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Effects on Transport of Rapidly Penetrating, Competing Substrates: Activation and Inhibition of the Choline Carrier in Erythrocytes by Imidazole

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Summary. The properties of the choline transport system are fundamentally altered in saline solution containing 5 mM imidazole buffer instead of 5 mM phosphate: (i) The system no longer exhibits accelerated exchange. (ii) Choline in the external compartment fails to increase the rate of inactivation of the carrier by N-ethylmaleimide. (iii) Depending on the relative concentrations of choline and imidazole, transport may be activated or inhibited. The maximum rates are increased more than fivefold by imidazole, but at moderate substrate concentrations activation is observed with low concentrations of imidazole and inhibition with high concentrations. (iv) The imidazole effect is asymmetric, there being a greater tendency to activate exit than entry. All this behavior is predicted by the carrier model if imidazole is a substrate of the choline carrier having a high maximum transport rate but a relatively low affinity, and if imidazole rapidly enters the cell by simple diffusion, so that it can add to carrier sites on both sides of the membrane. Addition at the *cis* side inhibits, and at the *trans* side activates. According to the carrier model, asymmetry is a necessary consequence of the potassium ion gradient in red cells, potassium ion being another substrate of the choline system.

Key Words choline transport · imidazole · carrier model · substrate analogs · transport kinetics

Introduction

In the course of experimental studies of choline transport in human erythrocytes, we have found that the properties of the carrier system are fundamentally altered in imidazole buffer as compared with phosphate buffer. In phosphate (5 mM) the system exhibits accelerated exchange; that is, exchange flux is faster than zero *trans* flux, implying that the carrier-substrate complex is more mobile than the free carrier [3, 10]. In imidazole (5 mM), accelerated exchange was not observed: zero *trans* flux was unaffected by the imidazole buffer, but exchange flux was only as fast as zero *trans* flux. That is, exchange flux, but not zero *trans* flux, was inhibited. In this experiment the rates were determined with a low concentration of labeled choline (well below the half-saturating level). When the zero *trans* rate was measured with a high (saturating) concentration of labeled choline, the imidazole buffer had an entirely different effect, increasing the rate of transport at least fivefold. As we show below, imidazole acted as an inhibitor or an activator, or was without effect, depending on the nature of the experiment and the relative concentrations of choline and imidazole.

It first seemed possible that imidazole might be an allosteric modifier of the choline carrier. If so, two allosteric sites were involved: one, activating and noncompetitive or uncompetitive with respect to the substrate; the other, inhibitory and competitive. But even this rather complicated hypothesis would not explain all the observations without the further postulate of different competitive allosteric sites on both sides of the membrane. The problem is greatly simplified when it is realized that imidazole is a substrate of the choline carrier. It is weakly bound, but, like substrate analogs no larger than choline, is rapidly transported [3]. The effect of imidazole on the system is far more complex than might be expected of a competing substrate, because it rapidly enters the cell by simple diffusion and is present at equilibrium across the cell membrane. It can therefore interact with both the inward-facing and outward-facing carrier sites, and in doing so it produces a variety of effects on transport, both activation and inhibition.

The results have a wider significance in revealing the effects on a carrier system of substances that act as substrates, but which rapidly diffuse across the cell membrane without the help of the carrier. Depending on the relative concentrations of the substrate analog and the true substrate, transport may be strongly activated or strongly inhibited. Even essential properties of the carrier system may

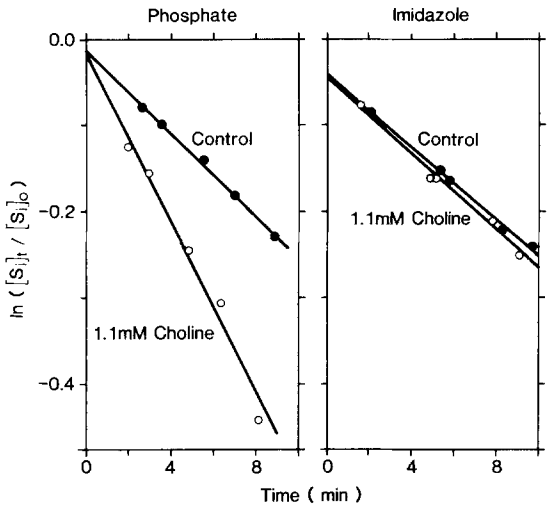

Fig. 1. Effect of imidazole on choline exit into a saline-buffer solution (control, $\bar{v}$) or into a solution of 1.1 mM unlabeled choline ($\bar{v}^T$). The medium was buffered with either 5 mM sodium phosphate, pH 6.8 (left), or 5 mM imidazole/HCl, pH 6.8 (right). Isotonicity was maintained with 150 mM NaCl. The cells initially contained 3.5 μmol ^{14}C-choline per liter of packed cells, $[S_i]_0$; $[S_i]_t$ is the concentration at the time of sampling. The rates (μmol · liter cells^{-1} · min^{-1}) calculated from the slopes, and the flux ratios for exit, are as follows: (a) phosphate: $\bar{v} = 0.024 \pm 0.0009$; $\bar{v}^T = 0.049 \pm 0.0044$; $(\bar{v}^T/\bar{v})_{S_i \to 0} = 2.06 \pm 0.20$; (*b*) imidazole: $\bar{v} = 0.021 \pm 0.0007$; $\bar{v}^T = 0.022 \pm 0.0013$; $(\bar{v}^T/\bar{v})_{S_i \to 0} = 1.06 \pm 0.07$

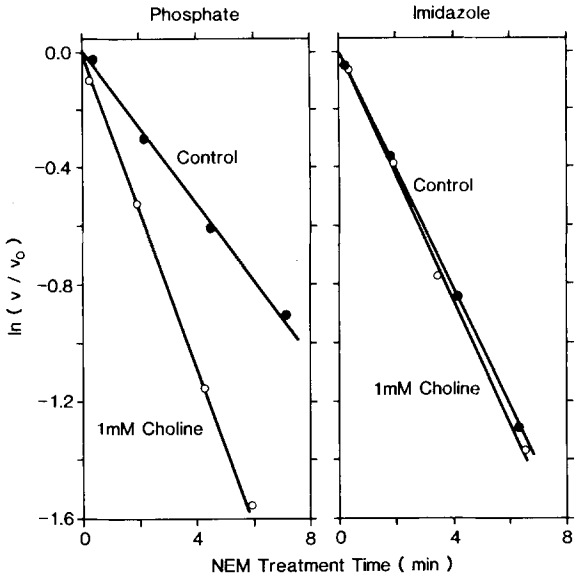

Fig. 2. Effect of imidazole on inactivation of transport by N-ethylmaleimide (pH 6.8, 37°C). The medium was buffered with either 5 mM sodium phosphate (left), or 5 mM imidazole (right). The rate of inactivation was determined in the absence (Q) or in the presence (Q_{To}) of 1 mM choline in the external medium. The natural logarithm of the relative transport activity is plotted against the time of treatment with NEM: v_0 is the initial activity of the system and v_t the activity after treatment for a period of time, t. The pseudo-first order rate constants calculated from the slopes of the lines are: (*a*) Phosphate: $Q = 0.129 \pm 0.0061$; $Q_{To} = 0.281 \pm 0.0107$; ratio $Q_{To}/Q = 2.18 \pm 0.13$; (*b*) Imidazole: $Q = 0.206 \pm 0.0022$; $Q_{To} = 0.212 \pm 0.0083$; ratio $Q_{To}/Q = 1.03 \pm 0.04$

seem to change. In the case of active transport, such substances could act as uncouplers of a novel kind.

