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EVIDENCE FOR THE CARRIER MODEL OF TRANSPORT FROM THE INHIBITION BY *N*-ETHYLMALEIMIDE OF CHOLINE TRANSPORT ACROSS THE HUMAN RED CELL MEMBRANE

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SUMMARY

1. The rate constant k of the inactivation of red cell choline transport by 1 mM *N*-ethylmaleimide has been measured under various conditions.

2. k has a control value between 0.17 and 0.30 min^{-1}; in the presence of a saturating concentration of a competitive inhibitor (decamethonium) outside the cell k is about 0.004 min^{-1}; when there is a saturating concentration of choline inside the cell k is about 0.04 min^{-1}; when there is a saturating concentration of substrate (choline, tetramethylammonium or *N*-methylpyridinium) outside the cell k is about 0.7 min^{-1}; in the presence of a saturating concentration of competitive inhibitor (metanephrine) inside the cell k is about 1.5 times greater still, and is the same as k in the presence simultaneously of a large concentration of substrate outside and of competitive inhibitor inside.

3. The concentrations of substrate and competitive inhibitor giving half-maximal changes in k are equal to K_m and K_i values for transport.

4. These results suggest that the transport system exists in two conformational states, which behave like the inward-facing and outward-facing states of a "carrier." The results are quantitatively consistent with the hypothesis that *N*-ethylmaleimide reacts with inward-facing carrier. Non-carrier models of transport proposed recently cannot explain the results.

INTRODUCTION

Little is known about the mechanism of the transport of small molecules across cell membranes. "Carrier" mechanisms have often been suggested: either a mobile carrier which shuttles between the inside and outside faces of the membrane (see for example Rosenberg and Wilbrandt[1]); or a macromolecule with a binding site for substrate that exists in inward-facing and outward-facing conformations, the transport step being the change between these conformations[2–4]. However, the kinetics of the glucose transport system of the human red cell are incompatible with all the carrier models of transport[5] and there is some evidence for the completely different mechanisms of transport postulated by Naftalin[6], and Lieb and Stein[5], though the evidence for Naftalin's mechanism is disputed[7].

One way to investigate the mechanism of transport is to study changes in the reactivity of the transport system toward irreversible inactivators of transport such as fluorodinitrobenzene and *N*-ethylmaleimide. These changes in reactivity can reflect changes in the conformation of the macromolecule(s) of the transport system, which might be part of the transport process[8]. Martin[9] showed qualitatively that the inactivation of choline transport in human red cells by *N*-ethylmaleimide proceeds faster when there is a substrate of the transport system outside the cell but, in the presence of a substrate inside the cell or a competitive inhibitor outside the cell, the inactivation reaction proceeds more slowly than in the control. To account for these effects he put forward the hypothesis that choline transport has a carrier mechanism and that *N*-ethylmaleimide reacts more rapidly with carrier in the inward-facing state than in the outward-facing state[9]. The inactivation of glucose transport in red cells by fluorodinitrobenzene shows the same effects and therefore could also have this mechanism[8,10].

I have made a quantitative study of the rate of inactivation of choline transport by *N*-ethylmaleimide in the presence of substrates or competitive inhibitors inside or outside the cell, to test predictions derived from Martin's hypothesis, and to see if the detailed properties of this reaction provide any information about the mechanism of transport.

A preliminary account of part of this work has been given[11].

THEORETICAL

The hypothesis to be tested is: (1) The choline transport system has a carrier mechanism. (2) *N*-Ethylmaleimide only attacks inward-facing carrier. (3) *N*-Ethylmaleimide attacks inward-facing carrier at the same rate whether it is free carrier or complexed with substrate or competitive inhibitor.

If the choline transport system is some type of carrier, carrier–substrate complex must cross the membrane much faster than free carrier, because[12] unidirectional choline flux is greatly accelerated by choline on the *trans* side of the membrane[13]. A consequence of this is that when there is substrate on only one side of the membrane, at any instant a large proportion of the carrier is present as free carrier at the opposite face of the membrane[9,12]. Therefore adding substrate outside the cell increases the proportion of carrier facing inwards and, according to Postulate 2, increases the rate of inactivation by *N*-ethylmaleimide[9]. Competitive inhibitor outside the cell decreases the rate of inactivation because it binds to the carrier and holds it in the outward-facing state.

The following theoretical treatment is based on a version of the carrier model that allows the carrier to be asymmetrical with respect to the outside and inside of the cell, and which has been discussed by Geck[14] and Miller[4].

Notation

Symbols for the model. S_{in} and S_{out}, substrate concentrations inside and outside; I_{in} and I_{out}, competitive inhibitor concentrations inside and outside; $K_{S(in)}$ and $K_{S(out)}$, true dissociation constants of inward-facing and outward-facing carrier–substrate complexes; $K_{I(in)}$ and $K_{I(out)}$, true dissociation constants of inward-facing and outward-facing carrier–competitive inhibitor complexes; a_{in} and

a_{out}, rate constant for empty carrier changing from inward-facing to outward-facing *vice versa*; $a_{in}r_{in}$, and $a_{out}r_{out}$, corresponding rate constants for loaded carrier; b, a_{in}/a_{out}; k^* rate constant of the reaction between *N*-ethylmaleimide and inward-facing carrier; X, a substrate or competitive inhibitor.

Experimental transport parameters. K_m, Michaelis constant; K_i, inhibition constant, *i.e.* apparent dissociation constant, of competitive inhibitor[15].

Experimental parameters of N-ethylmaleimide reaction. k, observed rate constant of *N*-ethylmaleimide attack on choline transport; k_0, k in the absence of added substrate or competitive inhibitor; k_∞, k in the presence of a saturating concentration of substrate or competitive inhibitor; K_p, apparent dissociation constant of a substrate or competitive inhibitor derived from its effect on the *N*-ethylmaleimide reaction.

