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THE BINDING AND TRANSLOCATION STEPS IN TRANSPORT AS RELATED TO SUBSTRATE STRUCTURE

A STUDY OF THE CHOLINE CARRIER OF ERYTHROCYTES

R. DEVÉS * and R.M. KRUPKA

Research Institute, Agriculture Canada, University Sub Post Office, London, Ontario N6A 5B7, and Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1 (Canada)

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Summary

The relationships between structure, affinity and transport activity in the choline transport system of erythrocytes have been investigated in order to (i) explore the nature of the carrier site and its surroundings, and (ii) determine the dependence of the carrier reorientation process on binding energies and steric restraints due to the substrate molecule. Affinity constants and maximum transport rates for a series of trialkyl derivatives of ethanolamine were obtained by a method that involves measuring the *trans* effect of unlabeled analogs upon the movement of radioactive choline. The main conclusions are as follows: (1) An analysis of transport kinetics shows that the affinity constants determined experimentally differ from the actual dissociation constants in a predictable way. The better the substrate, the higher the apparent affinity relative to the true value, whereas the affinity of non-transported inhibitors is underestimated by a constant factor. (2) The carrier-choline complex undergoes far more rapid reorientation (translocation) than the free carrier. (3) The carrier imposes a strict upper limit upon the size of a substrate molecule that can participate in the carrier reorientation process; this limit corresponds to the choline structure. A smaller substrate such as tetramethylammonium, despite relatively weak binding forces, is unhindered in its translocation, suggesting that a carrier conformational change, dependent upon substrate binding energy, is not required for transport. (4) Small increases in the size of the quaternary

* Present address: Department of Biochemistry, University of Southern California, Los Angeles, CA 90033, U.S.A.

ammonium head, as in triethylcholine, sharply lower affinity, consistent with a high degree of specificity for the trimethylammonium group. (5) Lengthening the alkyl substituent in derivatives of dimethyl- and diethylaminoethanol causes a regular increase in affinity, suggestive of unspecific hydrophobic bonding in a region very near the substrate site.

Introduction

Structural analogs of substrates have made valuable contributions to the understanding of enzyme catalysis, but their potential in elucidating transport mechanisms has been little exploited. In the field of transport, studies of analogs have most often been concerned with the physiological implications of having shared or multiple transport systems for groups of substrates, or with the discovery of powerful inhibitors that could be used as drugs or biochemical tools. In some cases, analogs have been employed as an aid in exploring the shape of the binding site, but seldom has their use been directed towards the understanding of the translocation process itself, the step that follows binding. Potentially, a 3-fold relationship may be established for a series of substrates: that between structure and affinity, between affinity and maximum transport rate, and between structure and rate. The first of these informs us about the shape and size of the binding site and the nature of its surroundings, the second about the dependence of the translocation process upon substrate binding forces within the site and in adjacent regions of the carrier, and the third about the effects of steric restraints upon penetration of the substrate into the channel through the membrane, which by some mechanism the carrier must provide.

We have now begun such an investigation. A facilitated (rather than an active) system has been chosen, because it involves the most fundamental form of a membrane carrier, uncomplicated by the superstructure of energy-channeling devices, but nevertheless possessing the essential features of great specificity, saturability, and the requirement for carrier reorientation steps in substrate translocation. It is to be noted that in the absence of an energy supply, active systems such as the lactose transporter of *Escherichia coli* [1] revert to this fundamental form, which has all the characteristics of the system when driven by energy except the capability of producing a concentration gradient.

Among facilitated systems, that for choline in erythrocytes is especially suitable, firstly because a great variety of structural analogs of the substrate may be prepared, and secondly because a potential means of probing carrier conformation, involving the irreversible inhibitor *N*-ethylmaleimide, has been described for this system [2].

