min}^{-1}$; *2*, control in pure saline solution, $0.197 \pm 0.015\ \text{min}^{-1}$; *3*, diethylmethyl(2-hydroxyethyl) ammonium iodide (385 μM), $0.245 \pm 0.013\ \text{min}^{-1}$; and *4*, choline chloride (50 μM), $0.357 \pm 0.026\ \text{min}^{-1}$. In each case the substrate analog is present at a saturating concentration (values of the experimental half-saturation constants in entry, $\overline{K}_{To}$, are given in Table 2). The inactivation rate constants and their standard deviations were calculated by a least squares analysis

Results

Experimental values of Q_{To}/Q and of $(\tilde{v}^T/\bar{v})_{Si\to 0}$ are recorded in Table 1. Values are also given for the ratio of inactivation rates in the presence of an analog and in the presence of choline (Q_{To}/Q_{cho}), together with the corresponding ratio of rates, $(\tilde{v}^T/\tilde{v}^{cho})_{Si\to 0}$. The latter is the ratio of exit rates of ^{14}C-choline with an analog or with unlabeled choline outside the cell. In every case the ratios $(\tilde{v}^T/\bar{v})_{Si\to 0}$ and Q_{To}/Q are equal, and in addition $(\tilde{v}^T/\tilde{v}^{cho})_{Si\to 0}$ is equal to Q_{To}/Q_{cho}. In these experiments both inactivation and transport rates were measured on the same sample of cells. In other experiments the rates were determined at different times and with cells from different donors. Here average values in a number of experiments were calculated and are given in Table 2. Again the two ratios are seen to be equal with all analogs. The ratios given in Tables 1 and 2 are plotted in Fig. 5, where the points are seen

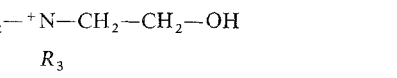
Table 1. Effects of choline analogs at saturating concentrations outside the cells on the rates of carrier inactivation by 1 mM N-ethylmaleimide and on rates of ^{14}C-choline efflux (2 μM)[a]

Analog	Concentration (μM)	$\bar{K}_{To}$ (μM)	Sample	Q_{To}/Q	$(\tilde{v}^T/\bar{v})_{Si\to 0}$	Q_{To}/Q_{cho}	$(\tilde{v}^T/v^{cho})_{Si\to 0}$
Choline	50	6.3 ±0.6	1	1.81 ±0.19	1.90±0.10		
Dimethylethyl(2-hydroxyethyl) ammonium iodide	80	12.4 ±0.6	1	1.71 ±0.15	1.84±0.09		
			2			1.06 ±0.11	0.93±0.04
			3			1.02 ±0.10	0.92±0.07
Diethylmethyl(2-hydroxyethyl) ammonium iodide	385	35.2 ±7.7	1	1.24 0.12	1.31±0.10		
			2			0.75 ±0.09	0.63±0.04
			3			0.68 ±0.09	0.62±0.03
Diethyl-*n*-decyl(2-hydroxyethyl) ammonium bromide	18	1.95±0.05	1	0.098±0.015	0.11±0.02		
			3			0.089±0.007	0.06±0.014

[a] Q is the inactivation rate in the absence of substrates or inhibitors. Q_{To} and Q_{cho} are the inactivation rates with an analog T or with choline, respectively, in the external solution at a saturating concentration. The corresponding transport rate measurements are $\bar{v}$, $\tilde{v}^T$ and $\tilde{v}^{cho}$; thus $(\tilde{v}^T/\bar{v})_{Si\to 0}$ is the ratio of ^{14}C-choline exit rates in the presence and absence of an analog T in the external solution, and $(\tilde{v}^T/v^{cho})_{Si\to 0}$ is the ratio with either the analog T or with choline (cho) outside the cell. Choline exit rates are determined with an internal concentration of ^{14}C-choline (~2 μM) which is much below its internal half-saturation constant, 30 μM. $\bar{K}_{To}$ is the external half-saturation constant for an analog [5]. Experiments were done with cells from three different donors, designated as 1, 2 and 3. Standard deviations of the measurements are included.

