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A SIMPLE EXPERIMENTAL APPROACH TO THE DETERMINATION OF CARRIER TRANSPORT PARAMETERS FOR UNLABELED SUBSTRATE ANALOGS

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Summary

A method is described by which affinities and transport rates for unlabeled substrate analogs are readily determined, and which is based on the effect of an unlabeled analog upon the rate of transport of a labeled substrate present at a low concentration on the *trans* side of the membrane. The procedure is widely applicable since it does not depend on assumptions about rate-limiting steps and holds for both active and non-active systems. Here it is applied in an experimental study of the facilitated diffusion system for choline in erythrocytes, and it is shown that the transport parameters for a test substrate obtained by this method are the same as those found when the transport of the substrate is followed directly.

Introduction

Transport studies often depend on the availability of labeled substrates, and this tends to limit their scope to substrate analogs that are conveniently obtained in an appropriate form. Here a method is described in which the transport of an unlabeled analog is monitored by its effect upon the movement of a labeled substrate present on the *trans* side of the membrane. By this means both the affinities and transport rates of an unlimited number of analogs may be determined through the use of a single radioactive substrate. The method is of general application, whether the system is active or non-active and whether the rate-limiting step in substrate movement is passage across the membrane or

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dissociation from the carrier. We shall present the kinetic theory upon which this method is based and show how it can be applied in the case of the choline transport system in erythrocytes.

Experimental approach

An equation for the rate of exit of substrate S under the conditions of the proposed experiment is given below. In the experiment, the efflux of labeled substrate S from pre-equilibrated cells is determined in the presence of varying concentrations of unlabeled analog T in the external solution. The internal concentration of S is set at a very low value ($[S_i]/\overline{K}_{Si} << 1$), for under this condition we can show that the apparent affinity and maximum rate constants for T are identical to those obtained when its influx is observed directly.

$$v = \frac{-d[S_i]}{dt} = \frac{[S_i]}{\overline{K}_{Si}} \left\{ \overline{V}_{Si} + \tilde{V}^{S}_{T_0} \frac{[T_0]}{\tilde{K}^{S}_{T_0}} \right\} \Big/ \left\{ 1 + \frac{[T_0]}{\overline{K}_{T_0}} \right\} \qquad (1)$$

This equation is readily obtained from the general treatment described previously [1], which was based on the transport scheme in Fig. 1, involving two substrates, S and T. The only assumptions made in deriving Eqn. 1 are that $[S_0] = [T_i] = 0$, and that $[S_i]/\overline{K}_{Si} << 1$, where the subscripts i and 0 designate the location of the substrate, either inside or outside the cell, respectively. The constants in the equation are experimental parameters whose equivalent expressions in terms of the individual rate constants in Fig. 1 are given in Table I, and whose definitions are as follows: $\overline{V}_{Si}$ is the maximum rate of exit of substrate S when no substrate is present in the external solution (zero *trans* efflux), and $\overline{K}_{Si}$ is the half-saturation constant for the same substrate in a zero *trans* efflux experiment. $\tilde{V}^{S}_{T_0}$ is the maximum entry rate for substrate T in the presence of a saturating concentration of substrate S inside the cell. $\overline{K}_{T_0}$ and $\tilde{K}^{S}_{T_0}$ are half-saturation constants for the entry of T into cells either free of substrate, or containing a saturating concentration of substrate S, respectively.