Materials and Methods

Preparation of Cells

Human blood was obtained fresh from donors, with heparin as an anticoagulant. Cells (5% suspension) were washed free of endogenous choline by incubation at 37°C for two periods of 3 hr with 5 mM phosphate buffer, pH 6.8, containing 154 mM NaCl. Alternatively, in some experiments the cells were washed by incubation overnight (14 hr) in a 2.5% suspension.

Loading with Radioactive Choline

Choline-free cells (50% hematocrit) were incubated overnight (14 hr) at 37°C in 5 mM sodium phosphate buffer, pH 6.8, containing [^{14}C]-choline chloride, and 0.02% chloramphenicol. The concentration of choline was varied, depending on the type of experiment.

Transport Rate Measurements

The initial rate of exit was measured by adding ^{14}C-choline-loaded cells to saline-buffer (10% hematocrit) and determining the concentration of choline in the supernatant. The initial rate of entry was measured by adding cells (10% hematocrit) to a solution containing radioactive choline and determining the concentration of choline in the cells at intervals. The temperature was 37°C, pH 6.8. Details of the methods were reported earlier [3, 6].

The measured transport rates were found to be unaffected by preincubation of the cells with imidazole. For example, the rates of entry of 1.2 μM choline in 5 mM imidazole were identical whether the cells had been incubated with the imidazole for 25 min or had had no preincubation. As rates were determined from the radioactivity in cell samples taken at various times between 2 and 10 min after addition of ^{14}C-choline, the agreement in the two rate measurements would indicate that imidazole equilibrates across the cell membrane in less than 2 min. The measured rates of exit were also found to be independent of the time of preincubation with imidazole.

The Rate of Inactivation by N-Ethylmaleimide (NEM)

Cells (2.5% suspensions) were treated with 1 mM NEM at 37°C with or without choline in the external medium. Samples were removed at intervals and placed in tubes containing mercaptoethanol (final concentration, 10.6 mM). The treated cells were separated, washed, and assayed for choline uptake. The method was described before [4].

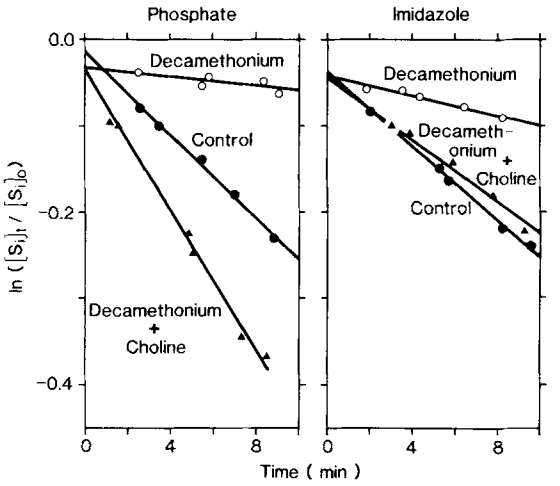

Fig. 3. Competitive inhibition of choline exit by decamethonium. Exit rates were determined in 150 mM NaCl buffered at pH 6.8 with either 5 mM phosphate (left), or 5 mM imidazole (right). In each case rates were measured in the saline-buffer solution alone (control), or with the addition of 0.33 mM decamethonium, or 0.33 mM decamethonium plus 1.1 mM unlabeled choline. The initial concentration of ^{14}C-choline in the cells, $[S_i]_0$, was 3.5 μmol per liter of packed cells; $[S_i]_t$ is the concentration at the time of sampling. The rates (μmol · liter cells^{-1} · min^{-1} are as follows: (*a*) phosphate : control, 0.024 ± 0.0009; decamethonium, 0.0033 ± 0.0013; choline, 0.049 ± 0.0044; decamethonium plus choline, 0.039 ± 0.0016; (*b*) imidazole : control, 0.021 ± 0.0007; decamethonium, 0.005 ± 0.0009; choline, 0.022 ± 0.0013; decamethonium plus choline, 0.018 ± 0.0018. (The data for the control and for exit in the presence of choline alone are the same as in Fig. 1)

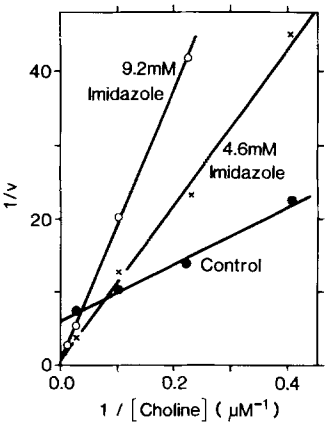

Fig. 4. Choline entry in the presence and absence of imidazole. Lineweaver-Burk plots are shown ($1/v$ against $1/[S]$), where the maximum rate is given by the reciprocal of the intercept on the vertical axis. Statistical calculations were based on the more reliable $[S]/v$ against $[S]$ plot: the maximum rates (μmol · liter cells^{-1} · min^{-1}) were found to be: control, 0.156 ± 0.003; 4.6 mM imidazole, 0.788 ± 0.135; 9.2 mM imidazole, 1.44 ± 0.20. The cells were incubated with imidazole for 1 hr before rates were determined

Results

Flux Ratio for Exit

The ratio of the unidirectional flux of a low-concentration of labeled substrate either into a solution containing a high concentration of unlabeled substrate ($\vec{v}^T$) or into pure buffer ($\vec{v}$) is referred to as flux ratio, $\vec{v}^T/\vec{v}$. Under standard assay conditions, in phosphate buffer, the ratio is about 2; i.e., there is accelerated exchange. In the presence of imidazole buffer, the ratio is equal to unity; i.e., accelerated exchange is no longer observed (Fig. 1). It was also found that imidazole abolishes accelerated exchange whether phosphate is present or not.

Inactivation by NEM

NEM has been shown to inactivate the carrier by reacting with a thiol group [13] exposed in the inward-facing carrier conformation [4, 5, 7, 14]. In phosphate buffer, choline in the external medium increases the rate of inactivation, but in the presence of imidazole it fails to do so (Fig. 2).

Interaction of Choline and Decamethonium at the External Site

Decamethonium, a nontransported choline analog [11], inhibits choline transport when present in the external medium, and choline added to the external medium reverses the inhibition; this is true both in phosphate buffer and imidazole buffer (Fig. 3).