Table 2. Effects of choline analogs on N-ethylmaleimide inactivation rates and ^{14}C-choline efflux, as in Table 1[a]

Structure



R_1	R_2	R_3	$\bar{K}_{To}$ (μM)	Concentration (μM)	Q_{To}/Q	Concentration (μM)	$(\tilde{v}^T/\bar{v})_{Si\to 0}$
Me	Me	Me	6.3	25 – 52	1.94±0.15 (3)	52	1.91±0.09 (6)
Me	Me	Et	12.4	42 – 80	1.70±0.02 (3)	80	1.81±0.06 (5)
Me	Et	Et	35.2	195 – 385	1.24±0.07 (4)	385	1.31±0.05 (5)
Me	Me	*n*-Prop	33.2	190	1.21±0.27 (2)	–	1.32±0.03[b]
Me	Me	iso-Prop	303.0	1,698	0.53±0.09 (2)	2,400	0.56±0.06 (4)
Me	Me	*n*-But	30.1	114	0.23±0.08 (2)	–	0.12±0.02[b]
Et	Et	*n*-Dec	1.95	12 – 18	0.08±0.03 (4)	18	0.08±0.04 (4)

[a] Average values for the ratios Q_{To}/Q and $(\tilde{v}^T/\bar{v})_{Si\to 0}$ are given for experiments carried out with red cells from different donors, and the standard deviations of the means are listed. The figure in brackets following each experimental ratio indicates the number of different experiments involved in its determination.

[b] Obtained by extrapolation to infinite concentration from at least 8 determinations of the rate at varying concentrations of the analogs in the external solution.

to be distributed along a line of unit slope passing through the origin.

Detailed Analysis

The essential features of the analysis were outlined above. We now present a more rigorous treatment, with the aim of deciding what carrier models are inconsistent with the observation that $Q_{To}/Q = (\tilde{v}^T/\bar{v})_{Si\to 0}$ for a whole series of substrate analogs, and of deciding what constraints may be imposed upon any adequate carrier model.

In testing the hypothesis that the carrier exists in different conformations on the inner and outer surface of the membrane, two additional observations will be taken into account, as follows.

1. The rate of inactivation with choline at saturating and equal concentrations inside and outside the

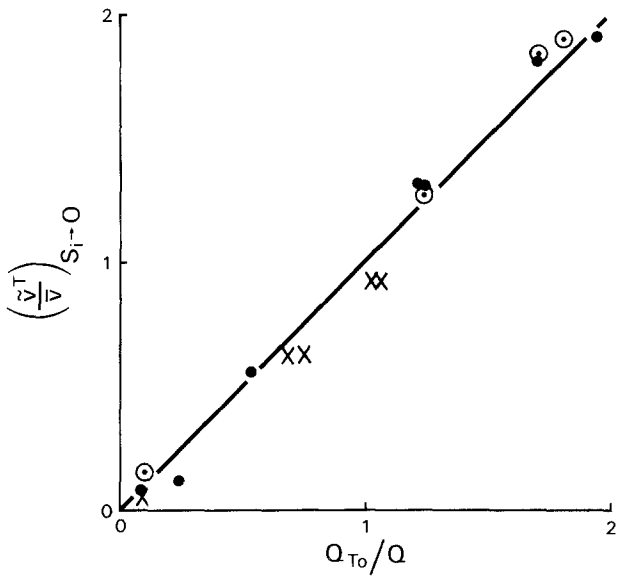


Fig. 5. The relationship between inactivation rate ratios and transport rate ratios in the presence or absence of an analog outside the cell. Solid circles, average values for cell samples derived from different donors (Table 2). Open circles, values obtained from a single cell sample (Table 1). Points marked with an x are ratios in relation to determinations in the presence of choline (Q_{To}/Q_{cho} and $(\tilde{v}^T/\tilde{v}^{cho})_{Si\to 0}$; Table 1)

cells, Q_{ToTi}, is similar to that in the absence of any substrate or inhibitor [1, 11]. The ratio of these rates, Q_{ToTi}/Q, equals 1.18.

2. With a nontransported substrate analog bound to the inner carrier form, the inactivation rate is roughly 3 times greater than in the absence of substrates or inhibitors [1, 11].