Eqn. 1 may be rearranged into the following linear form which is suitable

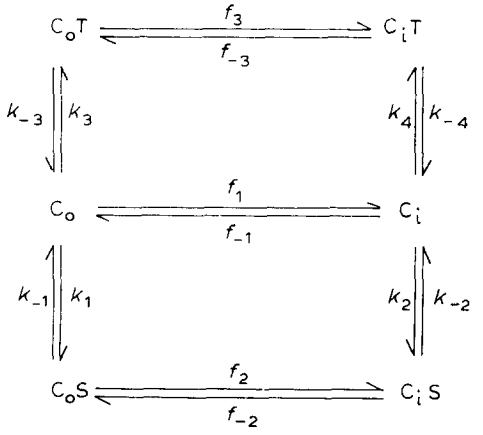

Fig. 1. Transport scheme for two substrates, S and T. Subscripts 0 and i refer to carrier forms on the outer and inner surfaces of the membrane, respectively; $f_{\pm i}$ are rate constants for reorientation of carrier in the membrane, and k_{+i} and k_{-i} are association and dissociation constants, respectively. $K_{T_0} = k_{-3}/k_3$; $K_{Ti} = k_{-4}/k_4$; $K_{S_0} = k_{-1}/k_1$; $K_{Si} = k_{-2}/k_2$.

TABLE I

EXPERIMENTAL PARAMETERS EXPRESSED IN TERMS OF INDIVIDUAL RATE CONSTANTS FOR THE TRANSPORT SCHEME IN FIG. 1

Values of the constants are listed both in the general case and when dissociation of the carrier-substrate complex is far more rapid than carrier reorientation. Equilibrium constants for complex formation are:

$K_{TO} = k_{-3}/k_3$; $K_{Si} = k_{-2}/k_2$.

C_t represents the total concentration of carrier in all forms, and the constants G and H are given by:

$G = k_{-3}f_{-3} + k_{-3}k_{-4} + k_{-4}f_3$; $H = k_{-1}f_{-2} + k_{-1}k_{-2} + k_{-2}f_2$.

Experimental parameter	Experiment	General expression	Rapid dissociation
$\bar{K}_{TO}$	Zero *trans* influx	$\frac{(f_1 + f_{-1})G/k_3}{k_{-4}(f_{-1} + f_3) + f_{-1}(f_3 + f_{-3})}$	$\frac{K_{TO}(f_1 + f_{-1})}{f_{-1} + f_3}$
$\bar{V}_{TO}$	Zero *trans* influx	$\frac{k_{-4}f_{-1}f_3C_t}{k_{-4}(f_{-1} + f_3) + f_{-1}(f_3 + f_{-1})}$	$\frac{f_{-1}f_3C_t}{f_{-1} + f_3}$
$\bar{K}_{Si}$	Zero *trans* efflux	$\frac{(f_1 + f_{-1})H/k_2}{k_{-1}(f_1 + f_{-2}) + f_1(f_2 + f_{-2})}$	$\frac{K_{Si}(f_1 + f_{-1})}{f_1 + f_{-2}}$
$\bar{V}_{Si}$	Zero *trans* efflux	$\frac{f_{-1}f_1f_{-2}C_t}{k_{-1}(f_1 + f_{-2}) + f_1(f_2 + f_{-2})}$	$\frac{f_1f_{-2}C_t}{f_1 + f_{-2}}$
$\tilde{K}^S_{To}$	Infinite *trans* influx	$\frac{\{k_{-1}(f_1 + f_{-2}) + f_1(f_2 + f_{-2})\}G/k_3}{k_{-1}f_{-2}(k_{-4} + f_3 + f_{-3}) + k_{-4}f_3(k_{-1} + f_2 + f_{-2})}$	$\frac{K_{To}(f_1 + f_{-2})}{f_{-2} + f_3}$
$\tilde{V}^S_{To}$	Infinite *trans* influx	$\frac{k_{-1}k_{-4}f_{-2}f_3C_t}{k_{-1}f_{-2}(k_{-4} + f_3 + f_{-3}) + k_{-4}f_3(k_{-1} + f_2 + f_{-2})}$	$\frac{f_{-2}f_3C_t}{f_{-2} + f_3}$
K_{Ti}	Zero *trans* efflux	$\frac{(f_1 + f_{-1})G/k_{-4}}{k_{-3}(f_1 + f_{-3}) + f_1(f_3 + f_{-3})}$	$\frac{K_{Ti}(f_1 + f_{-1})}{f_1 + f_{-3}}$

for the analysis of experiments:

$$\frac{v}{\bar{v}} = \left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_i \to 0} + \bar{K}_{T_0}\frac{(1 - v/\bar{v})}{[T_0]} \tag{2}$$