Rates of Choline Entry and Exit at Varying Choline Concentrations

The effect of imidazole on the rates of zero *trans* entry and exit as a function of choline concentration is shown in Figs. 4 and 5, where the results are presented as Lineweaver-Burk plots. Imidazole inhibits, activates, or is without effect, depending on the choline concentration. The straight lines in the plots (plus and minus imidazole) intersect; at substrate concentrations higher than that at which the lines intersect, transport is accelerated by imidazole, and at lower concentrations, inhibited. The maximum rate of entry is increased sixfold. The maximum rate of exit is also greatly increased, but by a factor which is hard to estimate, owing to reduced substrate affinity in the presence of imidazole, combined with difficulty in loading cells with a high choline concentration.

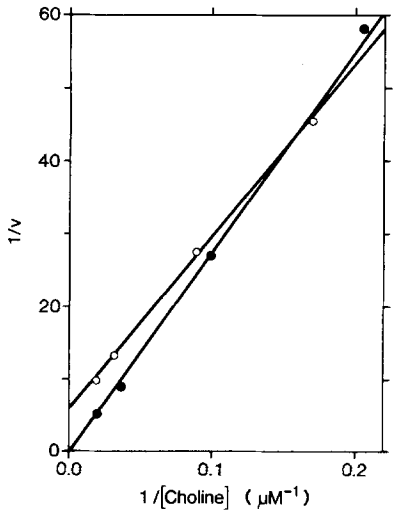

Fig. 5. Choline exit in the presence or absence of 4.6 mM imidazole. Plots and analysis are as in Fig. 4. The maximum rate (μmol · liter cells^{-1} · min^{-1}) of the control, 0.178 ± 0.004; the maximum rate in the presence of imidazole is higher, but the exact value cannot be determined from these data (*see* Discussion). The cells were incubated with imidazole for 1 hr before rates were determined. Open circles, control; filled circles, imidazole

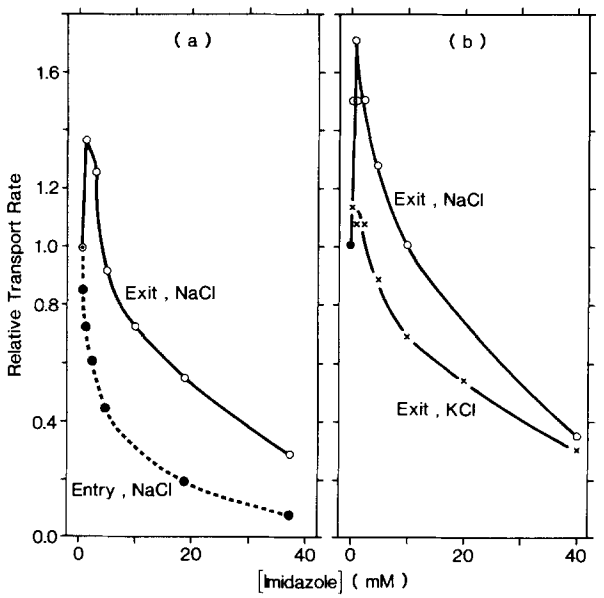

Fig. 6. Effects of varying concentrations of imidazole on exit and entry at a low substrate concentration. The initial ^{14}C-choline concentrations were 3.7 μM in exit and 2.5 μM in entry. Exit rates were determined in either 150 mM NaCl or 150 mM KCl, both buffered at pH 6.8 with 5 mM phosphate; entry rates were determined in 150 mM NaCl with 5 mM phosphate, pH 6.8. (*a*) Exit and entry rates, with cells suspended in a NaCl solution. (*b*) Exit into solutions of NaCl or KCl. The exit rate in the absence of imidazole is actually faster in KCl solution than in NaCl by a factor of 1.4

Dependence of the Transport Rate on the Concentration of Imidazole

Figure 6 shows the effect of increasing imidazole concentrations on transport at a choline concentration which is low in relation to the half-saturation constant. In entry, imidazole progressively inhibits. In exit, it first activates, increasing the rate up to a maximum, beyond which the rate falls. For reasons explained below, an exit experiment was also carried out in KCl (instead of NaCl); in this solution there is almost no activation, but the inhibition persists (Fig. 6).

At a higher choline concentration (29 μM, about 4 times K_m), entry is first accelerated as the imidazole concentration is raised (Fig. 7).

The Effect of Dimethylaminoethanol on the Flux Ratio

Dimethylaminoethanol is a close analog of choline, which, being a tertiary amine, rapidly penetrates the membrane by simple diffusion [8]. This analog was found to have much the same effect on the flux ratio as imidazole. The flux ratio for exit, $(\tilde{v}^T/\bar{v})_{S_i \to 0}$, was 2.05 in the control; in the presence of 5 μM dimethylaminoethanol the ratio was 1.47, and in the presence of 10 μM, 1.20.

Discussion

The flux ratio, a measure of the effect of a high concentration of unlabeled substrate in the *trans* compartment on the rate of transport of a low concentration of labeled substrate, is an informative characteristic of a transport system. The ratio depends on the relative rates of translocation of the carrier-substrate complex and the free carrier, governed by f_2, f_{-2}, and f_1, f_{-1}, respectively, in the kinetic scheme in Fig. 8*a*. The flux ratio for exit[1] is found to be [2]

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_i \to 0} = \frac{1 + f_{-1}/f_1}{1 + f_{-1}/f_2}. \quad (1)$$

When the complex is the more rapidly transported carrier form ($f_2 > f_1$), the ratio is greater than unity, and the system is said to exhibit accelerated exchange; if $f_2 \gg f_{-1}$, while $f_1 \simeq f_{-1}$, the ratio is about

[1] $\tilde{v}^T$ is the rate of exit of labeled choline (S) into a medium containing a saturating concentration of unlabeled choline (T), and $\bar{v}$ is the rate of exit of labeled choline into a medium of pure saline buffer.