I. The Classical Carrier Mechanism Having Inner and Outer Forms of Substrate Complex: An Adequate model

A. From a general kinetic treatment of transport and inactivation rates for the model in Fig. 1 (*see* Appendix), it may be shown that if $Q_{To}/Q = (\tilde{v}_T/\tilde{v})_{Si\to 0}$, then the following relationship must hold:

$$\frac{\frac{q_1}{f_{-1}}+\frac{q_3}{k_{-4}}+\frac{q_4}{f_3}\left(1+\frac{f_{-3}}{k_{-4}}\right)}{\frac{q_1}{f_{-1}}+\frac{q_2}{f_1}}=1. \tag{6}$$

In Eq. (6), q represents rate constants for inactivation, f rate constants for carrier reorientation, and k rate constants for substrate dissociation.

There are only two possible solutions to Eq. (6). First, the inactivation constants for the outward-facing free carrier and for the carrier-substrate complex in either conformation (q_2, q_3 and q_4) may be insignificant compared to that for the inward-facing free carrier (q_1). Second, some or all of these constants, q_2, q_3 and q_4, may be nonzero, with the equality in Eq. (6) preserved because of compensating values of the transport rate constants, f_3, f_{-3}, f_1, f_{-1} and k_{-4}. We shall consider each of these possibilities in turn.

(a) $q_1 \gg q_2, q_3, q_4$

Two different observations clearly rule out this case. First, the inactivation rate with saturating choline in both compartments, inside and outside the cell (Q_{ToTi}), is roughly equal to that in pure salt solution (Q), as was noted above. Since

$$Q_{ToTi}=(f_3q_3+f_{-3}q_4)/(f_3+f_{-3}) \tag{7}$$

and

$$Q=(f_1q_1+f_{-1}q_2)/(f_1+f_{-1}) \tag{8}$$

it follows that q_3 and q_4 cannot both be negligibly small relative to q_1. Second, although the inactivation rate is very low with a reversible inhibitor present outside (Q_{To}), the rate with an inhibitor bound to the inner carrier form (Q_{Ti}) is 3 times higher than in the absence of substrates or inhibitors (Q). The measured inactivation rate with such an inhibitor, which does not undergo transport and for which

$f_3 \simeq f_{-3} \simeq 0$, is

$$Q_{Ti}=q_3. \tag{9}$$

Therefore q_3 cannot equal zero[2], and the first solution, where $q_2, q_3, q_4 \ll q_1$, must be ruled out.

(b)

$$\frac{q_3}{k_{-4}}+\frac{q_4}{f_3}\left(1+\frac{f_{-3}}{k_{-4}}\right)=\frac{q_2}{f_1}. \tag{11}$$

In considering how this equality may hold, we will take up each term separately.

(i) q_2/f_1

The following analysis shows that q_2 is negligibly small relative to q_1. As seen in Table 2, $Q_{To}/Q=1.94$ for choline. The general expression for this ratio is

[2] An alternative explanation for failure of an analog to undergo transport is rate-limiting dissociation of the substrate complex ($k_{-3}, k_{-4} \ll f_3, f_{-3}$). In this case a nonpenetrating inhibitor should exert the same effect whether located in the internal or external compartment (*see* Appendix):

$$Q_{To}=Q_{Ti}=(f_3q_3+f_{-3}q_4)/(f_3+f_{-3}). \tag{10}$$

Since an external inhibitor protects against inactivation while an internal inhibitor makes the system more vulnerable, the hypothesis of rate-limiting dissociation must be rejected.

found from Table 3 (Appendix).

$$\frac{Q_{To}}{Q}=\frac{\left(q_1+\frac{f_{-1}}{k_{-4}}q_3+\frac{f_{-1}}{f_3}\left(1+\frac{f_{-3}}{k_{-4}}\right)q_4\right)(f_1+f_{-1})}{\left(1+\frac{f_{-1}}{f_3}+\frac{f_{-1}}{k_{-4}}\left(1+\frac{f_{-3}}{f_3}\right)\right)(f_1q_1+f_{-1}q_2)}. \quad (12)$$

With the use of the inequalities previously demonstrated in the case of choline [8]. $f_{-1}/f_3 \ll 1$ and $f_{-1}(1+f_{-3}/f_3)/k_{-4} \ll 1$, Eq. (12) reduces to the following form:

$$\frac{Q_{To}}{Q}=\frac{q_1(f_1+f_{-1})}{f_1q_1+f_{-1}q_2}=1.94. \quad (13)$$

Since f_1 and f_{-1} were shown [8] to be approximately equal ($f_{-1}/f_1 \simeq 0.9$), Q_{To}/Q can approach a value of 2 only if q_2 is very much smaller than q_1.