In this equation, $\bar{v}$ is defined as the rate of exit of labeled substrate S into pure buffer solution ($[T_0] = 0$), and $\tilde{v}^T$ its exit rate when an analog T has been added to the external medium at a saturating concentration ($[T_0]/\bar{K}_{T_0} >> 1$). From Eqn. 1 these two rates are as follows:

$$\bar{v} = \bar{V}_{Si}[S_i]/\bar{K}_{Si} \tag{3}$$

$$\tilde{v}^T = \frac{\tilde{V}^S_{T_0}\bar{K}_{T_0}[S_i]}{\tilde{K}^S_{T_0}\bar{K}_{Si}} \tag{4}$$

Two experimental parameters, $(\tilde{v}^T/\bar{v})_{Si \to 0}$ and $\bar{K}_{T_0}$, are found from a plot of the experimental data in accordance with Eqn. 2. For a substrate, $\bar{K}_{T_0}$ is the affinity constant in a zero *trans* influx experiment, as noted above. In the case of an inhibitor, where f_3 and $\tilde{v}^T$ equal zero, $\bar{K}_{T_0}$ is equal to the half-saturation constant for inhibition of substrate entry [1].

The intercept, $(\tilde{v}^T/\bar{v})_{Si \to 0}$, is directly proportional to the maximum transport rate of the analog under investigation, T, and is independent of the rate of

transport of the labeled substrate, S. Substitution of the expressions for experimental parameters (Table I) yields

$$(\tilde{v}^T/\bar{v})_{Si\to 0} = \overline{V}_{T_0}(1/f_1 + 1/f_{-1})/C_t \tag{5}$$

where C_t is the total carrier concentration, and f_1 and f_{-1} are the rates of reorientation of the free carrier inward and outward, respectively (see Fig. 1). The ratio of the parameters $(\tilde{v}^T/\bar{v})_{Si\to 0}$ for two different substrates is equal to the ratio of the maximum zero *trans* entry rates.

$$\frac{\{(\tilde{v}^T/\bar{v})_{Si\to 0}\}_1}{\{(\tilde{v}^T/\bar{v})_{Si\to 0}\}_2} = \frac{(\overline{V}_{T_0})_1}{(\overline{V}_{T_0})_2} \tag{6}$$

where the subscripts 1 and 2 refer to the two substrates.

Application to the choline transport system

Methods

1. Chemicals. The synthesis and characterization of choline analogs, as well as other materials used, are described in another communication (2).

2. Preparation of cells. (a) Washing. Human blood obtained from an outdated blood bank supply was spun for 7 min at top speed in a clinical centrifuge and the plasma and buffy coat removed by aspiration. The cells were thoroughly washed with sterile salt solution (154 mM NaCl containing 5 mM sodium phosphate buffer, pH 6.8) and twice incubated for 3-h periods at 37°C in a shaking bath to allow for exit of endogenous choline (13 μM [3]). The same salt/buffer solution was used at all stages of the experiment. Cells were then packed by spinning for 15—20 min in a clinical centrifuge. *(b) Loading with radioactive choline.* Cells (40% hematocrit) were incubated for 6 h at 37°C in a buffered saline solution containing [^{14}C]choline chloride (5—6 μM, 30 or 53 Ci/mol), 0.1% glucose and 0.02% chloramphenicol. The cells were centrifuged down and the external radioactivity removed by washing four times in ice-cold buffer. The resulting concentration of choline was 3—4 μmol/l of packed cells. This is approximately 1/8 of the affinity constant on the inner side of the cell membrane [4].