A series of analogs was examined in which the fundamental ethanolamine structure is preserved, and in which the three methyl groups on the quaternary nitrogen atom may be replaced by alkyl chains of various lengths. It is therefore expected that enlargement should produce crowding in the presumed cation-binding locus, but not in the region of the carrier site that interacts with the hydroxyethyl chain.

Methods

(I) Materials

Choline chloride (*methyl*-^{14}C, 30 Ci/mol) was obtained from New England Nuclear. The isotope was stored at −10° C and its purity examined by paper chromatography (Butanol/HCl/H_2O (4 : 1 : 1)), with choline and trimethylammonium as standards. Other chemicals were of commercial reagent grade.

(II) Preparation of choline analogs

Trialkyl(2-hydroxyethyl)ammonium halides (Table I) were prepared by quaternarization of tertiary amino alcohols with alkyl iodides or bromides. Equimolar quantities of reactants were refluxed in dry methyl ethyl ketone at 80° C for approximately 8 h [3]. The products were purified by recrystallization in various solvents, followed by washing in ether and drying under vacuum, and were then characterized and shown to be homogeneous by means of melting points, paper chromatography (Table I) and NMR spectroscopy (Table II).

One-dimensional ascending-paper chromatography was carried out in butanol/acetic acid/water, (4 : 1 : 5) [4], and chromatograms were sprayed with an iodoplatinate reagent [5]. In every case a single spot was observed having the R_F value given in Table I.

NMR spectra (Table II) were obtained in ^2H_2O with tetramethylsilane as external standard. Chemical shifts (δ) are given in ppm. In the case of multiplets, the chemical shifts given correspond to the center of the signal. The number of protons was calculated by multiplying the relative area of the signal by the assumed number of protons in the molecule.

The chloride of dimethyl-*n*-propyl(2-hydroxyethyl)ammonium was prepared from the iodide, which did not crystallize. Equimolar amounts of analog and AgCl were shaken in ethanol for 2 days. The mixture was then filtered, the solvent evaporated and the product recrystallized from acetone.

(III) Transport studies

(1) Determination of affinity and transport rate. The method used in determining the transport parameters for the analogs, as well as the theoretical basis of the analysis, are described in detail in our previous paper [6]. In brief, rates of efflux of [^{14}C]choline from pre-loaded cells were measured in the presence of various concentrations of an unlabeled choline analog in the external solution, and the results were plotted in accordance with the following equation:

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

where v is the experimental rate of choline exit in the presence of a given concentration of analog, $[T_0]$, in the external solution, and $\bar{v}$ the measured exit rate in the absence of the analog. From the intercept of a plot of $v/\bar{v}$ against $(1 - v/\bar{v})/[T_0]$ we find $(\tilde{v}^T/\bar{v})S_{i\to 0}$, which was previously shown to be directly proportional to the maximum transport rate in zero *trans* entry. From the slope, we obtain $\bar{K}_{T_0}$, which is identical to the apparent dissociation constant when entry of the analog is studied directly. In terms of the present experi-

TABLE I

SYNTHESIS OF CHOLINE ANALOGS

Solvents used in recrystallization, melting points, and R_F values in ascending paper chromatography (butanol/acetic acid/water, 4 : 1 : 5).

Series	R	Anion	Melting point (°C)		R_F	Recrystallization solvent
			Determined	Reported [3]		
$R\text{-}^{+}N(CH_3)_2\text{-}CH_2\text{-}CH_2\text{-}OH$	Ethyl	I^-	272	259	0.48	ethanol
	n-Propyl	Cl^-	124.5		0.54	acetone
	iso-Propyl	Br^-	278 (decompn.)		0.50	ethanol
	n-Butyl	I^-	117	117	0.69	ethanol
	n-Pentyl	I^-	136		0.82	methyl ethyl ketone
	n-Decyl	Br^-	139		0.93	acetone
	n-Dodecyl	Br^-	166		0.91	acetone
$R\text{-}^{+}N(C_2H_5)_2\text{-}CH_2\text{-}CH_2\text{-}OH$	Methyl	I^-	278	279	0.54	ethanol
	Ethyl	I^-	283	283	0.57	ethanol
	n-Propyl	I^-	178.5	178	0.68	ethanol
	n-Butyl	I^-	116	112	0.75	methyl ethyl ketone
	n-Pentyl	I^-	75—79		0.83	methyl ethyl ketone
	n-Hexyl	Br^-	61.5		0.93	methyl ethyl ketone + ether
	n-Decyl	Br^-	83.5		0.93	acetone
$R\text{-}^{+}N(C_3H_7)_2\text{-}CH_2\text{-}CH_2\text{-}OH$	*n*-Propyl	I^-	166.7		0.83	methyl ethyl ketone