(ii)

$q_4(1+f_{-3}/k_{-4})/f_3$

The above analysis shows that q_2/f_1, the term on the right-hand side of Eq. (11), is very small relative to q_1/f_{-1}, and it follows that the terms on the left-hand side must also be very small, including the term above. With nontransported inhibitors in the external solution, for which f_3 and f_{-3} approach zero, the expression for Q_{To} (Table 3) reduces to q_4, and in these cases Q_{To} is experimentally found to be equal to zero (Fig. 4, Tables 1 and 2). Hence with inhibitors, q_4 equals zero. There is the possibility with a rapidly transported substrate, where k_{-4} and $f_3 \gg f_{-1}$ or f_1, that q_4 is nonzero but that nevertheless $q_4/f_3 \ll q_1/f_{-1}$. This cannot be true for substrates having intermediate transport rates, such as diethylmethyl-, dimethyl-*n*-propyl- and dimethylisopropyl-(2-hydroxyethyl) ammonium ions, whose maximum transport rates are lower than choline's (Table 2; [5]). Here f_3 or k_{-4} must be comparable in size to f_{-1} and f_1 (*see* Appendix, Eq. (18)) so that unless $q_4 \ll q_1$, $q_4(1+f_{-3}/k_{-4})/f_3$ would not be negligible relative to q_1/f_{-1}. We conclude therefore that q_4 must be small relative to q_1 with both inhibitors and substrates.

(iii) q_3/k_{-4}

This is the last of the three terms in Eq. (11), and as seen in the preceding section, it too must be small relative to q_1/f_{-1}. However q_3 itself is not small. It was shown in section (a) that q_3 and q_4 cannot both be small with choline, and since q_4 is small, q_3 must be large. It was also shown in section (a) that q_3 is large with a nontransported analog as well. Despite this, the term q_3/k_{-4} is small with all analogs, and this can only happen if $k_{-4} \gg f_{-1}$, i.e., if the rate of substrate dissociation is far more rapid than reorientation of the free carrier. In fact, rapid substrate dissociation was previously demonstrated in the case of choline [8]. We may now extend this conclusion to all substrate analogs, including inhibitors, since the relationship $Q_{To}/Q=(\tilde{v}^T/\tilde{v})_{Si\to 0}$ holds throughout the series of substrates (*see also* footnote 2).

In summary, the following conditions are required to satisfy Eq. (11):

(a) q_2, $q_4 \ll q_1$, q_3

(b) $k_{-4} \gg f_{-1}$.

B. A second experimental relationship demonstrated in Fig. 4 is the following:

$$Q_{To}/Q_{\mathrm{cho}}=(\tilde{v}^T/\tilde{v}^{\mathrm{cho}})_{Si\to 0}$$

where cho designates the experimental value with a saturating concentration of choline in the external medium. The condition required for this equality is

$$\frac{q_3}{k_{-4}}-\frac{q'_3}{k'_{-4}}+\frac{q_4}{f_3}\left(1+\frac{f_{-3}}{k_{-4}}\right)-\frac{q'_4}{f'_3}\left(1+\frac{f'_{-3}}{k'_{-4}}\right)=0 \quad (14)$$

Table 3. Experimental inactivation rate constants expressed in terms of individual constants for the two-complex formulation of the carrier model in Fig. 1 and for the one-complex formulation in Fig. 2

Experimental inactivation rate constant	Analog location (saturating)		Two-complex model	One-complex model
	Inside	Outside		
Q	–	–	$\frac{f_1q_1+f_{-1}q_2}{f_1+f_{-1}}$	$\frac{f_1q_1+f_{-1}q_2}{f_1+f_{-1}}$
Q_{To}	–	+	$\frac{k_{-4}f_3q_1+f_{-1}f_3q_3+f_{-1}(k_{-4}+f_{-3})q_4}{k_{-4}(f_{-1}+f_3)+f_{-1}(f_3+f_{-3})}$	$\frac{k_{-4}q_1+f_{-1}q_3}{k_{-4}+f_{-1}}$
Q_{Ti}	+	–	$\frac{k_{-3}f_{-3}q_2+f_1f_{-3}q_4+f_1(k_{-3}+f_3)q_3}{k_{-3}(f_1+f_{-3})+f_1(f_3+f_{-3})}$	$\frac{k_{-3}q_2+f_1q_3}{f_1+k_{-3}}$
Q_{ToTi}	+	+	$\frac{f_3q_3+f_{-3}q_4}{f_3+f_{-3}}$	q_3

where the primed constants are those for choline. A detailed analysis of this expression will not be given here, since following the procedure above leads to essentially the same conclusions: that q_4 and q'_4 are negligibly small compared to q_3 and q'_3, and that k_{-4} and k'_{-4} are very much larger than f_{-1}.