3. Assay. (a) Rate measurements. Ice-cold packed cells (1 ml) containing radioactive choline were added to 9 ml of buffer at 41°C with or without a choline analog; the resulting suspension, whose temperature is found to be 37°C, was immediately transferred to a shaking bath at 37°C. Samples (3 ml) were removed at intervals and immediately centrifuged in the cold for 5 min. The supernatant (2 ml) was counted for radioactivity in 8 ml Aquasol. A correction for hemolysis was routinely applied, though it was almost always extremely small (less than 0.5%). The correction factor was obtained indirectly from the counting efficiency which was shown to be proportional to the absorbance at 540 nm. Corrections for counting efficiency were also applied. *(b) Total radioactivity in efflux suspension: $[S_i]_0$.* 2 ml of 5% trichloroacetic acid was added to 1 ml of the cell suspension [5]. After centrifugation, the radioactivity in 2 ml of supernatant was counted in 8 ml of Aquasol.

Treatment of experimental data

1. Calculation of rates. Rate constants for transport are obtained from the

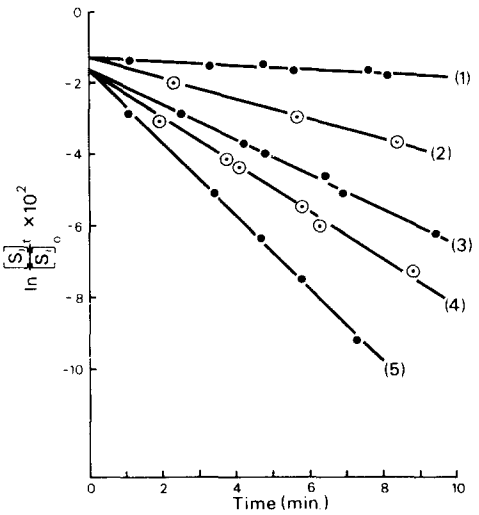

Fig. 2. [^{14}C]Choline exit into solutions containing various unlabeled choline analogs. (1) Diethyl-*n*-decyl-(2-hydroxyethyl)ammonium bromide, 0.018 mM. (2) Dimethyl isopropyl(2-hydroxyethyl)ammonium bromide, 2.3 mM. (3) Control: no analog present. (4) Diethyl methyl(2-hydroxyethyl)ammonium iodide, 0.38 mM. (5) Choline chloride, 0.052 mM. Cells were preloaded with 4 μmol [^{14}C]choline/l of cell water, 53 Ci/mol.

slope of a plot of $[\ln([S_i]_t/[S_i]_0)]$ against time, calculated by means of a least-squares fit to the data (Fig. 2) *. $[S_i]_0$ is the initial substrate concentration and is calculated from the total radioactivity in the efflux suspension. $[S_i]_t$ is the concentration at the time of sampling and is found from the difference between $[S_i]_0$ and the concentration in the supernatant.

2. *Determination of affinities and transport rates.* The ratio of choline efflux rates in the presence or absence of an external substrate at a given concentration, $(v/\bar{v})$, is plotted against the absolute value of a function of the reciprocal of the analog concentration, $(1 - v/\bar{v})/[S_0]$, according to Eqn. 2. Plots of this kind are shown in Fig. 3. All analogs give the predicted straight line, the slope being negative with analogs that increase the rate of choline exit above the control with pure buffer in the external medium, and positive with other analogs that inhibit choline efflux. Apparent affinities $(\bar{K}_{T_0})$ are calculated from the slope, and relative transport rates, $(\tilde{v}^T/\bar{v})_{Si\to 0}$, from the intercept in the ordinate.

All calculations were carried out automatically with the aid of a computer program written for the purpose: the data were first corrected for counting efficiency and hemolysis, and initial rates were determined from the experimental points by the method of least squares; then the experimental half-saturation and velocity constants, together with their standard deviations, were determined by a least-squares fit to Eqn. 2. Estimates of the calculated constants with probable errors are summarized in Tables II and III.

* Exit rates are expected to be logarithmic when the internal substrate concentration is well below the half-saturation constant.