General equation for the rate of inactivation of transport by N-ethylmaleimide

From Postulates 2 and 3, the rate of inactivation k is given by $k = k^* \times$ (fraction of carrier facing inwards). An expression for the fraction of carrier facing inwards can be derived from the carrier model by the procedures given, for example, in refs 4 and 14. Substituting this expression,

$$k = k^* \frac{a_{out}(1 + r_{out}S_{out}/K_{S(out)}),(1 + S_{in}/K_{S(in)} + I_{in}/K_{I(in)})}{a_{out}(1 + r_{out}S_{out}/K_{S(out)})\,(1 + S_{in}/K_{S(in)} + I_{in}/K_{I(in)}) + a_{in}(1 + r_{in}S_{in}/K_{S(in)})}$$

$$(1 + S_{out}/K_{S(out)} + I_{out}/K_{I(out)}) \qquad (1)$$

As loaded carrier crosses the membrane much faster than empty carrier, r_{in} and r_{out} are large compared to 1. This enables us to deduce from the equation the effect of substrate on the rate of inactivation.

Double-reciprocal plot and dissociation constants

The effect of various concentrations of a substrate or competitive inhibitor X on the rate of inactivation by *N*-ethylmaleimide can be described by k_∞, the rate of inactivation in the presence of a saturating concentration of X, and an apparent dissociation constant or half-saturation constant for X. Consider for example the case of substrate outside the cell, when rearranging Eqn 1 gives:

$$1 - k/k_0 = \frac{S_{out}(1 - k_\infty/k_0)}{S_{out} + K_p} \qquad (2)$$

where

$$k_\infty = \frac{k^* r_{out}}{r_{out} + b} \qquad (3)$$

and

$$K_p = \frac{K_{S(out)}(1 + b)}{r_{out} + b} \qquad (4)$$

The equation for the analogous transport situation, substrate influx into substrate-free cells, is:

$$\text{influx, } v = \frac{S_{out}V}{S_{out} + K_m} \tag{5}$$

Comparing this with Eqn 2 shows that K_p is analogous to K_m and is an apparent dissociation constant for substrate outside the cell. Furthermore, the expression for K_p (Eqn 4) is the same as the expression for K_m derived from the asymmetrical carrier model[4]; in other words the hypothesis predicts that K_p for substrate outside the cell will be equal to the K_m for influx of substrate into substrate-free cells. Similarly, K_p for substrate inside is predicted to be equal to the K_m for efflux into substrate-free medium. For competitive inhibitor outside the cell, K_p will be equal to the K_i[15] for the inhibition of substrate influx into substrate-free cells by the competitive inhibitor outside the cell.

Eqn 2 can be rearranged to give a double-reciprocal plot, analogous to the Lineweaver–Burk plot, and the same double-reciprocal plot is obtained for a substrate or competitive inhibitor X inside or outside the cell:

$$\frac{1}{1 - k/k_0} \textit{ versus } \frac{1}{[X]} \tag{6}$$

will be a straight line intercepting the axes at

$$\frac{1}{1 - k_\infty/k_0} \text{ and } -\frac{1}{K_p}$$

This graph can be used to obtain k_∞ and K_p from experimental results.

Rates of inactivation by N-ethylmaleimide in the presence of saturating concentrations of substrate or competitive inhibitor

From Eqn 1 the predicted values of k_∞ are, in order of size:

$$\text{competitive inhibitor outside } 0 \tag{7}$$

$$\text{substrate inside } \frac{k^*}{1 + br_{in}} \tag{8}$$

$$\text{control, } k_0, \frac{k^*}{1 + b} \tag{9}$$

$$\text{substrate outside } \frac{k^* r_{out}}{r_{out} + b} \tag{10}$$

$$\text{competitive inhibitor inside } k^* \tag{11}$$

Variations in the control rate k_0

It was found experimentally that although k_0 is constant for a given batch of cells, it is higher in cells that have been cold-stored for a few days. This increase might be explained by the fall in the K^+ concentration inside cold-stored cells[16],

as K^+ is a weak substrate of the choline transport system [17]. An allowance can be made in the formal treatment for the presence of K^+, but it does not alter the predictions to be tested.

Predictions to be tested

(a) Competitive inhibitors inside the cell (in the absence of substrate) will accelerate inactivation of transport by *N*-ethylmaleimide. (b) When the rate of inactivation k is measured in the presence of various concentrations of a substrate or competitive inhibitor X, the results will give a straight line on the double-reciprocal Plot 6. (c) The apparent dissociation constants for X, K_p, derived from this plot will be equal to particular K_m or K_i values for X.

The double-reciprocal plot will also give the rate of inactivation by *N*-ethylmaleimide in the presence of a saturating concentration of X, k_∞. From Expressions 7–11, remembering that r_{in} and r_{out} are large compared to 1:

(d) k_∞ for competitive inhibitor outside will be the smallest rate of inactivation and approximately zero, since all the carrier will be bound at the outside face of the membrane; for substrate inside the cell k_∞ will be larger but still small compared to the control rate, k_0; for substrate outside the cell k_∞ will be much larger than the control; but k_∞ for competitive inhibitor inside will be larger still and will be the highest rate of inactivation, since all the carrier will be bound in the inward-facing state.

(e) If, as seems reasonable, carrier–substrate complex crosses the membrane at about the same rate for various different substrates, *i.e.* r_{out} values are similar for these substrates, then k_∞ for substrate outside the cell will be the same for all these substrates (Expression 10).

Since in the presence of a saturating concentration of competitive inhibitor inside the cell all the carrier will face inwards, it will not be possible to increase the rate of inactivation by adding substrate outside the cell. Putting this in a form which is easier to test:

(f) The rate of inactivation in the presence simultaneously of a near-saturating concentration of substrate outside and a large concentration of competitive inhibitor inside the cell will be equal to, and not greater than, k_∞ for competitive inhibitor inside.