II. A Model with only One Form of Carrier-Substrate Complex: An Inadequate Model

The one-complex model (Fig. 2) predicts that with a nontransported inhibitor, where $k_{-4}=k_{-3}=0$, $Q_{To}=Q_{Ti}$ (Table 3). This contradicts the experimental results, since with inhibitors $Q_{To}\simeq 0$ while Q_{Ti} is the highest inactivation rate observed. The two-complex scheme reduces to a one-complex model if the rate of reorientation of the carrier-substrate complex is very much faster than the rate of substrate dissociation, and in this case too $Q_{To}=Q_{Ti}$ for a nontransported analog, as was pointed out in Footnote 2.

While the above is sufficient to rule out the one-complex scheme, we may note that in general it fails to explain how $Q_{To}/Q=(\tilde{v}^T/\vec{v})_{Si\to 0}$ for a complete series of substrate analogs. For this to be true, it may be shown from the expressions for the constants given in the Appendix that $q_3/k_{-4}=q_2/f_1$. This equality would hold if $q_2=q_3=0$, but in this case substrates and inhibitors at saturating concentrations should completely protect the system (for a substrate at equilibrium $Q_{ToTi}=q_3$, and for inhibitors also, $Q_{To}=Q_{Ti}=q_3$) but this is not found experimentally. If q_2 and q_3 are nonzero, the term q_2/f_1 will be substrate-independent, while k_{-4} in the term q_3/k_{-4} varies with different substrates. Accordingly, it would be necessary to assume that q_3/k_{-4} is a constant equal to q_2/f_1, and that q_3 is proportional to k_{-4}. Again, with a nontransported analog, for which $k_{-4}=0$, q_3 must also be zero. Hence inhibitors should always protect, but they do not.

Conclusions

The conclusions to be drawn from the present experiments hinge upon the quantitative relationships between transport and inactivation rates found in the presence of a series of substrate analogs having varying maximum transport rates. The results confirm and extend the hypothesis of Martin [11] and Edwards [6]. Based on this evidence the main features of the choline transport mechanism are as follows:

1) The process of substrate transfer across the membrane requires a change in conformation in the carrier of such magnitude that a substrate site which has been exposed on one surface of the membrane disappears, after which a substrate site becomes exposed on the other side. The mechanism is therefore that of a "mobile carrier", defined previously [2] as "any specific saturable transport system dependent on the operation of a mobile element."

2) Distinct inward-facing and outward-facing carrier conformations are detected experimentally in the free carrier, in the carrier-inhibitor complex, and in the carrier-substrate complex. The basis for this conclusion is the finding that the inward-facing forms of the free carrier and of the carrier complex with substrates or inhibitors react far more rapidly with NEM than the outward-facing forms. Indeed, a model which takes account of only one form of carrier-substrate complex (Fig. 2) cannot explain the observations on substrates or inhibitors, as we have seen. This one-complex model, which was developed by Lieb and Stein [9, 10, 12] and shown to be consistent with a number of observations on transport kinetics, must therefore be rejected, at least in the case of the choline system. Further, the conclusion reached by Lieb and Stein [9, 10, 12] that steady-state data on either substrates or irreversible inhibitors cannot distinguish between a one-complex (Fig. 1) and a two-complex (Fig. 2) formulation of the carrier must also be revised.

3) The rate of dissociation of the substrate from the carrier-substrate complex is in all cases rapid compared to the rate of reorientation of the free carrier ($k_{-4}\gg f_{-1}$). This was previously demonstrated for choline [8], and is now extended to other substrates, including those that are slowly transported. Accordingly the rate-limiting step in the transport of substrates having a low maximum velocity must be reorientation of the carrier-substrate complex rather than substrate dissociation ($k_{-4}\gg f_3 < f_{-1}$).