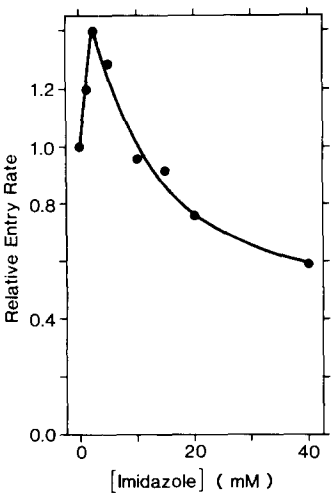

Fig. 7. Effects of varying concentrations of imidazole on entry at a high choline concentration (29 μM). Rates were determined in 150 mM NaCl buffered with 5 mM phosphate, pH 6.8. The cells were preincubated for 30 min with imidazole

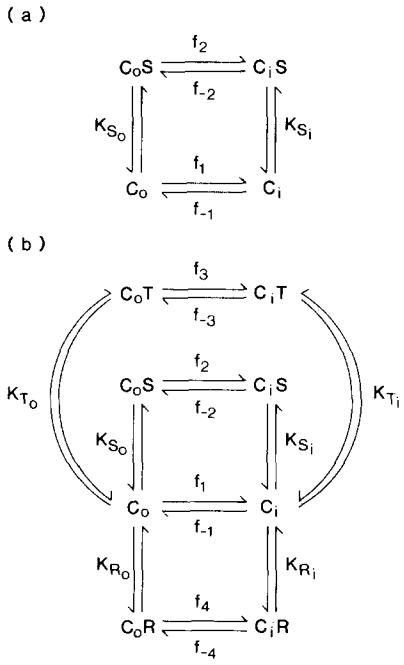

Fig. 8. Kinetic scheme for the carrier model. The upper diagram (*a*) shows the basic features of the model, involving a single substrate, *S*. The conformation of the carrier alternates between inward-facing and outward-facing forms, C_i and C_o respectively, and substrate molecules in the internal and external solutions, S_i and S_o, add to these two forms of the carrier. The lower diagram (*b*) shows the extended scheme for three substrates, *S*, *R*, and *T*

2, as in the choline system (Fig. 1). This property is lost in imidazole buffer. Our problem is to understand why.

Conceivably, imidazole could abolish accelerated exchange by preventing unlabeled choline in the trans (external) compartment from binding to the carrier, but the following observation rules out this mechanism. Decamethonium, a competitive inhibitor restricted to the external solution, inhibits choline exit, and choline in the external solution reverses the inhibition, both in the presence and absence of imidazole. It follows that the inhibitor and the substrate bind at the outer carrier site under either condition (*see* Fig. 3).

The strong activation of transport under some conditions might suggest another possibility, that imidazole is an allosteric modifier of the choline transport system. An allosteric modifier could eliminate accelerated exchange by causing the rates of movement of the free carrier (f_1 and f_{-1}) and the complex (f_2 and f_{-2}) to be equal, thus bringing the flux ratio down to unity [Eq. (1)]. As well, the postulated increase in f_1 and f_{-1} up to the level of f_2 and f_{-2} would explain the increased maximum rates; for in choline transport [3], movement of the free carrier is a rate-limiting step ($f_1, f_{-1}, \ll f_2, f_{-2}$) determining the maximum rates of entry ($\bar{V}_{So}$) and exit ($\bar{V}_{Si}$) [2]:

$$\bar{V}_{So} = f_{-1}f_2C_t/(f_{-1} + f_2) \simeq f_{-1}C_t \quad (2)$$

$$\bar{V}_{Si} = f_1f_{-2}C_t/(f_1 + f_{-2}) \simeq f_1C_t \quad (3)$$

where C_t is the total carrier concentration.

The hypothesis of an activating allosteric site does not explain all the observations, however. Imidazole is an activator under some conditions, but an inhibitor under others, and sometimes has no effect at all. Accordingly, both activating and inhibitory allosteric sites would have to be postulated, whose effects can balance. The activating site has the higher affinity, judging by activation seen at low and inhibition at high imidazole concentrations (Figs. 6 and 7).

A simpler interpretation comes into view when we consider that there is increasing inhibition as the substrate concentration falls (Fig. 4). In this sense there is a competitive interaction between imidazole and the substrate, which could imply that imidazole actually binds at the substrate site. The hypothesis is not unreasonable, since imidazole in the protonated form (pK_a = 7.0) is a cation, like choline, and is about the same size as choline.

The whole problem would be simplified if the binding of imidazole at the substrate site could also account for activation, eliminating the need to postulate allosteric sites. Indeed, a competing substrate could produce the observed behavior if it were present in both compartments, and this is expected of imidazole, which in its neutral form would pene-

trate the cell membrane by simple diffusion. It should then be able to add to both the inward-facing and outward-facing carrier forms.

Briefly stated, the consequence of having a competing substrate—imidazole—in both compartments is as follows. When bound at the *cis* site, in the same compartment as labeled choline, imidazole would compete with the substrate and inhibit. But when bound at the *trans* site, in the opposite compartment, it would accelerate, for the same reason that unlabeled choline in the *trans* compartment accelerates, by facilitating the return of the carrier site to the *cis* compartment. Thus the contrary effects of imidazole would result from its binding to two different forms of the carrier, rather than to two different sites.

Can this simple hypothesis account for all the observations? We now turn to a more detailed examination of the problem.

Flux Ratios

As Eq. (1) shows, the flux ratio for exit is dependent on the relative rates of inward translocation of the free carrier (f_1) and the carrier-substrate complex (f_2). In the presence of an equilibrated competing substrate, the ratio depends on the relative rates of translocation of the complex with the competing substrate (the rate constant for which now replaces f_1) and with the substrate itself (f_2 as before). Hence the flux ratio should equal unity if the two substrates are translocated at the same rate. The proof of this follows.

From an analysis of the carrier model [Eq. (A12), Appendix], the effect of a competing substrate R (imidazole) on the flux ratio for another substrate T (choline) is given by Eq. (4)

$$\left(\frac{\vec{v}^T}{\vec{v}}\right)^R_{S_i\to 0} = \frac{\left(\frac{\vec{v}^T}{\vec{v}}\right)_{S_i\to 0}\left\{1 + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}} + \frac{[R_i][R_o]}{\bar{K}_{R_i}\tilde{K}^R_{R_o}}\right\}}{\left(1 + \frac{[R_i]}{\tilde{K}^T_{R_i}}\right)\left\{1 + \frac{[R_o]}{\bar{K}_{R_o}}\left(\frac{\vec{v}^R}{\vec{v}}\right)_{S_i\to 0}\right\}}. \tag{4}$$

R_o and R_i stand for imidazole outside and inside the cell, respectively; T_o is unlabeled choline outside, and S_i is labeled choline inside; $(\vec{v}^T/\vec{v})^R_{S_i\to 0}$ is the flux ratio for choline in the presence of imidazole, and $(\vec{v}^R/\vec{v})_{S_i\to 0}$ is the flux ratio which would be found for imidazole (R) if it could be restricted to the external solution.

The limiting value of the flux ratio, Eq. (4), with saturating $R([R_i] = [R_o] \to \infty)$ is

$$\left(\frac{\vec{v}^T}{\vec{v}}\right)^{R\to\infty}_{S_i\to 0} = \frac{\tilde{K}^T_{R_i}}{\tilde{K}^R_{R_i}}\frac{(\vec{v}^T/\vec{v})_{S_i\to 0}}{(\vec{v}^R/\vec{v})_{S_i\to 0}}. \tag{5}$$

From expressions for the two flux ratios and the two half-saturation constants[2] on the right-hand side of Eq. (5), the ratio is found to be unity, or very close to unity, in two cases: (i) the carrier complexes with choline and imidazole move at the same rate (i.e., $f_2 = f_4$ in Fig. 8*b*); or (ii) both these complexes move much faster than the free carrier. Since the complex with choline was shown previously [3] to move at least 10 times faster than the free carrier, we conclude that the complex with imidazole is also rapidly translocated.