TABLE II

CHARACTERIZATION OF CHOLINE ANALOGS BY NMR SPECTROSCOPY

The relative positions of proton adsorption signals (δ) are assigned for individual groups in the molecule, as shown in the upper row in the table. The observed chemical shifts (ppm) and the number of protons calculated on the basis of the integrated spectrum are listed; in every case, the numbers of protons calculated for individual chemical groups agree with the expected number for a given structure. External standard, tetramethylsilane. Spectrometers: Varian A60A for all spectra except those for the dimethyl-propyl, dimethyl-butyl and dimethyl-dodecyl analogs, where a Varian XL100 spectrometer was used.

Series	R	CH_3-C		CH_3-CH_2-C		C-CH_2-C		CH_3-N		C-CH_2-N		CH-N		C-CH_2-O	
		δ	*H*	δ	*H*	δ	*H*	δ	*H*	δ	*H*	δ	*H*	δ	*H*
CH_3	Ethyl	1.85	3.00					3.65	6.20	3.95	3.70			4.52	2.10
R-$^+$N-CH_2-CH_2-OH	*n*-Propyl	1.54	2.83			2.35	1.93	3.65	6.15	3.94	3.98			4.53	2.17
CH_3	iso-Propyl	1.90	5.97					3.56	5.97	3.95	2.10	4.15	0.82	4.52	2.10
	n-Butyl	1.46	2.95			1.87	2.00	3.65	5.90	3.97	4.07			4.56	2.00
						2.25	2.00								
	n-Pentyl	1.40	3.20			1.92	6.06	3.65	5.98	3.95	4.06			4.54	1.72
	n-Decyl	1.40	2.90			1.80	15.80	3.65	6.00	3.95	4.00			4.52	2.20
	n-Dodecyl	1.36	2.74			1.79	17.70	3.65	6.10	4.01	4.00			4.52	2.32
						2.29	2.10								
C_2H_5	Methyl	1.84	5.89					3.58	3.04	3.95	5.98			4.52	2.14
R-$^+$N-CH_2-CH_2-OH	Ethyl	1.80	8.90							3.86	8.14			4.48	1.97
C_2H_5	*n*-Propyl			1.80	11.10					3.90	7.80			4.50	2.16
	n-Butyl			1.85	13.10					3.90	7.90			4.52	1.90
	n-Pentyl	1.36	2.80	1.80	12.30					3.85	7.96			4.49	1.88
	n-Hexyl	1.35	3.30	1.80	14.00					3.85	7.60			4.48	1.96
	n-Decyl	1.38	3.20	1.82	22.00					3.90	7.30			4.48	2.20
C_3H_7	*n*-Propyl	1.45	8.80			2.20	6.20			3.83	8.03			4.50	1.94
R-$^+$N-CH_2-CH_2-OH															
C_3H_7															

ment, $\tilde{v}^T$ is the choline exit rate when the analog concentration is saturating.

(2) Distribution of choline analogs during the efflux experiment. In the present assay the exit rates are measured under conditions where the analog is assumed to be restricted to the external solution. If analogs could enter the cell with sufficient rapidity, either via the carrier in the case of substrates or by passive diffusion through the membrane in the case of inhibitors, they would compete with [^{14}C]choline inside the cell and give a misleading result.