4) The transport step which fails to occur with nontransported substrate analogs is not complex formation (since such substrates can be competitive inhibitors of high affinity [5]) and it is not dissociation of the complex either. Instead, reorientation of the carrier-inhibitor complex is blocked ($k_{-4}\gg f_3$). If this were not true, then inhibitors on either side of the membrane would have identical effects on NEM inactivation rates (*see* Footnote 2 above).

Appendix

I. Inactivation Rate Equations

A general equation for the inactivation rate may be written on the basis of the transport scheme in Fig. 1.

$$Q_{app}=(q_1[C_i]+q_2[C_0]+q_3[C_iT]+q_4[C_0T])/C_t \qquad (A1)$$

where Q_{app} is the measured inactivation rate constant and q_1, q_2, q_3 and q_4 are inactivation rate constants for the various carrier forms and where C_t is the total carrier concentration. An analogous rate equation can be written for the transport scheme involving a single form of substrate complex, as shown in Fig. 2:

$$Q_{app}=(q_1[C_i]+q_2[C_o]+q_3[CT])/C_t. \tag{A2}$$

Expressions for the individual concentration terms, $[C_i]$ etc., may be determined by means of the King-Altman schematic method, and when these are substituted into Eqs. (A1) and (A2) the following general expression for the inactivation rate is found:

$$Q_{app}=\frac{Q+Q_{To}\frac{[T_o]}{\bar{K}_{To}}+Q_{Ti}\frac{[T_i]}{\bar{K}_{Ti}}+Q_{ToTi}\frac{[T_o][T_i]}{\bar{K}_{Ti}\tilde{K}^T_{To}}}{1+\frac{[T_o]}{\bar{K}_{To}}+\frac{[T_i]}{\bar{K}_{Ti}}+\frac{[T_o][T_i]}{\bar{K}_{Ti}\tilde{K}^T_{To}}}. \tag{A3}$$

$\bar{K}_{To}$ and $\bar{K}_{Ti}$ are half-saturation constants for the effect of an analog on the inactivation rate when it is present either outside or inside the cell, respectively. These constants are identical to the transport half-saturation constants determined in zero trans entry and zero trans exit, respectively, and their definitions in terms of individual transport constants in Fig. 1 have been given previously [4]. $\tilde{K}^T_{To}$ is the half-saturation constant for the effect of an external substrate on the inactivation rate when the substrate is also present inside the cell at a saturating concentration. This constant is identical to the transport constant found in infinite trans entry experiments [4].

The full expressions for the experimental inactivation rate constants are listed in Table 3 and their definitions are given below.

a. Definitions of Experimental Inactivation constants

Q: Inactivation rate constant in the absence of substrates or inhibitors, for a given concentration of N-ethylmaleimide.
Q_{To}: Inactivation rate constant in the presence of a saturating concentration of substrate T outside the cells.
Q_{Ti}: Inactivation rate constant in the presence of a saturating concentration of substrate T inside the cells.
Q_{ToTi}: Inactivation rate constant with saturating, and equal, concentrations of substrate T inside and outside the cells.

b. Method of Derivation

In deriving Eq. (A3) for the case of the two-complex model, expressions are first written down for the steady-state concentrations of each of the four carrier species in the reaction scheme in Fig. 1, following the schematic method of King and Altman [7]. The concentrations, as a fraction of the total carrier C_t, are found to be:

$$[C_o]/C_t=\{f_3k_3k_{-4}[T_o]+f_1G\}/\Sigma \tag{A4}$$

$$[C_i]/C_t=\{f_{-3}k_{-3}k_4[T_i]+f_{-1}G\}/\Sigma \tag{A5}$$

$$[C_oT]/C_t=\{f_{-1}k_3(f_{-3}+k_{-4})[T_o]+f_1f_{-3}k_4[T_i]+f_{-3}k_3k_4[T_o][T_i]\}/\Sigma \tag{A6}$$

$$[C_iT]/C_t=\{f_{-1}f_3k_3[T_o]+f_1k_4(f_3+k_{-3})[T_i]+f_3k_3k_4[T_o]/T_i]\}/\Sigma \tag{A7}$$

where $G=f_{-3}k_{-3}+k_{-3}k_{-4}+f_3k_{-4}$, and where Σ is the sum of all terms in the numerators on the right-hand sides of Eqs. (A4)–(A7). The expressions for the concentrations of individual carrier forms are then substituted into Eq. (A1), which gives the inactivation rate, Q_{app}. As a first step in simplifying this equation, the numerator and denominator are divided by $G(f_1+f_{-1})$, which is the constant term in the denominator, Σ. Next, substitution is made of the substrate dissociation constants found in transport experiments. These were previously shown to be [4]:

$$\bar{K}_{To}=\frac{(f_1+f_{-1})G}{k_3\{k_{-4}(f_{-1}+f_3)+f_{-1}(f_3+f_{-3})\}} \tag{A8}$$

$$\bar{K}_{Ti}=\frac{(f_1+f_{-1})G}{k_4\{k_{-3}(f_1+f_{-3})+f_1(f_3+f_{-3})\}} \tag{A9}$$

$$\tilde{K}^T_{To}=\frac{k_{-3}(f_1+f_{-3})+f_1(f_3+f_{-3})}{k_3(f_3+f_{-3})} \tag{A10}$$

where the transport rate is governed by the equation:

$$\frac{-d[T_i]}{dt}=\frac{\bar{V}_{Ti}([T_i]-[T_o])/\bar{K}_{Ti}}{1+\frac{[T_o]}{\bar{K}_{To}}+\frac{[T_i]}{\bar{K}_{Ti}}+\frac{[T_o][T_i]}{\bar{K}_{Ti}\tilde{K}^T_{To}}}. \tag{A11}$$

When these substrate constants have been taken into account, the equation for Q_{app} is found to be identical to Eq. (A3), with Q, Q_{To}, Q_{Ti} and Q_{ToTi} equal to the expressions shown in Table 3 for the two-complex model.

In the case of the one-complex model in Fig. 2, the equations for the concentrations of each of the three carrier forms are found by the method of King and Altman to be:

$$[C_o]/C_t=(f_{-1}(k_{-3}+k_{-4})+k_{-3}k_4[T_i])/\Sigma \tag{A12}$$

$$[C_i]/C_t=(f_1(k_{-3}+k_{-4})+k_3k_{-4}[T_o])/\Sigma \tag{A13}$$

$$[CT]/C_t=(f_{-1}k_3[T_o]+f_1k_4[T_i]+k_3k_4[T_o][T_i])/\Sigma \tag{A14}$$

where Σ is now the sum of the denominator terms on the right-hand sides of Eqs. (A12)–(A14). These equations for the concentrations of the carrier species are substituted into Eq. (A2), following which the equation is simplified by inserting the substrate dissociation constants in transport:

$$\bar{K}_{To}=\frac{(f_1+f_{-1})(k_{-3}+k_{-4})}{k_3(f_{-1}+k_{-4})} \tag{A15}$$

$$\bar{K}_{Ti}=\frac{(f_1+f_{-1})(k_{-3}+k_{-4})}{k_4(f_1+k_{-3})} \tag{A16}$$

$$\tilde{K}^T_{To}=\frac{f_1+k_{-3}}{k_3}. \tag{A17}$$

These constants are found in a general analysis of the one-complex model in Fig. 2, where the general rate expression is identical in form to Eq. (A11) [1, 9, 12]. The simplified equation for the inactivation rate found in this way is identical to Eq. (A3), where the constants Q, Q_{To}, Q_{Ti}, Q_{ToTi} are now defined by the expressions shown in Table 3 for the one-complex model.

II. Transport Rate Equations

A general treatment of the two-complex carrier scheme in Figs. 1 and 3 was presented earlier [4], and Stein and Lieb [9, 12] have treated the one-complex scheme in Fig. 2. The only expressions required in the present analysis are those for the flux ratio, $(\tilde{v}^T/\bar{v})_{Si\to 0}$. This is the ratio of the exit rates for labeled substrate S, present in the cell at a concentration well below its half-saturating level, either into a solution containing a saturating concentration of a substrate analog T, or into pure buffer. In the ordinary two-complex scheme this ratio is

$$(\tilde{v}^T/\bar{v})_{Si\to 0}=\frac{f_3k_{-4}(1+f_{-1}/f_1)}{k_{-4}(f_{-1}+f_3)+f_{-1}(f_3+f_{-3})} \tag{A18}$$

while in the one-complex scheme it is given by

$$(\tilde{v}^T/\bar{v})_{Si\to 0}=\frac{1+f_{-1}/f_1}{1+f_{-1}/k_{-4}}. \tag{A19}$$

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