METHODS AND MATERIALS

The buffer used was 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Tris–HCl (pH 7.7 at 25 °C). It was sterilised before use. Bank blood cells 2–4 days old were used except where indicated. They were washed in buffer according to Martin[13].

Measurement of the rate at which N-ethylmaleimide irreversibly inactivates choline transport

Cells were prepared with or without a competitive inhibitor or substrate inside or outside. For example, cells with no choline inside were suspended in a solution of a competitive inhibitor, or cells loaded with choline were washed free of external choline with ice-cold buffer, then suspended in plain buffer at 37 °C. They were

exposed to *N*-ethylmaleimide for periods long enough to inactivate 30 to 90% of their choline transport. The *N*-ethylmaleimide and substrate or competitive inhibitor were then removed and the ability of the cells to transport choline was measured, to determine the extent to which the *N*-ethylmaleimide had inactivated the transport system.

Preparation of cells

Circulating red blood cells contain about 13 μM choline[18], so cells were either loaded with a known concentration of choline or made choline-free by incubating them overnight at room temperature in buffer containing 0.2% glucose and 0.01% penicillin, with or without choline, respectively.

Provided sterile buffer was used bacterial contamination was negligible. The amount of choline taken up by the cells was measured in a duplicate incubation with [^{14}C]choline.

Treatment of cells with N-ethylmaleimide

A stock solution of 100 or 200 mM *N*-ethylmaleimide was made up just before use in 150 mM NaCl, 1 mM sodium phosphate (pH 6.5). This solution was diluted with buffer at 37 °C not more than 10 min before it was reacted with the cells.

Cells were treated with *N*-ethylmaleimide in the presence and absence of substrates or competitive inhibitors at 37 °C, pH 7.4, 2–3% hematocrit, in 10 ml of buffer. 1 mM *N*-ethylmaleimide was normally used. Incubation was for 3–12 min. In a given experiment the hematocrits of all samples were the same to within a 20% range, and the time of incubation was the same, to avoid errors introduced by small decreases of the *N*-ethylmaleimide reaction rate with time, caused by consumption of the *N*-ethylmaleimide by non-specific reaction with the cells. About a quarter of the reagent was found to be used up in 12 min from 1 mM *N*-ethylmaleimide at 2.5% hematocrit; *N*-ethylmaleimide was estimated by reaction with mercaptoethanol and back titration with 5,5-dithio-bis-(2-nitrobenzoic acid)[19]. Hydrolysis of *N*-ethylmaleimide[20] was not significant.

Reaction with *N*-ethylmaleimide was stopped by adding 30 ml of ice-cold buffer containing 10 mM mercaptoethanol; this stops the reaction in under 2 s even when the concentration of *N*-ethylmaleimide is 10 mM.

Measurement of transport

The cells were extensively washed and prepared for the measurement of transport in one of two ways. (i) Cells which had been loaded with choline were washed with the concentration of choline that was inside them, to give a distribution of choline approximately in the steady state[13], since the concentration was always 50 μM or more. Transport was then assayed by measuring influx from the same concentration of [^{14}C]choline, that is, exchange influx was measured. [^{14}C]Choline influx was measured according to Martin[13,17]. (ii) Cells which had initially been choline-free were washed in buffer. If they had been incubated with *N*-ethylmaleimide in the presence of metanephrine (a competitive inhibitor of choline transport which can enter the red cell) or of external substrate, it was necessary to remove the metanephrine or the substrate which had entered the cells, so that it would not interfere with the subsequent influx measurement, by incubating them at 37 °C in buffer for up to 2 h. Net [^{14}C]choline influx into choline-free cells was then measured[13,17] from 3 μM [^{14}C]choline in substrate experiments, or, in experiments where competitive inhibitors had been used, from 50 μM [^{14}C]choline.

Choline transport was taken to be the hemicholinium-sensitive choline flux[21]. The hemicholinium-insensitive flux was less than 0.5% of the total choline flux before *N*-ethylmaleimide treatment; it was not increased by *N*-ethylmaleimide under these conditions, and it was always less than 10% of the total choline flux after inhibition by *N*-ethylmaleimide.

N-Ethylmaleimide did not increase cell lysis under these conditions.

Treatment of decamethonium-loaded ghosts with N-ethylmaleimide

Decamethonium is a competitive inhibitor of choline transport that does not penetrate the red cell membrane[21]. The effect of intracellular decamethonium on the rate of inactivation by *N*-ethylmaleimide was investigated by preparing resealed ghosts containing decamethonium, incubating them with *N*-ethylmaleimide, and measuring the inactivation of choline transport produced by the *N*-ethylmaleimide.

Resealed ghosts were prepared from fresh blood cells according to Martin[9], by hypotonic lysis at 0–4 °C, restoration of tonicity to 320 ideal mosM with NaCl, and incubation at 37 °C. Ghosts were loaded with decamethonium by including it in the lysing medium. The resealed ghosts were spun down at $20000 \times g$ for 5 min. They were then washed with Ca^{2+}-free buffer containing the concentration of decamethonium that was inside the ghosts, then again in plain buffer.

Treatment with *N*-ethylmaleimide. Ghosts from 0.75 ml intact cells were suspended at 37 °C in 5 ml of buffer. If the sample of ghosts was to have decamethonium outside during exposure to *N*-ethylmaleimide, the buffer contained decamethonium. Reaction with *N*-ethylmaleimide was started by addition of concentrated *N*-ethylmaleimide solution and stopped with 30 ml ice-cold buffer containing 10 mM merecaptoethanol. The ghosts were then washed three times in buffer.

Transport was assayed by [^{14}C]choline efflux. The ghosts were loaded with [^{14}C]choline by incubation for 1.5–2 h at 37 °C with 15 μM [^{14}C]choline (this incubation was found to load all the samples to a similar extent). [^{14}C]Choline efflux was then measured as described by Martin[9]. Each sample of ghosts was halved before the measurement and one half used to determine efflux into plain buffer, the other to determine efflux into 2 mM hemicholinium-3.