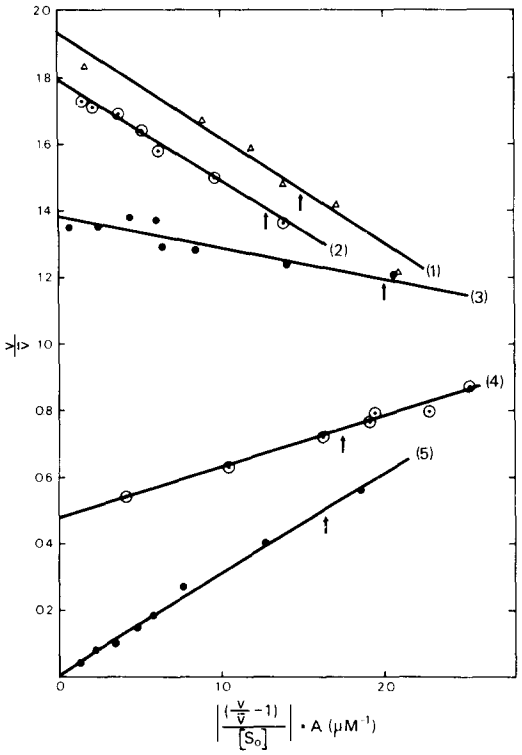

Fig. 3. Determination of affinities and transport rates for choline analogs (see Eqn. 2). The factor A which multiplies the units in the abscissa was introduced so that different concentration ranges for various analogs could be plotted on the same scale. (1) Choline chloride; A = 20. (2) Dimethyl ethyl(2-hydroxyethyl)ammonium iodide; A = 40. (3) Diethyl methyl(2-hydroxyethyl)ammonium iodide; A = 400. (4) Dimethyl isopropyl(2-hydroxyethyl)ammonium bromide; A = 2000. (5) Dimethyl-*n*-decyl(2-hydroxyethyl)ammonium bromide; A = 1. In each case the arrow marks the half-saturation concentration for the analog.

TABLE II

RATE CONSTANTS WITH STANDARD DEVIATIONS FOR [^{14}C] CHOLINE EXIT INTO SOLUTIONS OF UNLABELED CHOLINE ANALOGS PRESENT AT SATURATING CONCENTRATIONS

Calculated from the data in Fig. 2.

Analog	Rate constant (min^{-1})	Standard deviation of measurement
Choline chloride	$1.01 \cdot 10^{-2}$	$1.86 \cdot 10^{-4}$
Diethyl methyl(2-hydroxyethyl)ammonium iodide	$6.30 \cdot 10^{-3}$	$2.34 \cdot 10^{-4}$
Dimethyl isopropyl(2-hydroxyethyl)ammonium bromide	$2.81 \cdot 10^{-3}$	$1.00 \cdot 10^{-4}$
Diethyl-*n*-decyl(2-hydroxyethyl)-ammonium bromide	$6.03 \cdot 10^{-4}$	$1.36 \cdot 10^{-4}$
—	$4.90 \cdot 10^{-3}$	$1.36 \cdot 10^{-4}$

TABLE III

TRANSPORT PARAMETERS FOR CHOLINE ANALOGS DERIVED FROM THE DATA IN FIG. 3

Analog	$\bar{K}_{T0}$ (μM)	$(\tilde{V}^T/\bar{V})_{Si \to 0}$
Choline chloride	6.33 ± 0.62	1.93 ± 0.043
Dimethyl ethyl(2-hydroxyethyl)ammonium iodide	12.4 ± 0.61	1.79 ± 0.012
Diethyl methyl(2-hydroxyethyl)ammonium iodide	35.2 ± 7.7	1.38 ± 0.019
Dimethyl isopropyl(2-hydroxyethyl)ammonium bromide	303 ± 19	0.48 ± 0.017
Dimethyl-*n*-decyl(2-hydroxyethyl)ammonium bromide	0.031 ± 0.009	0.012 ± 0.008

Discussion

Since the experimental approach is based on completely general rate equations [1], the analysis must be valid whatever the rate-limiting steps in transport, whether diffusion through the membrane or dissociation of the carrier-substrate complex; and in both active and facilitated transport. It does not depend on the system's exhibiting accelerated exchange, which is seen in the object of our present investigation, choline transport. It should therefore broaden the scope of transport studies in all systems to include a great variety of substrate analogs.