That this analysis is essentially correct is supported by the results with dimethylaminoethanol. This tertiary analog of choline equilibrates across the cell membrane [8], and it is almost certainly a transported substrate, based on the following evidence: (i) its affinity for the carrier is close to choline's [8]; (ii) it has the same effect on NEM inactivation as equilibrated choline (*5*); (iii) substrate analogs no larger than choline are transported as fast as choline [3]. Dimethylaminoethanol, therefore, should have the same effect on the flux ratio as imidazole, as observed.

Inhibition by NEM

NEM has been shown to react with a thiol group which is exposed in the inward-facing, and masked in the outward-facing, carrier [4, 5, 7, 12, 14]. The rate at which NEM inactivates the transport system therefore depends on the proportion of the carrier in the inward-facing form: there is little or no inactivation when all the carrier faces out, and the inactivation rate is maximal when all the carrier faces in. Choline, when present on only one side of the membrane, alters the steady-state distribution between these two forms and therefore affects the inactivation rate: substrate outside shifts the carrier inward (because the complex moves in faster than the free carrier moves out) and therefore accelerates inactivation; substrate inside shifts the carrier outward and protects against inactivation. What are the implications for imidazole?

By hypothesis, imidazole reduces the flux ratio to unity because in its presence external choline does not alter the carrier partition, as seen above. Hence external choline should fail to alter the NEM

[2] Analysis of the carrier model (2) shows that $\tilde{K}^T_{R_i} = K_{R_i}(f_{-1} + f_3)/(f_{-4} + f_3)$; $\tilde{K}^R_{R_i} = K_{R_i}(f_{-1} + f_4)/(f_{-4} + f_4)$; $(\vec{v}^T/\vec{v})_{S_i\to 0} = (1 + f_{-1}/f_1)/(1 + f_{-1}/f_3)$; $(\vec{v}^R/\vec{v})_{S_i\to 0} = (1 + f_{-1}/f_1)/(1 + f_{-1}/f_4)$.

inactivation rate in the presence of imidazole, which is the observed result (Fig. 2).

Zero Trans Flux

Imidazole increases the maximum rates of both entry and exit (Figs. 4 and 5). According to the carrier model [*see* Eq. (A3) and (A4), Appendix] the maximum rate of entry of substrate S, in the presence of an equilibrated substrate R, is

$$(\bar{V}_{So})_R = \frac{\left\{1 + \frac{\tilde{V}^R_{So}[R_i]}{\bar{V}_{So}\tilde{K}^S_{R_i}}\right\}\bar{V}_{So}}{1 + \frac{[R_i]}{\tilde{K}^S_{R_i}}} \tag{6}$$

where $\tilde{V}^R_{So} = f_2 f_{-4}/(f_2 + f_{-4})$ and $\bar{V}_{So} = f_2 f_{-1}/(f_2 + f_{-1})$. $\tilde{V}^R_{So}$ and $\bar{V}_{So}$ are the maximum rates of entry in the presence of saturating R or in the absence of R, respectively, and the increase in the maximum rate produced by R is given by their ratio:

$$\frac{\tilde{V}^R_{So}}{\bar{V}_{So}} = \frac{f_{-4}}{f_{-1}(1 + f_{-4}/f_2)}. \tag{7}$$

As seen in Fig. 8*b*, f_{-4} is the rate constant for outward movement of the complex with R (imidazole), f_2 the constant for inward movement of the complex with S (choline), and f_{-1} the constant for outward movement of the free carrier.

The expected value of this ratio may be found from our previous estimate of f_2/f_{-1}, which was 10 or more [3]. As seen above, the observed flux ratio of unity in the presence of imidazole requires that the complex with imidazole should move at least as fast as that with choline ($f_{-4} \geq f_2$). If $f_{-4} = f_2$, the ratio of maximum rates [Eq. (7)] equals $(f_2/f_{-1})/2$, and if $f_{-4} \gg f_2$ it equals (f_2/f_{-1}). Hence the maximum rate could be increased by a factor of between 5 and 10, as found. The analysis applies specifically to entry rates, but the equations for exit have the same form and a similar conclusion would be reached.

The increase in the maximum rate is easily understood, since choline at a saturating concentration competes with imidazole in the same compartment, completely displacing it from the carrier site and overcoming the competitive inhibition. In the *trans* compartment, where there is no choline, imidazole freely adds to the carrier and should produce a *trans* acceleration if its complex is more mobile than the free carrier. (Movement of the free carrier is ordinarily rate limiting, as the observation of accelerated exchange shows.)

Following this argument, it may be expected that, at declining concentrations, choline should compete less and less effectively with imidazole in the same (*cis*) compartment, until a point is reached where the growing inhibition balances the acceleration by imidazole in the *trans* compartment. Here imidazole has no effect. At still lower choline concentrations, inhibition should predominate [*see* Eq. (A4), Appendix].

We can now explain the effect of imidazole on double-reciprocal plots for choline entry, where intersecting straight lines are found (Fig. 4); to the left of the intersection point, i.e., at higher substrate concentrations, there is activation, and to the right, at lower concentrations, inhibition. The intersection point (where imidazole has no effect) should depend on the imidazole concentration, as confirmed by the different effects of 5 and 9 mM imidazole on entry [Fig. 4; *see* Eq. (A5), Appendix].

Surprisingly, 5 mM imidazole has somewhat different effects on entry and exit, in that in exit the intersection point is shifted to a lower substrate concentration (relative to the half-saturating level). The cause of asymmetry in the imidazole effect is considered below.

The effect of Increasing Imidazole Concentrations

Assuming that imidazole competes with choline for the carrier, it should activate zero *trans* flux at low concentrations, but should inhibit at high concentrations. At a low concentration it activates for the same reason that it increases the maximum transport rate—activation at the *trans* site outweighs inhibition at the *cis* site. At sufficiently high imidazole concentrations, inhibition predominates because the inhibitory effect of competing imidazole steadily increases with rising concentration, whereas the activation reaches a limit when the *trans* site is saturated. The transport rate should therefore pass through a maximum as the imidazole concentration is raised and then fall steadily. Such behavior is observed (Figs. 6 and 7).

The Asymmetry of the Imidazole Effect

A difference was noted in the double-reciprocal plots for exit and entry (Figs. 4 and 5). In exit the lines with and without imidazole are nearly parallel, but in entry they differ markedly in slope; the plots show an activation of exit at substrate concentrations as much as six times lower than the substrate half-saturation constant, but an inhibition of entry at all concentrations below the half-saturation constant.