K_i for the inhibition of choline influx by decamethonium

Decamethonium is a competitive inhibitor of choline transport[21]. The apparent inhibition constant, K_i, for the inhibition by decamethonium of choline influx into choline-free cells was derived from triplicate measurements of influx from 1 μM [^{14}C]choline in the presence and in the absence of 80 or 100 μM decamethonium outside the cell. K_i was calculated from [15]

$$v/v_0 = \frac{S + K_m}{S + K_m\left(1 + \frac{I}{K_i}\right)}$$

where v is the inhibited and v_0 is the control influx, S is the concentration of substrate, K_m the Michaelis constant for influx —about 25 μM (see Results)— and I is the concentration of decamethonium.

The permeability of the red cell membrane to metanephrine

In some experiments cells were loaded with metanephrine, so the rate at which metanephrine crosses the cell membrane was measured (metanephrine is not a substrate of choline transport; it presumably crosses the membrane by the same route as adrenalin[22]). Its rate of influx into red cells was determined by incubating cells at 60–70% hematocrit with 2 or 4 or 30 mM metanephrine at 37 °C. Samples were taken at 1-min intervals, rapidly centrifuged, and the concentration of metanephrine remaining in the supernatant estimated from the ultraviolet absorbance at 278 nm of the supernatant. A correction was made for the absorbance due to traces of haemoglobin by measuring absorbance also at 540 nm.

Liquid scintillation counting

1 ml aqueous samples were mixed with 10 ml of 4 g/l 'butyl PBD' (2-(4-*t*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole obtained from CIBA) in toluene–Triton X-100 (2:1, v/v) and counted at room temperature. Counting efficiency was estimated by the sample–channels-ratio method.

Materials

Metanephrine was obtained from Sigma Chemical Co. *N*-Methylpyridinium was a gift from the Molecular Pharmacology Unit, National Institute for Medical Research, London, N.W.7. Other materials as Martin[9,21].

RESULTS

The time-course of the inactivation of choline transport by N-ethylmaleimide

Fig. 1 shows the time-course of the inactivation of choline transport by *N*-ethylmaleimide. The results suggest that the reaction between *N*-ethylmaleimide and the transport system is first-order with respect to the transport system, that is, the inhibition of transport proceeds according to the equation:

$$v/v_0 = e^{-kt}$$

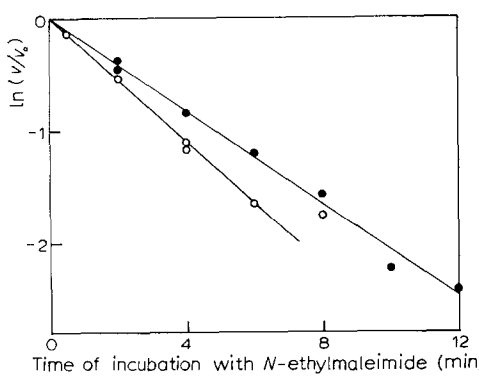


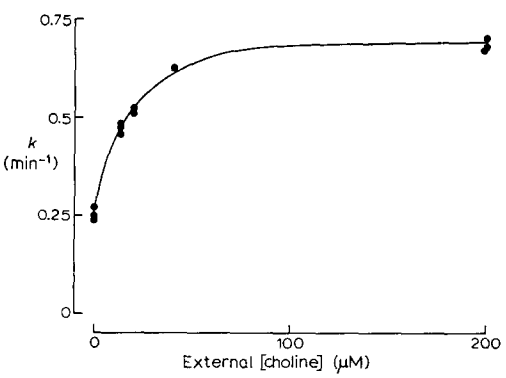
Fig. 1. The time course of the inactivation of choline transport by *N*-ethylmaleimide. Choline-free cells were incubated in the absence of choline with 1 mM *N*-ethylmaleimide, washed, and choline transport assayed. v/v_0 is transport rate of *N*-ethylmaleimide-treated cells divided by transport rate in untreated cells. Two experiments: ●—●, cells from bank blood stored for 1 day; ○—○, cells stored for 2 days.

where v is the rate of choline transport in cells treated with *N*-ethylmaleimide for a time t, v_0 is the rate of transport in untreated cells, and k is the rate constant of the reaction with *N*-ethylmaleimide. In all other experiments the rate of inactivation by *N*-ethylmaleimide was taken to be the value of k obtained by measuring the degree of inactivation v/v_0 after incubation with *N*-ethylmaleimide for a single time t, which was the same for all the incubations in a given experiment.

For a given batch of cells measurements of k_0, the rate of inactivation by *N*-ethylmaleimide in the absence of added substrate or competitive inhibitor, had a standard deviation of 5% but k_0 varied between batches of cells with the age of the cell sample, from 0.17 and 0.18 min^{-1} in two experiments on fresh blood, using 1 mM *N*-ethylmaleimide, to 0.28 to 0.30 min^{-1} in three experiments on 4-day-old bank blood. On the other hand, other k values, for example, the rate of inactivation in the presence of a saturating concentration of choline outside the cell (Table I), are constant to within the limits of error regardless of the age of the cells. A possible explanation of this is given in Theoretical.

Effects of substrate on the rate of inactivation by N-ethylmaleimide

Substrate outside. Fig. 2 shows how the presence of various concentrations of choline outside the cell accelerate inactivation by *N*-ethylmaleimide. When these results are plotted on the double-reciprocal plot they give a straight line (Fig. 3), in agreement with Prediction b. Values for the apparent dissociation constant for choline outside the cell obtained from Fig. 3 and two similar experiments were 16, 22 and 18 μM (Table I). In agreement with the hypothesis (Prediction c), K_m for choline influx into choline-free cells, measured by the method of Martin[17] on three separate occasions, was 20, 22 and 29 μM, not significantly different from the dissociation constant but perhaps slightly larger. The rate of inactivation by *N*-ethylmaleimide in the presence of a saturating concentration of choline outside the cell was about 0.7 min^{-1}, which was large compared to the control rate (Table I).