Rationale of the method

When expressions for choline exit rates in the presence or absence of an external substrate are examined, it becomes evident that the analog exerts its effect by altering the distribution of the free carrier on either side of the membrane, and either increasing or diminishing the supply of the internal carrier form, which combines with internal substrate and moves it out of the cell. This is most simply demonstrated by recourse to a form of Eqn. 1 which contains individual rate constants rather than experimental parameters, and where dissociation of the carrier-substrate complex is assumed to be rapid compared to carrier reorientation steps:

$$v = \frac{-\mathrm{d}[S_i]}{\mathrm{d}t} = \frac{f_{-2}C_t\,(f_1 + f_3[T_0]/K_{T_0})[S_i]/K_{Si}}{f_1 + f_{-1} + (f_{-1} + f_3)[T_0]/K_{T_0}} \tag{7}$$

where K_{Si} and K_{T_0} are dissociation constants for internal S and external T, respectively. Rates in the presence of a saturating concentration of T or in its absence are given by:

$$\tilde{v}^T = f_{-2}[CS_i] = \frac{f_{-2}[S_i][C_i]}{K_{Si}} = \frac{f_{-2}[S_i]}{K_{Si}}\left(\frac{f_3 C_t}{f_3 + f_{-1}}\right) \tag{8}$$

$$\bar{v} = f_{-2}[CS_i] = \frac{f_{-2}[S_i][C_i]}{K_{Si}} = \frac{f_{-2}[S_i]}{K_{Si}}\left(\frac{f_1 C_t}{f_1 + f_{-1}}\right) \tag{9}$$

$[C_i]$ is seen to be equal to a fraction of the total amount of carrier, C_t; in the first case this fraction is $f_3/(f_3 + f_{-1})$, and in the second $f_1/(f_1 + f_{-1})$. The value of $(\tilde{v}^T/\bar{v})_{Si\to 0}$ therefore depends on the ratio of f_3 to f_1:

$$(\tilde{v}^T/\bar{v})_{Si\to 0} = (1 + f_{-1}/f_1)/(1 + f_{-1}/f_3) \tag{10}$$

When this ratio is greater than unity, the analog accelerates the exit of internal substrate. The maximum possible acceleration, however, depends upon the distribution of the free carrier in the membrane in the absence of substrates or inhibitors, which is governed by the ratio of f_1/f_{-1}. For example if $f_1 = f_{-1}$ the maximum possible rate increase due to an analog is two-fold, whereas if $f_1 << f_{-1}$, so that the undisturbed carrier is mainly in the form of C_0, the rate increase may be very much larger than this. If the analog does not undergo transport ($f_3 = 0$), $(\tilde{v}^T/\bar{v})_{Si\to 0}$ is zero in all cases.

Because of the upper limit on the value of $(\tilde{v}^T/\bar{v})_{Si\to 0}$ this parameter is insensitive to differences in the value of f_3 (and hence to the rate of reorienta-

tion of carrier-substrate complex) when $f_3 >> f_1$. Exactly the same limitation inheres in zero *trans* entry experiments with the same substrates, however, as is seen from the value of $\overline{V}_{T_0}$ in Table I. The only experiment capable of revealing the rate of movement of the carrier-substrate complex is equilibrium exchange, where reorientation of free carrier cannot limit the rate.

The experimental constant $(\tilde{v}^T/\bar{v})_{Si\to o}$ may also be intuitively understood as the ratio of maximum zero *trans* influx rates for the substrate T and for a hypothetical substrate whose complex with the carrier moves inward at exactly the same rate as free carrier, again assuming rapid dissociation of the carrier-substrate complex. For this substrate, $f_3 = f_1$ and $\overline{V}_c = C_t/(1/f_1 + 1/f_{-1})$, while for substrate T, $\overline{V}_{T_0} = C_t\ (1/f_3 + 1/f_{-1})$ as may be verified from the rate expressions in Table I. The ratio of $\overline{V}_{T_0}$ and $\overline{V}_c$ is exactly equal to $(\tilde{v}^T/\bar{v})_{Si\to o}$, given in Eqn. 10.