This asymmetry depends on a property of the choline system not yet discussed, which is due to the potassium ion gradient in red cells. K^+ is present at a high concentration (~130 mM) inside the cells, and it has been shown to be a substrate of the choline carrier; the gradient in K^+ therefore produces, by countertransport, a gradient in choline, whose internal concentration rises to a higher level than the external concentration: $([S_i]/[S_o])_{final} > 2$ [10, 13]. We show in the Appendix that the observed asymmetry in the imidazole effect is a necessary consequence of this induced asymmetry in the transport system and is unrelated to any intrinsic asymmetry that may exist in the binding constants for either choline or imidazole. In effect, internal K^+ decreases the apparent affinity of another substrate, internal imidazole, by competing with it for the inner carrier site. In the same way, internal K^+ gives rise to asymmetry in the measured affinity constants for choline in entry and exit, as well as accumulation of choline in the final steady state.

Another aspect of the asymmetry is seen in the effects of increasing imidazole concentrations at a low, fixed, substrate concentration (Fig. 6). Here imidazole progressively inhibits entry, with no sign of activation, but it first accelerates exit and then inhibits. If the effect were symmetrical, only inhibition would be expected in both entry and exit at a disappearingly low substrate concentration [as may be deduced from Eq. (A8), Appendix]. It follows that elimination of the K^+ gradient should abolish the activation of choline exit by imidazole at a sufficiently low substrate concentration. In an experiment run in KCl instead of NaCl, where K^+ is present on both sides of the membrane, the expected effect was found (Fig. 6). (In the experiment, a small activation is still seen, because in order to measure rates a higher substrate concentration was employed than the theoretical, $[S_i] \to 0$.)

The Carrier Model

Our analysis has been based on the carrier model in Fig. 8*a*, the evidence for which should now be summarized. (i) Accelerated exchange [10] rules out simple channel mechanisms in which the transport protein is an open canal [1]. Accelerated exchange also rules out carrier mechanisms in which substrate sites are simultaneously exposed on both sides of the membrane, where binding at one site induces a conformational change in the carrier that blocks binding at the opposite site. All such models *require* that exchange flux be slower than zero *trans* flux [1]. (ii) Two different conformational states of the carrier are involved in transport, one inward-facing (i.e., having a substrate site exposed on the inner surface of the membrane) and one outward-facing [4, 5, 7, 12, 14]. These two carrier forms are stabilized by nontransported substrate analogs bound in competition with choline on the inner and outer surfaces of the cell membrane, respectively. NEM reacts with a thiol group exposed in the inward-facing form. (iii) Substrate analogs which are bound to the inner and outer sites cannot add to the carrier simultaneously, showing that a site is only exposed on one surface of the membrane at a time [9]. (iv) The steady-state partition of the carrier between these forms (as monitored by the rate of reaction of the system with NEM) is related in an exactly predictable way to the substrate distribution across the membrane and to the maximum rate of translocation of the substrate (where a series of substrates is tested). Models with substrate sites exposed on both sides of the membrane simultaneously would not predict these relationships [4]. (v) The linearity of Dixon plots for competitive inhibitors [8] and of double reciprocal plots for substrates (Figs. 4 and 5) rules out models involving interacting substrate or inhibitor sites on the same side of the membrane. (vi) The carrier model provides a simple explanation for the effect of pH on transport, where increasing hydrogen ion concentrations increase the maximum rate of exit but decrease the maximum rate of entry, behavior not accounted for by various other models [6].

To this list we can now add the ability of the model to explain the complicated effects of imidazole.

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Appendix

Using methods outlined before [2], we can derive general kinetic equations for the transport of one substrate in the presence of others. An appropriate kinetic scheme for this case, based on the carrier model, is shown in Fig. 8b, where the movement of substrate S is followed in the presence of two competing substrates, T and R. For present purposes S is radioactive choline, R is imidazole, and T may be either unlabeled choline or another substrate, potassium ion. The rate equation takes the following form

$$v = \frac{\dfrac{\bar{V}_{S_i}}{\bar{K}_{S_i}}(\alpha[S_o] - [S_i]) + \dfrac{\tilde{V}^T_{S_i}}{\bar{K}_{S_i}\tilde{K}^S_{T_o}}([S_o][T_i] - [S_i][T_o]) + \dfrac{\tilde{V}^R_{S_i}}{\bar{K}_{S_i}\tilde{K}^S_{R_o}}([S_o][R_i] - [S_i][R_o])}{1 + \dfrac{[S_i]}{\bar{K}_{S_i}} + \dfrac{[S_o]}{\bar{K}_{S_o}} + \dfrac{[T_i]}{\bar{K}_{T_i}} + \dfrac{[T_o]}{\bar{K}_{T_o}} + \dfrac{[R_i]}{\bar{K}_{R_i}} + \dfrac{[R_o]}{\bar{K}_{R_o}} + \dfrac{[S_i][S_o]}{\bar{K}_{S_i}\tilde{K}^S_{S_o}} + \dfrac{[T_i][T_o]}{\bar{K}_{T_i}\tilde{K}^T_{T_o}} + \dfrac{[R_i][R_o]}{\bar{K}_{R_i}\tilde{K}^R_{R_o}} + \dfrac{[S_i][T_o]}{\bar{K}_{S_i}\tilde{K}^S_{T_o}} + \dfrac{[S_i][R_o]}{\bar{K}_{S_i}\tilde{K}^S_{R_o}} + \dfrac{[S_o][T_i]}{\bar{K}_{S_o}\tilde{K}^S_{T_i}} + \dfrac{[S_o][R_i]}{\bar{K}_{S_o}\tilde{K}^S_{R_i}} + \dfrac{[R_i][T_o]}{\bar{K}_{R_i}\tilde{K}^R_{T_o}} + \dfrac{[R_o][T_i]}{\bar{K}_{R_o}\tilde{K}^R_{T_i}}}. \quad \text{(A1)}$$

All the constants in the equation are experimental parameters. V and K, with subscripts and superscripts denoting the experimental arrangement, represent a maximum transport rate and a half-saturation constant, respectively. For example $\bar{V}_{S_i}$ is the maximum zero *trans* rate of exit for S, and $\tilde{V}^T_{S_i}$ and $\tilde{V}^R_{S_i}$ are the maximum rates of exit for S in infinite *trans* experiments, where substrate T or R, respectively, is present at as saturating concentration in the *trans* compartment. The experimental parameters can be written in terms of individual rate constants in Fig. 8*b* (*see* ref. 2).

The constant α is the ratio of substrate concentrations inside and outside the cell in the final steady state:

$$\alpha = \left(\frac{[S_i]}{[S_o]}\right)_{\text{final}} = \left(\frac{[T_i]}{[T_o]}\right)_{\text{final}} = \left(\frac{[R_i]}{[R_o]}\right)_{\text{final}}. \quad \text{(A2)}$$

Equation (A2) assumes that transport is entirely carrier mediated, with no leak due to simple diffusion.

We now deal with experimental arrangements relevant to the imidazole effect.