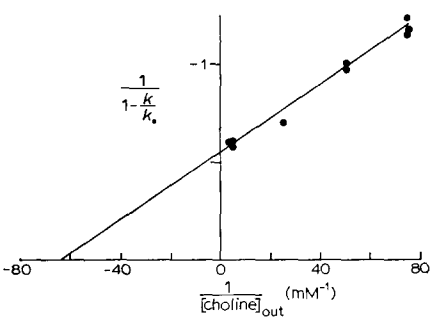


Fig. 2. The effect of external choline on the rate of inactivation by *N*-ethylmaleimide. Choline-free cells were incubated in 1 mM *N*-ethylmaleimide with various concentrations of choline for 4 min. They were then washed and incubated in buffer for 2 h at 37 °C to remove the small amount of choline that had entered the cells. Transport was then assayed. k is the rate constant of inactivation by *N*-ethylmaleimide.

Fig. 3. Double-reciprocal plot of the effect of external choline. Data from Fig. 2. k/k_0 is rate of inactivation divided by the rate in the absence of choline.

TABLE I

THE EFFECT OF EXTERNAL SUBSTRATE ON THE RATE OF INACTIVATION OF CHOLINE TRANSPORT BY *N*-ETHYLMALEIMIDE

Methods as in Fig. 2. Except for choline (1 mM only), the results were obtained ± S.E. from double-reciprocal plots like Fig. 3, which is Expt 8/12. k_∞ is the rate of inactivation in the presence of a saturating concentration of substrate outside the cell, given by the intercept on the ordinate of the double-reciprocal plot; K_p is the apparent dissocation constant of the substrate given by the intercept on the abscissa. k_0 is the rate of inactivation in the absence of added substrate (mean of triplicates; S.D. 5% in a given experiment). For choline (1 mM only) the rate of inactivation was measured in the presence of 1 mM choline and k_∞ calculated by extrapolation using the results of the first three experiments.

Experiment	*Substrate outside*	k_∞ (min^{-1})	k_0 (min^{-1})	K_p (μM)
3/9	choline	0.65 ± 0.1	0.22	22 ± 8
8/12	choline	0.73 ± 0.03	0.26	16 ± 2
12/1	choline	0.68 ± 0.04	0.28	18 ± 3
8/9	*N*-methylpyridinium	0.68 ± 0.03	0.18	380 ± 50
	choline (1 mM only)	0.73 ± 0.02		
7/9	tetramethylammonium	0.62 ± 0.06	0.23	140 ± 30
	choline (2 mM only)	0.65 ± 0.02		

It did not vary significantly between experiments. As predicted the rates of inactivation in the presence of a saturating concentration of two other substrates, tetramethylammonium[21] and *N*-methylpyridinium (Edwards, P.A.W., unpublished), outside the cell, were the same (Table I).

Substrate inside. Fig. 4 shows an experiment to estimate the apparent dissociation constant for choline inside the cell. The value obtained was $80 \pm 10\,\mu$M, fulfilling the prediction that it would be equal to the K_m of choline efflux into a choline-free medium, which is $89 \pm 8\,\mu$M (mean ±S.E.; Edwards, P.A.W. and Martin, K., unpublished).

The rate of inactivation by *N*-ethylmaleimide in the presence of a saturating concentration of choline inside the cell k_∞ obtained from Fig. 4 was 0.02 ± 0.02 min^{-1}, about 1/8 of the control rate 0.17 min^{-1}. In a further experiment, cells were loaded with $326 \pm 6\,\mu$M choline (mean ±S.E.) by incubation overnight at 37 °C in 2 mM choline, washed free of extracellular choline with ice-old buffer, and the rate of inactivation by *N*-ethylmaleimide determined as in Fig. 4. From two determinations of this rate and the control rate, k_∞ was calculated to be 0.05, 0.065 min^{-1}, assuming that the apparent dissociation constant for choline inside the cell is $80\,\mu$M. So k_∞ for choline inside the cell is about 0.02 to 0.06 min^{-1}.

Substrate on both sides of the membrane at once. The rate of inactivation of transport in the presence on both sides of the membrane of $50\,\mu$M choline was determined in three experiments. It was about twice the control rate but always less than the rate in the presence of $50\,\mu$M choline outside the cell.

Effects of competitive inhibitor on the inactivation by N-ethylmaleimide

Two competitive inhibitors were used in this study: decamethonium[21], which

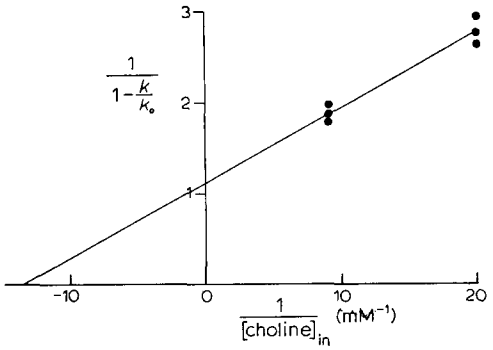


Fig. 4. The slowing effect of internal substrate on the rate of inactivation by *N*-ethylmaleimide. Cells from fresh blood were loaded with choline by incubation overnight with choline in 75 mM $MgCl_2$, 75 mM sucrose, 5 mM Tris–HCl instead of buffer as this gives an increased loading with choline[17]. The cells were then washed free of extracellular choline with ice-cold buffer, incubated at 37 °C with 1 mM *N*-ethylmaleimide for 8 min, and the inactivation of transport determined. Double-reciprocal plot as Fig. 3.

does not penetrate red cells[21], and metanephrine (Edwards, P. A. W., unpublished) which enters red cells rapidly.