Fortunately, this does not happen, as the following facts show. Firstly, the plots of exit rate are linear with time in the case of both substrates and inhibitors [6]. Secondly, the inhibition of choline entry by dimethylpentyl(2-hydroxyethyl)ammonium bromide is purely competitive (see below). Competitive kinetics are expected only if the inhibitor is restricted to the external solution [7]. Were the inhibitor present inside the cell as well, inhibition would necessarily be non-competitive. Thirdly, and most conclusively, neither the pentyl nor the decyl analog penetrated the cells even after a 12-h incubation period (see below).

(3) Uptake assay. Cells (hematocrit 10%) were incubated at 37°C in a solution of 154 mM NaCl and 5 mM sodium phosphate buffer, pH 6.8, containing various concentrations of [^{14}C]choline. Samples (2 ml) were taken at intervals and added to tubes containing 5 ml of dibutyl phthalate and 10 ml of ice cold phosphate buffer. The cells were immediately spun in a clinical centrifuge for 10 min, and were found to sediment below the organic layer, which separated them from the aqueous radioactive solution above [8]. The latter was removed by aspiration and the walls of the tube thoroughly washed with isotonic NaCl to eliminate contaminating radioactivity. Then, after removal of the dibutyl phthalate, the cells were precipitated [9] by adding 0.8 ml of buffer and 2.0 ml of 5% trichloroacetic acid containing 1 mM choline. After centrifugation, 2 ml of the supernatant were removed and counted in 8 ml Aquasol.

The linearity of [^{14}C]choline (0.65 μM, 53 Ci/mol) uptake with time is illustrated by the results in Fig. 1. The almost complete inhibition by the

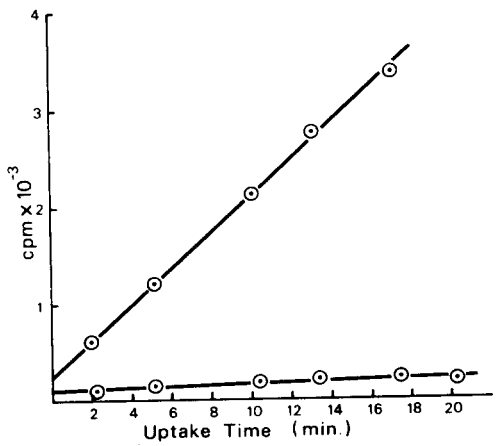

Fig. 1. The uptake of [^{14}C]choline (0.65 μM, 53 Ci/mol) either in the presence or absence (lower and upper lines respectively) of dimethylaminoethanol (100 μM), as a function of time.

choline analog, dimethylaminoethanol (100 μM), whose half-saturation constant is of the order of 10 μM, demonstrates that influx is entirely due to the operation of the carrier, rather than to passive diffusion.

Results

(I) Affinities and transport rates

The experimental dissociation constants, $\overline{K}_{T_0}$, and the relative transport rates, $(\tilde{v}^T/\bar{v})S_{i\to 0}$, for choline and its analogs are shown in Table III (see Methods). The ratio f_3/f_{-1}, the relative rate of translocation of the carrier-substrate complex and the free carrier, as represented in the transport scheme in Fig. 2, was estimated from the $(\tilde{v}^T/\bar{v})S_{i\to 0}$ values, on the basis of an equation derived previously [6]:

$$\left(\frac{\tilde{v}^T}{\bar{v}}\right)_{S_i\to 0} = \frac{(1 + f_{-1}/f_1)}{(1 + f_{-1}/f_3)} \tag{2}$$

For this purpose, the assumption was made that in the absence of substrate the free carrier is equally distributed in the inward and outward facing forms, so that $f_1 = f_{-1}$. Probable limits for f_3/f_{-1} were calculated from $(\tilde{v}^T/\bar{v})S_{i\to 0}$ plus or minus one standard deviation. Using this value of f_3/f_{-1}, the true substrate dissociation constant, K_{T_0}, was estimated from the expression for $\overline{K}_{T_0}$ [6]:

$$\overline{K}_{T_0} = K_{T_0}\frac{(1 + f_1/f_{-1})}{(1 + f_3/f_{-1})} \tag{3}$$

Again, f_1 and f_{-1} were assumed to be equal. Probable upper and lower limits for K_{T_0} were calculated from the upper and lower limits of f_3/f_{-1} *.