Zero Trans Entry

Here the labeled substrate S is initially present in only the external medium ($[S_i] = 0$), and entry is followed in the presence of a competing substrate, R (imidazole). Equation (A1) reduces to

$$v = \frac{\dfrac{\bar{V}_{S_o}}{\bar{K}_{S_o}}\left(1 + \dfrac{\tilde{V}^R_{S_o}[R_i]}{\bar{V}_{S_o}\tilde{K}^S_{R_i}}\right)[S_o]}{1 + \dfrac{[R_i]}{\bar{K}_{R_i}} + \dfrac{[R_o]}{\bar{K}_{R_o}}\left(1 + \dfrac{[R_i]}{\tilde{K}^R_{R_i}}\right) + \dfrac{[S_o]}{\bar{K}_{S_o}}\left(1 + \dfrac{[R_i]}{\tilde{K}^S_{R_i}}\right)}. \quad \text{(A3)}$$

$\bar{V}_{S_o}$ is the maximum rate of zero *trans* entry in the absence of R, and $\tilde{V}^R_{S_o}$ the corresponding maximum rate in the presence of a saturating concentration of R. In reciprocal form, Eq. (A3) becomes

$$\frac{1}{v} = \frac{1 + \dfrac{[R_i]}{\tilde{K}^S_{R_i}} + \dfrac{\bar{K}_{S_o}}{[S_o]}\left\{1 + \dfrac{[R_i]}{\bar{K}_{R_0}} + \dfrac{[R_o]}{\bar{K}_{R_o}}\left(1 + \dfrac{[R_i]}{\tilde{K}^R_{R_i}}\right)\right\}}{\bar{V}_{S_o}\left(1 + \dfrac{\tilde{V}^R_{S_o}[R_i]}{\bar{V}_{S_o}\tilde{K}^S_{R_i}}\right)}. \quad \text{(A4)}$$

The substrate concentration at the point where the lines in double reciprocal plots intersect is found from Eq. (A4); here v is the same in the presence and absence of R, and therefore

$$[S_0] = \bar{K}_{S_0}\left\{\frac{\dfrac{\tilde{K}^S_{R_i}}{\bar{K}_{R_i}} + \dfrac{\tilde{K}^S_{R_i}}{\bar{K}_{R_0}} - \dfrac{\tilde{V}^R_{S_0}}{\bar{V}_{S_0}} + \dfrac{\tilde{K}^S_{R_i}[R_o]}{\tilde{K}^R_{R_i}\bar{K}_{R_o}}}{\dfrac{\tilde{V}^R_{S_o}}{\bar{V}_{S_o}} - 1}\right\}. \quad \text{(A5)}$$

Zero *Trans* Exit

The equations correspond to those for zero *trans* entry, with the parameters for exit substituted for those for entry. In reciprocal form the equation is [*cf.* Eq. (A4)]

$$\frac{1}{v} = \frac{1 + \frac{[R_o]}{\bar{K}^S_{R_o}} + \frac{\bar{K}_{S_i}}{[S_i]}\left\{1 + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}}\left(1 + \frac{[R_i]}{\bar{K}^R_{R_i}}\right)\right\}}{\bar{V}_{S_i}\left(1 + \frac{\bar{V}^R_{S_i}}{\bar{V}_{S_i}}\frac{[R_o]}{\bar{K}^S_{R_o}}\right)}. \tag{A6}$$

Flux Ratios

The flux ratio for exit, $(\bar{v}^T/\bar{v})S_i \to 0$, is the ratio of the rates of exit of a low concentration of labeled substrate S into a suspending medium containing either (a) a saturating concentration of unlabeled substrate T ($\bar{v}^T$), or (b) pure buffer ($\bar{v}$). These rates, in the presence of imidazole, R, are found from Eq. (A1).

(a) $[S_i] \ll \bar{K}_{S_i}$; $[T_o] \to \infty$; $[S_o] = [T_i] = 0$

$$\bar{v}^T = \frac{\bar{V}^T_{S_i}[S_i]/\bar{K}^T_{S_i}}{1 + [R_i]/\bar{K}^T_{R_i}} \tag{A7}$$

(b) $[S_i] \ll \bar{K}_{S_i}$; $[T_o] = [S_o] = [T_i] = 0$

$$v = \frac{\frac{\bar{V}_{S_i}}{\bar{K}_{S_i}}[S_i] + \frac{\bar{V}^R_{S_i}[S_i][R_o]}{\bar{K}_{S_i}\bar{K}^S_{R_o}}}{1 + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}} + \frac{[R_i][R_o]}{\bar{K}_{R_i}\bar{K}^R_{R_o}}}. \tag{A8}$$

The ratio of these two rates is

$$\left(\frac{\bar{v}^T}{\bar{v}}\right)^R_{S_i \to 0} = \frac{\frac{\bar{V}^T_{S_i}\bar{K}_{S_i}}{\bar{V}_{S_i}\bar{K}^T_{S_i}}\left\{1 + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}} + \frac{[R_i][R_o]}{\bar{K}_{R_i}\bar{K}^R_{R_o}}\right\}}{\left(1 + \frac{[R_i]}{\bar{K}^T_{R_i}}\right)\left(1 + \frac{\bar{V}^R_{S_i}[R_o]}{\bar{V}_{S_i}\bar{K}^S_{R_o}}\right)}. \tag{A9}$$

In the absence of the competing substrate ($[R] = 0$) the flux ratio is

$$(\bar{v}^T/\bar{v})_{S_i \to 0} = \frac{\bar{V}^T_{S_i}\bar{K}_{S_i}}{\bar{V}_{S_i}\bar{K}^T_{S_i}} \tag{A10}$$

and by analogy:

$$(\bar{v}^R/\bar{v})_{S_i \to 0} = \frac{\bar{V}^R_{S_i}\bar{K}_{S_i}}{\bar{V}_{S_i}\bar{K}^R_{S_i}}. \tag{A11}$$

After substitution of Eqs. (A10) and (A11), Eq. (A9) can be rewritten as

$$\left(\frac{\bar{v}^T}{\bar{v}}\right)^R_{S_i \to 0} = \frac{\left(\frac{\bar{v}^T}{\bar{v}}\right)_{S_i \to 0}\left\{1 + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}} + \frac{[R_i][R_o]}{\bar{K}_{R_i}\bar{K}^R_{R_o}}\right\}}{\left(1 + \frac{[R_i]}{\bar{K}^T_{R_i}}\right)\left\{1 + \frac{[R_o]}{\bar{K}_{R_o}}\left(\frac{\bar{v}^R}{\bar{v}}\right)_{S_i \to 0}\right\}}. \tag{A12}$$