The effect of the competitive inhibitor decamethonium outside the cell. The effect of decamethonium outside the cell on the rate of inactivation of transport by *N*-ethylmaleimide was exactly that predicted by the hypothesis: the rate of inactivation in the presence of various concentrations of decamethonium gave a straight line on the double-reciprocal plot (Fig. 5); the apparent dissociation constant for decamethonium obtained from such double-reciprocal plots was equal to the K_i for the inhibition of net choline influx by decamethonium outside the cell (Table II); and the rate of inactivation in the presence of a saturating concentration of decamethonium outside the cell (Expression 7) was about 0.004 min^{-1} (Table II). This is for example 0.5% of the rate of inactivation in the presence of a saturating concentration of choline outside the cell, and it is smaller than the rate in the presence of a saturating concentration of choline inside the cell.

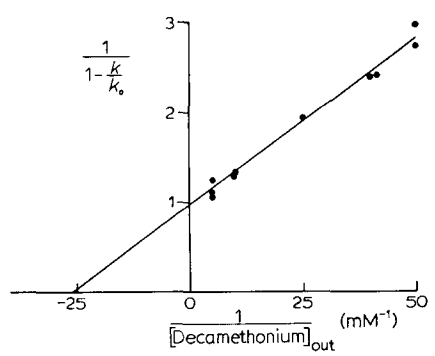


Fig. 5. Double-reciprocal plot of the slowing effect of external decamethonium on the rate of inactivation by *N*-ethylmaleimide. Choline-free cells were incubated with decamethonium and 1 mM *N*-ethylmaleimide for 8 min, washed to remove all the decamethonium, and transport assayed.

TABLE II

THE EFFECT OF THE COMPETITIVE INHIBITOR DECAMETHONIUM OUTSIDE THE CELL ON THE *N*-ETHYLMALEIMIDE REACTION

Methods as in Fig. 5 except that in the undated experiments cells were incubated for 12 min with decamethonium and *N*-ethylmaleimide. In the first three experiments k_∞, the rate of *N*-ethylmaleimide attack in the presence of a saturating concentration of external decamethonium, and K_p, the apparent dissociation constant of external decamethonium, were obtained, as described in Table I, from the intercepts of double-reciprocal plots, Fig. 5 being the double-reciprocal plot for Expt 21/12. In the four other experiments k was measured with 1 mM decamethonium outside the cells, then k_∞ calculated by extrapolation using the value of K_p obtained in the first three experiments. K_i is the inhibition constant for external decamethonium inhibiting choline influx into choline-free cells (see Methods and Materials), measured in the same experiments.

Experiment	k_∞ (min^{-1})	K_p (μM)	K_i (μM)
9/9	-0.004 ± 0.04	45 ± 11	41 ± 6
9/12	-0.03 ± 0.05	50 ± 7	40 ± 1.5
21/12	-0.014 ± 0.03	42 ± 5	36 ± 1
Four others (total 7 determinations)	0.004 ± 0.001		

The effect of decamethonium on the inactivation by N-ethylmaleimide of choline transport in ghosts. The effect of decamethonium inside the cell was investigated by preparing resealed ghosts with and without decamethonium inside, and measuring the rate at which their choline transport was inactivated by *N*-ethylmaleimide. The effect of decamethonium outside the ghosts was also measured.

Choline transport is usually taken to be the choline flux sensitive to hemicholinium-3[21]. Choline efflux from ghosts includes a substantial leak component insensitive to hemicholinium-3 which is larger after *N*-ethylmaleimide treatment. Typical values of efflux rate constants were ($\times 10^3$ in min^{-1}): efflux from ghosts not treated with *N*-ethylmaleimide into plain buffer, 16; into buffer containing 2 mM hemicholinium, 3; efflux from *N*-ethylmaleimide-treated ghosts into buffer, 6–16; from *N*-ethylmaleimide-treated ghosts into 2 mM hemicholinium, 8. Ghosts which had had nearly all their choline transport inactivated by *N*-ethylmaleimide gave a higher choline efflux into hemicholinium than into plain buffer, implying that hemicholinium itself or some impurity increases the leak efflux. The apparent hemicholinium-sensitive efflux, calculated by subtracting the efflux into hemicholinium from the efflux into buffer, is therefore an underestimate of the efflux mediated by the choline transport system and can be negative. The increase in leak induced by hemicholinium should, however, be the same for all ghosts which received the same *N*-ethylmaleimide treatment, so the efflux values for *N*-ethylmaleimide-treated ghosts given in Table III will all be underestimated to the same extent, as will the efflux values given for untreated ghosts. The changes caused by decamethonium in the rate of inactivation of transport by *N*-ethylmaleimide deduced from Table III will therefore be qualitatively correct. The results for the untreated samples show that the ghosts that had been loaded with decamethonium still contained enough decamethonium when efflux was measured to inhibit efflux by 25% or less (depending on how much

TABLE III

THE EFFECT OF THE COMPETITIVE INHIBITOR DECAMETHONIUM ON *N*-ETHYLMALEIMIDE INACTIVATION OF CHOLINE TRANSPORT IN GHOSTS

Resealed ghosts with or without 100 μM or 500 μM decamethonium inside or outside were treated with *N*-ethylmaleimide. They were then loaded with [^{14}C] choline by incubation in [^{14}C]choline at 37 °C, in preparation for the measurement of transport as choline efflux. For details see Methods and Materials and text. All experiments are shown.

Decamethonium		*N-Ethyl-maleimide*	*10^3 × apparent hemicholinium-sensitive efflux* (min^{-1})*			
Inside	*Outside*		*100 μM decamethonium (Expt 5/11)*	*500 μM decamethonium*		
				Expt 4/2	*Expt 16/2*	
−	−	−	14.1	13.8	5.3	5.5
−	+	−	13.5	12.9		
+	−	−	11.5	9.0	4.6	
+	+	−	11.5	11.0		
−	−	+	2.2	2.7	−0.8	−0.2
−	+	+	3.3	7.9		
+	−	+	0.2	−4.8	−2.5	−1.8
+	+	+	2.2	4.9		

* Efflux rate constant obtained by subtracting the rate constant of efflux into 2 mM hemicholinium from the rate constant of efflux into buffer; this gives negative values for samples with almost completely inhibited transport (see text).

the given efflux rates underestimate the efflux mediated by the choline transport system). The rest of the decamethonium had presumably leaked out during the 1.5–2-h incubation at 37 °C necessary to load the ghosts with [^{14}C]choline before the measurement of efflux (see Methods and Materials). Correcting the results for this competitive inhibition would raise the efflux values for the decamethonium-loaded ghosts, but not enough to change the qualitative differences between the different situations.