Trends in affinity and transport rates with increasing chain length for the dimethyl and diethyl series of choline analogs are shown in Figs. 3 and 4, respectively. The difference in affinity between corresponding members of the dimethyl and diethyl series, based on calculated K_{T_0} values, is shown in Fig. 5 as a function of the number of carbon atoms in the alkyl chain. The difference is seen to be constant throughout the series.

(II) Competitive inhibition by a non-transported choline analog

Choline entry was found to be competitively inhibited by the dimethyl-*n*-pentyl analog (Fig. 6). The $\overline{K}_{T_0}$ values for both choline (6.54 ± 0.15 μM) and the inhibitor (8.2 ± 1.6 μM) agree with those determined indirectly.

(III) Comparison of the sensitivity of glucose and choline transport to detergents and choline analogs

The effects of detergents and choline analogs on the transport of both choline and glucose are recorded in Table IV. The glucose system is the more

* The interpretation of $\overline{K}_{T_0}$ and $(\tilde{v}^T/\bar{v})S_{i\to 0}$ is independent of assumptions about rate-limiting steps in transport. Eqns. 2 and 3 and the calculated values of f_3/f_{-1} and K_{T_0}, however, depend on the condition that dissociation of the carrier-substrate complex is far more rapid than carrier reorientation steps. This assumption is supported by accelerated exchange for choline, and by observations to be reported later on non-competitive inhibition by a *trans* inhibitor, and on inactivation rates for *N*-ethylmaleimide in the presence of choline analogs.

TABLE III

TRANSPORT PARAMETERS FOR CHOLINE ANALOGS

$\overline{K}_{T_0}$ is the observed half-saturation constant, and $(\tilde{v}^T/\overline{v})_{S_i \to 0}$ is proportional to the maximum rate of transport (in zero *trans* entry): $(\tilde{v}^T/\overline{v})_{S_i \to 0} = \overline{V}_{T_0}(1/f_1 + 1/f_{-1})$. The ratio f_3/f_{-1} is the calculated rate of reorientation of the carrier-substrate complex relative to the free carrier (Fig. 2), and K_{T_0} the calculated dissociation constant for the carrier-substrate complex (see text).