Rate determinations with the distribution of substrates reversed

If with certain substrates the efficiency of the transport system is very great, it could be inconvenient to follow the strategy described here, which involves measuring exit in the presence of high external concentrations of an unlabeled substrate, for the latter would rapidly enter the cells and interfere with the efflux of the labeled substrate inside. In this circumstance it might be better to reverse the locations of the substrates; first to incubate the cells with unlabeled analog and then to measure rates of influx of labeled substrate present externally at a very low concentration. This procedure has another advantage in extremely efficient systems where sufficiently rapid sampling and quenching is difficult to achieve, for there is a transient build-up in the internal concentration of the labeled substrate, described as 'counterflow', and this prolongs the period during which the entry of label is linear with time. The principles involved in analysis with the substrates reversed are the same, and the appropriate equation now has the form:

$$v/\bar{v} = (\tilde{v}^T/\bar{v})_{So\to o} + \overline{K}_{Ti}\,\frac{1 - v/\bar{v}}{[T_i]} \tag{11}$$

where $\overline{K}_{Ti}$ is defined in Table I, and where

$$(\tilde{v}^T/\bar{v})_{So\to o} = \overline{V}_{Ti}(1/f_1 + 1/f_{-1})/C_t \tag{12}$$

The maximum velocities of entry for two substrates may be shown to be related to $(\tilde{v}^T/\bar{v})_{So\to o}$ by an equation of exactly the same form as Eqn. 6.

Important information on the symmetry of the system may be obtained if both $(\tilde{v}^T/\bar{v})_{So\to o}$ and $(\tilde{v}^T/\bar{v})_{Si\to o}$ are determined for the same substrate. These parameters have the following relationship [1]:

$$\frac{(\tilde{v}^T/\bar{v})_{Si\to o}}{(\tilde{v}^T/\bar{v})_{So\to o}} = \left(\frac{\overline{K}_{T_0}}{\overline{K}_{Ti}}\right)\beta = \frac{\overline{V}_{T_0}}{\overline{V}_{Ti}} \tag{13}$$

where β is the ratio of the final substrate concentrations attained inside and outside the cell. In an equilibrating (non-active) system, $\beta = 1$, and the expression is equal to both the ratio of the apparent affinity constants, and the related ratio of maximum velocities of entry and exit [1]. In active transport,

$\beta >> 1$; if $\overline{K}_{T_0}$ and $\overline{K}_{Ti}$ are determined independently, by the methods described here, then β may be calculated from Eqn. 13.

Experimental tests of the method

The validity of the method is demonstrated by several different observations, the first of which is that the relatively complex relationship between independent and dependent variables predicted by Eqn. 2 is found to hold experimentally, as shown by linear plots for all analogs, ranging from good substrates to inhibitors (Fig. 3). Next, the affinity constants obtained here may be compared with those measured directly. The half-saturation constant, $\overline{K}_{T_0}$, determined from the rates of uptake of [^{14}C]choline into choline-free cells (zero *trans* entry) was found to be 6.54 ± 0.15 μM [2]. The result obtained by the present method is practically identical to this, 6.33 ± 0.62 μM. The affinity constant for an inhibitor was also determined directly: dimethyl-*n*-pentyl(2-hydroxyethyl)ammonium iodide placed in the external medium competitively inhibited the uptake of [^{14}C]choline into choline-free cells, as expected, and the inhibition constant was found to be 8.2 ± 1.6 μM [2]. The value of $\overline{K}_{T_0}$ obtained by the present method [2] agrees well with this: 7.3 ± 1.2 μM. In quantitative terms, therefore, the method produces results which are true measures of the constants desired. Rates of transport have been checked only for the case of inhibitors. The present findings indicated that neither the dimethyl-*n*-pentyl nor diethyl-*n*-decyl analog undergoes transport. Even after an incubation with cells for 12 h at 37°C, at a concentration which was roughly five or nine times their $\overline{K}_{T_0}$ values, respectively, these compounds failed to penetrate the cell membrane [2], in complete agreement with the present results.

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