In spite of these technical difficulties, the results show reproducible qualitative changes in the rate of inactivation by *N*-ethylmaleimide. Because different concentrations of decamethonium were used, and the rate of choline transport varies between batches of cells[17,23] the three experiments in Table III should be considered individually.

The most important result is that in each experiment the inactivation of transport by *N*-ethylmaleimide was greater in the ghosts with decamethonium inside only than in the ghosts without decamethonium (Table III). This is very important support for the hypothesis. Decamethonium outside ghosts reduces the inactivation by *N*-ethylmaleimide (Table III) as it does in intact cells, supporting the assumption that the reaction of *N*-ethylmaleimide with ghosts is similar to its reaction with intact cells.

Effects of the competitive inhibitor metanephrine inside and outside the cell. Metanephrine is not transported by the choline transport system (Edwards, P. A. W., unpublished) but crosses the membrane by some other route, perhaps in the same way

as adrenalin[22]. In order to design the experiments, the permeability of red cells to metanephrine was measured. The rate constant of influx of 2, 4 and 30 mM metanephrine was similar, 0.30 ± 0.05 min^{-1} at 37 °C, so the half-time of metanephrine movement into or out of red cells, at low hematocrit, is about 2 min.

The effect of metanephrine outside the cell was measured by suspending cells in *N*-ethylmaleimide with 2 mM metanephrine and incubating for 2 min. After this time there would have been about 1 mM metanephrine inside the cells, which would have caused the effect of metanephrine outside the cells to be underestimated since the metanephrine inside would tend to increase the rate of inactivation (see below). The rate of inactivation was reduced to $43 \pm 10\%$ of the control (mean ± S.E.), suggesting that metanephrine behaves like other competitive inhibitors[9].

To study the effect of metanephrine inside the cell, packed cells which had been equilibrated with metanephrine were suspended in *N*-ethylmaleimide and incubated for 40 s. Although a little of the metanephrine would have come out of the cells during this incubation, it would have been diluted too much to have had any effect on the outside, and it does not come out on the choline transport system. Table IV and Fig. 6 show that metanephrine inside the cell accelerated inactivation by *N*-ethylmaleimide, in agreement with the prediction of the hypothesis and with the results of the experiments on decamethonium-loaded ghosts. This effect was measured for a series of concentrations of metanephrine and Fig. 6 shows that the results give a straight line on the double-reciprocal plot.

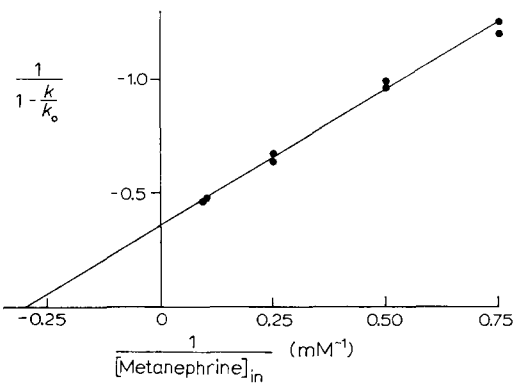


Fig. 6. The effect of internal metanephrine. Cells were pre-equilibrated for 15 min at 37 °C with metanephrine, centrifuged, resuspended in 8 mM *N*-ethylmaleimide for a 40-s incubation, washed and incubated in buffer at 37 °C to remove the metanephrine, then transport was assayed.

The rate of inactivation of transport by 3 mM *N*-ethylmaleimide in the presence of a saturating concentration of metanephrine inside the cell was 3.0 ± 0.12 min^{-1} (mean ± S.E. from Table IV and two other experiments) which can be compared with the rate in the presence of 1 mM choline outside the cell, 2.0 ± 0.08 min^{-1} in the same experiments (1 mM choline is virtually a saturating concentration, see Table I). The rate in the presence of choline was three times the rate with 1 mM *N*-ethylmaleimide, suggesting that the rate of inactivation by *N*-ethylmaleimide is proportional to its concentration, as might be expected.

These results complete the survey of the rates of inactivation in the presence of saturating concentrations of substrate and competitive inhibitor, k_∞. In agreement with Prediction d, k_∞ for competitive inhibitor outside is virtually zero; k_∞ for substrate inside is larger but still very small compared to the control rate; k_∞ for substrate outside is large; and k_∞ for competitive inhibitor is larger still.

TABLE IV

THE EFFECTS OF METANEPHRINE INSIDE, CHOLINE OUTSIDE, AND BOTH TOGETHER

Methods as in Fig. 6, except that 3 mM *N*-ethylmaleimide was used, and some cell samples were incubated with *N*-ethylmaleimide in the presence of external choline. k_∞, the rate of *N*-ethylmaleimide attack in the presence of a saturating concentration of metanephrine inside, was obtained ±S.E. from a double-reciprocal plot of k for 3 and 30 mM metanephrine inside. k and k_0 values are averages of duplicates or triplicates ±S.E.