Series	R	$\overline{K}_{T_0}$ (μM)	$(\tilde{v}^T/\overline{v})_{S_i \to 0}$	f_3/f_{-1}	K_{T_0} (μM)
$R\text{-}^{+}N(CH_3)_2\text{-}CH_2CH_2OH$	Methyl	6.33 ± 0.62	1.93 ± 0.043	17 —73	57.0 —234
	Ethyl	12.4 ± 0.61	1.79 ± 0.011	8 — 9	55.8 — 62.0
	n-Propyl	33.2 ± 6.4	1.32 ± 0.026	1.8 — 2.1	46.5 — 51.5
	iso-Propyl	303 ± 19	0.48 ± 0.017	0.30 — 0.33	197 —202
	n-Butyl	30.1 ± 1.8	0.12 ± 0.022	0.05 — 0.08	15.8 — 16.3
	n-Pentyl	7.3 ± 1.2	0.04 ± 0.08	0.00 — 0.06	3.65 — 3.87
	n-Decyl	0.30 ± 0.009	0.012 ± 0.008	0.00 — 0.01	0.150 — 0.152
	n-Dodecyl	0.095 ± 0.010	0.019 ± 0.019	0.00 — 0.02	0.0475— 0.0485
$R\text{-}^{+}N(C_2H_5)_2\text{-}CH_2CH_2OH$	Methyl	35.2 ± 7.7	1.38 ± 0.019	2.1 — 2.3	54.6 — 58.1
	Ethyl	693 ± 65	0.12 ± 0.028	0.05 — 0.08	364 —374
	n-Propyl	590 ± 44	0.004 ± 0.03	0.00 — 0.02	295 —301
	n-Butyl	203 ± 32	0.009 ± 0.08	0.00 — 0.05	102 —107
	n-Pentyl	51.8 ± 3.2	—0.07 ± 0.04	0.00	25.9
	n-Hexyl	17.1 ± 0.14	0.07 ± 0.003	0.035—0.038	8.85 — 8.88
	n-Decyl	1.95 ± 0.05	0.02 ± 0.006	0.007—0.013	0.98 — 0.99
$R\text{-}^{+}N(C_3H_7)_2\text{-}CH_2\text{-}CH_2OH$	*n*-Propyl	1230 ± 170	0.049 ± 0.093	0.00 —0.08	615 —664
$CH_3\text{-}{}^{+}N(CH_3)_2\text{-}CH_3$	—	206 ± 25 *	1.74 ± 0.13 *		

* The apparent affinity constant for tetramethyl ammonium was determined from its effect at varying concentrations outside the cell on the rate of [^{14}C]choline entry. $(\tilde{v}^T/\overline{v})_{S_i \to 0}$ was obtained by measuring choline efflux in the presence of a single saturating concentration (1 mM) of analog on the outside. In the same experiment $(\tilde{v}^T/\overline{v})_{S_i \to 0}$ for choline was 1.88 ± 0.17.

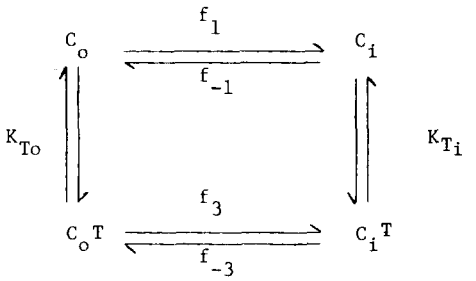

Fig. 2. Kinetic scheme for the transport of substrate T. C_o and C_i represent the free carrier forms on the outer and inner membrane surfaces respectively and C_oS and C_iS the corresponding carrier-substrate complexes. Rate constants for carrier reorientation steps are represented by $f \pm i$, and K_{T_o} and K_{T_i} are dissociation constants.

sensitive to detergents but is not significantly inhibited by the choline analogs at concentrations which strongly inhibit choline transport.

(IV) Failure of large choline analogs to penetrate into the cells

According to the results given in Table III, many of the choline analogs, though bound to the carrier, do not undergo transport at a significant rate. This result was subjected to an independent proof in the case of two such inhibitors. Cells (17% hematocrit) were incubated for 12 h at 37°C with either 34.1 μM dimethyl-*n*-pentyl(2-hydroxyethyl)ammonium or 17.7 μM diethyl-*n*-decyl(2-hydroxyethyl)ammonium ions (4.7 or 8.8 times the K_{T_o} value, respectively). Cells were then harvested, chilled and washed as in experiments on choline efflux. Uptake of [^{14}C]choline into cells preincubated with the inhibitor was found to be as rapid as in the control. Relative rates with and without inhibitor were 1.12 ± 0.02 and 0.93 ± 0.08, respectively. Since an inhibitor present inside the cells at these concentrations should have caused strong inhibition, it