The Problem of Asymmetry: Transport in the Presence of a Potassium Ion Gradient

Rate equations for this case may be written by substitution into Eq. (A1): T_i now represents internal K^+; $[T_o] = 0$; R, as before, stands for imidazole. The equations for entry and exit, in reciprocal form, are found to be (respectively)

$$\frac{1}{v} = \frac{1 + \frac{[R_o]}{\bar{K}^S_{R_o}} + \frac{\bar{K}_{S_i}}{[S_i]}\left\{1 + \frac{[T_i]}{\bar{K}_{T_i}} + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}}\left(1 + \frac{[T_i]}{\bar{K}^R_{T_i}} + \frac{[R_i]}{\bar{K}^R_{R_i}}\right)\right\}}{\bar{V}_{S_i}\left(1 + \frac{\bar{V}^R_{S_i}[R_o]}{\bar{V}_{S_i}\bar{K}^S_{R_o}}\right)} \tag{A13}$$

$$\frac{1}{v} = \frac{1 + \frac{[T_i]}{\bar{K}^S_{T_i}} + \frac{[R_i]}{\bar{K}^S_{R_i}} + \frac{\bar{K}_{S_o}}{[S_o]}\left\{1 + \frac{[T_i]}{\bar{K}_{T_i}} + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}}\left(1 + \frac{[T_i]}{\bar{K}^R_{T_i}} + \frac{[R_i]}{\bar{K}^R_{R_i}}\right)\right\}}{\bar{V}_{S_o}\left(1 + \frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}} + \frac{\bar{V}^R_{S_o}[R_i]}{\bar{V}_{S_o}\bar{K}^S_{R_i}}\right)}. \tag{A14}$$

From these equations, the ratio of slopes of double-reciprocal plots with and without equilibrated imidazole (R), and with K^+ (T) inside the cell but not outside, is, for exit

$$\frac{1 + \frac{[T_i]}{\bar{K}_{T_i}} + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}}\left\{1 + \frac{[T_i]}{\bar{K}^R_{T_i}} + \frac{[R_i]}{\bar{K}^R_{R_i}}\right\}}{\left(1 + \frac{\bar{V}^R_{S_i}[R_o]}{\bar{V}_{S_i}\bar{K}^S_{R_o}}\right)\left(1 + \frac{[T_i]}{\bar{K}_{T_i}}\right)} \tag{A15}$$

and for entry

$$\frac{\left\{1 + \frac{[T_i]}{\bar{K}_{T_i}} + \frac{[R_i]}{\bar{K}_{R_i}} + \frac{[R_o]}{\bar{K}_{R_o}}\left(1 + \frac{[T_i]}{\bar{K}^R_{T_i}} + \frac{[R_i]}{\bar{K}^R_{R_i}}\right)\right\}\left(1 + \frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}}\right)}{\left(1 + \frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}} + \frac{\bar{V}^R_{S_o}[R_i]}{\bar{V}_{S_o}\bar{K}^S_{R_i}}\right)\left(1 + \frac{[T_i]}{\bar{K}_{T_i}}\right)}. \tag{A16}$$

Experimentally, the ratio of slopes for entry is larger than the ratio for exit; i.e., expression (A16) is larger than (A15); after cancelling like terms it is found that

$$\frac{1 + \frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}}}{1 + \frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}} + \frac{\bar{V}^R_{S_o}[R_i]}{\bar{V}_{S_o}\bar{K}^S_{R_i}}} > \frac{1}{1 + \frac{\bar{V}^R_{S_i}[R_o]}{\bar{V}_{S_i}\bar{K}^S_{R_o}}}. \tag{A17}$$

After substitution into Eq. (A17) of the following relationship

$$\frac{\bar{V}_{S_o}\bar{V}^R_{S_i}\bar{K}^S_{R_i}}{\bar{V}_{S_i}\bar{V}^R_{S_o}\bar{K}^S_{R_o}} = \frac{f_{-1}f_4K_{R_i}}{f_1f_{-4}K_{R_o}} = 1 \tag{A18}$$

Eq. (A17) reduces to

$$1 + \frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}} > \frac{[R_i]}{[R_o]}. \tag{A19}$$

(Equation (A18) is found from expressions for the transport parameters $\bar{V}_{S_o}$, $\bar{V}_{S_i}$, etc., in terms of constants in the carrier scheme[3]; note that, because the transport scheme is a closed loop, the constants are necessarily related to one another: $f_{-1}f_4K_{R_i}/f_1f_{-4}K_{R_o} = 1$ in a facilitated transport system.) As imidazole, R, equilibrates across the cell membrane, $[R_i] = [R_o]$, and Eq. (A19) reduces to

$$\frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}} > 0. \tag{A20}$$

These are the conditions, then, under which the observed asymmetry in the imidazole effect is predicted by the carrier model: (i) the internal and external imidazole concentrations are equal [Eq.

[3] In terms of individual rate constants in Fig. 8*b*, the constants in Eq. (A18) may be written as follows: $\bar{V}_{S_o} = f_{-1}f_2C_t/(f_{-1} + f_2)$; $\bar{V}_{S_i} = f_1f_{-2}C_t/(f_1 + f_{-2})$; $\bar{V}^R_{S_i} = f_{-2}f_4C_t/(f_4 + f_{-2})$; $\bar{V}^R_{S_o} = f_2f_{-4}C_t/(f_2 + f_{-4})$; $\bar{K}^S_{R_i} = K_{R_i}(f_{-1} + f_2)/(f_2 + f_{-4})$; $\bar{K}^S_{R_o} = K_{R_o}(f_1 + f_{-2})/(f_{-2} + f_4)$. ($C_t$ is the total concentration of carrier sites.)

(A19); *see below*); (ii) the concentration of K^+ (T_i) is greater than zero; and (iii) K^+ is a transported substrate ($\bar{V}^T_{S_o}$ is greater than zero if T is transported, but equals zero if it is not: $\bar{V}^T_{S_o} = f_2 f_{-3} C_t / (f_2 + f_{-3})$).

The asymmetry, it will be noted, cannot result from an asymmetry in the transport parameters for imidazole, such as an intrinsically lower affinity on the inside than the outside. Asymmetry results because imidazole, freely permeating the lipid bilayer, fails to accumulate inside the cell, and is therefore present at equal concentrations inside and outside. The gradient in substrate concentration would ordinarily be determined by the K^+ gradient, as follows:

$$\left(\frac{[S_i]}{[S_o]}\right)_{\text{final}} = 1 + \frac{\bar{V}^T_{S_o}[T_i]}{\bar{V}_{S_o}\bar{K}^S_{T_i}} \tag{A21}$$

where T_i is internal K^+. If imidazole did accumulate in the same way, i.e., if $[R_i]/[R_o] = ([S_i]/[S_o])_{\text{final}}$, the terms in Eq. (A19) would be equal rather than unequal [*compare* Eqs. (A19) and (A21)], and imidazole would have a perfectly symmetrical effect.[4]

[4] An equivalent analysis of the asymmetry seen with imidazole could have been carried out without any explicit reference to internal K^+, on the basis of general rate equations containing the constant α [such as Eq. (A1), Appendix]: α is the ratio of internal to external substrate concentrations in the final steady state and is therefore a measure of the asymmetry of the system. Its experimental value would then be a function of the potassium ion concentration, according to Eq. (A21). Hence our analysis of the imidazole effects based on Eq. (A1) is valid, even though K^+ is not explicitly considered.