Metanephrine inside (mM)	*Choline outside (mM)*	*Rate constants of inactivation (min^{-1})*
		k_∞
3 and 30	—	3.24 ± 0.15
		k
30	1	3.32 ± 0.05
30	—	2.70 ± 0.06
—	1	2.16 ± 0.05
		k_0
—	—	0.72 ± 0.04

The combined effect of substrate outside and competitive inhibitor inside the cell

Prediction f was also fulfilled; in the presence simultaneously of a large concentration of the competitive inhibitor metanephrine inside the cell and a near-saturating concentration of choline outside the cell, the rate of inactivation was 1.02±0.05 and 0.99±0.035 (mean±S.E. in two experiments, respectively) times the rate in the presence alone of a saturating concentration of competitive inhibitor inside the cell. The first of these experiments is shown in Table IV.

DISCUSSION

Two questions can be asked about the results: (1) what do they suggest about the mechanism of transport, and (2) which mechanisms of transport postulated in the literature can account for them?

Inferences from the results about the mechanism of transport

Increases in the rate of inactivation of transport are thought to be caused by conformational changes in the macromolecule(s) of the transport system[8]. Since substrate outside the cell and competitive inhibitor inside the cell increase the rate of inactivation of choline transport by *N*-ethylmaleimide, they must each induce such a conformational change. Two observations suggest that these two conforma-

tional changes are one and the same: (i) the maximum increases in the rate of *N*-ethylmaleimide attack produced by substrate outside and competitive inhibitor inside are similar; (ii) these increases in the rate of *N*-ethylmaleimide attack are not independent because, when there is both a near-saturating concentration of substrate outside the cell and of competitive inhibitor inside the cell at the same time, the rate of *N*-ethylmaleimide attack is no more than when competitive inhibitor only is present inside at a saturating concentration. Competitive inhibitor outside and substrate inside, respectively, occupy the same binding sites as substrate outside and competitive inhibitor inside, and therefore they would also be expected either to promote or reverse the same conformational change. It is probably such a reversal of the conformational change that causes the reductions in the rate of inactivation by *N*-ethylmaleimide which occur in the presence of substrate inside or competitive inhibitor outside the cell. In support of this deduction, the maximum reduction of the rate of inactivation by *N*-ethylmaleimide produced by competitive inhibitor outside is slightly more than that produced by substrate inside the cell, just as the maximum increase in inactivation rate produced by competitive inhibitor inside is more than that produced by substrate outside.

In summary, the results suggest that the mechanism of choline transport involves a particular conformational change in the transport macromolecule(s): both substrate outside and competitive inhibitor inside induce one conformation, and substrate inside and competitive inhibitor outside induce the other conformation, competitive inhibitor being more effective in both cases. The carrier model of transport is the only model so far proposed that postulates such a conformational change[3,4].

Another aspect of the results, which suggests a close connection between the transport process and this conformational change is the good agreement between particular K_m and K_i values of choline transport kinetics and the apparent dissociation constants of substrate and competitive inhibitor measured by their effects on inhibition by *N*-ethylmaleimide. This is not self-evident: for example, in a transport system that had binding sites of high and low affinity, as postulated by Lieb and Stein[24], the site determining the K_m and K_i values could be different from the site controlling the conformational change.

The ability of specific models of transport to account for the results

Non-carrier models. Non-carrier models of transport have recently been proposed by Naftalin[25], and Lieb and Stein[24]. Neither of these models includes the type of conformational change which, according to the preceding argument, would provide a simple explanation of the experimental results. On the contrary, both mechanisms would be expected to give quite different patterns of reactivity toward reagents such as *N*-ethylmaleimide.

In the Lieb and Stein[24] model the transport step involves a change in the conformation of a tetrameric protein. This change transfers substrate to a pool inside the tetramer, then the conformational change is reversed to release substrate the other side of the membrane. The same pair of conformational changes occurs in influx and efflux, and is promoted by substrate[24] but must be prevented by competitive inhibitor. This conformational change cannot account for the changes in the rate of *N*-ethylmaleimide inactivation of choline transport, since substrate outside (corresponding to influx) increases the rate of *N*-ethylmaleimide attack but substrate

inside (corresponding to efflux) reduces the rate, and both competitive inhibitor and substrate can increase or reduce the rate of inactivation. This model would therefore require some *ad hoc* postulates to account for the properties of *N*-ethylmaleimide reaction.

Naftalin's[25] model postulates a pore through the membrane containing binding sites for substrate and, to account for the phenomenon of accelerative exchange diffusion (acceleration of unidirectional substrate flux by substrate at the *trans* face), postulates unstirred layers at the faces of the membrane. Choline transport shows a strong accelerative exchange effect[13], so that when there is a large concentration of substrate at one face of the membrane the unstirred layer at the *trans* face would have to be the rate-limiting step of transport, and all the binding sites in the pore would be virtually saturated with substrate (illustrated by Fig. 5 of Naftalin's paper[25]). These sites will be saturated whether the substrate is on the inside or outside of the membrane, so it is difficult to see how a saturating concentration of substrate outside could induce a state of maximum reactivity to *N*-ethylmaleimide while a saturating concentration of substrate inside induces a state of minimum reactivity to *N*-ethylmaleimide.

In conclusion, the models of transport proposed by Naftalin[25], and Lieb and Stein[24] cannot account for the properties of the inhibition of choline transport by *N*-ethylmaleimide without the addition of elaborate *ad hoc* hypotheses.

The carrier model. A plausible molecular interpretation of the carrier model is that inward-facing and outward-facing carrier are two conformational states of the transporting macromolecule[2–4]. The transport step is then a conformational change of the type suggested by the results. The original interpretation of the carrier model, that the whole carrier moved between the faces of the membrane[1], is also consistent with the results, since it is not unreasonable that a mobile carrier should show different reactivities to *N*-ethylmaleimide at the two faces of the membrane (*N*-ethylmaleimide penetrates the membrane easily[26]).

All the properties of the inactivation of choline transport by *N*-ethylmaleimide that have been investigated are quantitatively consistent with the hypothesis that choline transport has a carrier mechanism and that *N*-ethylmaleimide reacts much faster with carrier in the inward-facing state than in the outward-facing state. The results presented therefore strongly favour a carrier mechanism of transport.

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