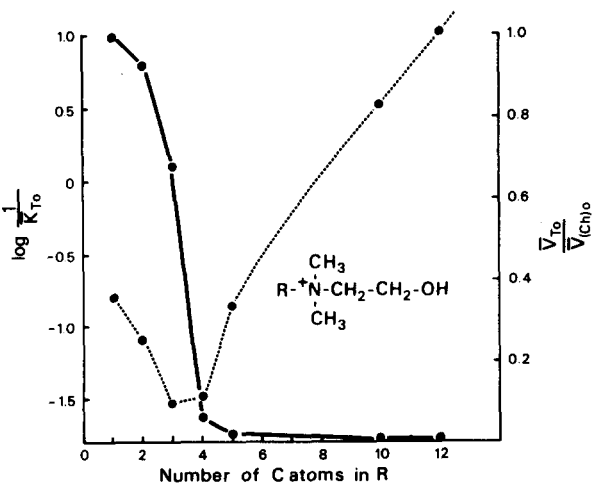

Fig. 3. Observed half-saturation constants and maximum transport rates (zero trans influx, relative to choline) for choline analogs in the series of diethyl-*n*-alkyl(2-hydroxyethyl)ammonium halides. $\overline{K}_{T_o}$ is expressed in μM units. Dotted line, affinities; solid line, rates.

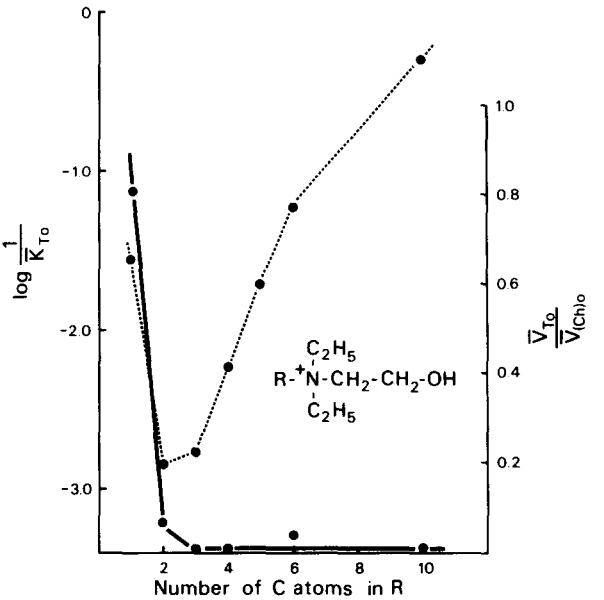

Fig. 4. Observed half-saturation constants and maximum transport rates (zero trans influx, relative to choline) for choline analogs in the series of dimethyl-*n*-alkyl(2-hydroxyethyl)ammonium halides. $\bar{K}_{T_0}$ is expressed in μM units. Dotted line, affinities; solid line, rates.

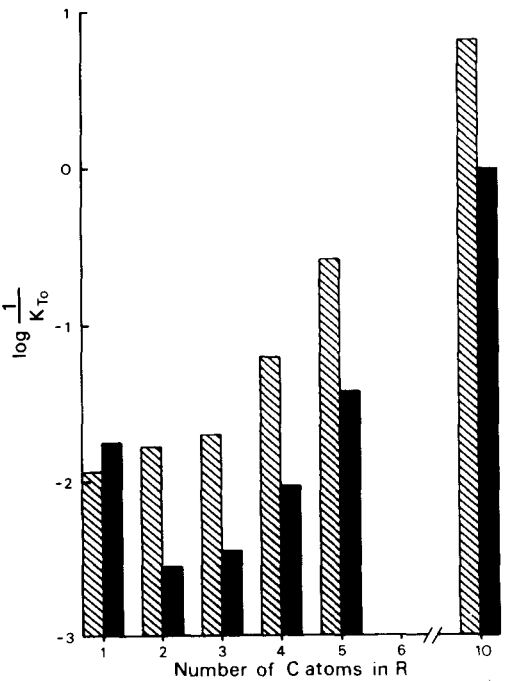

Fig. 5. Binding strengths of dimethyl- and diethyl-*n*-alkyl(2-hydroxyethyl)ammonium ions, as a function of the length of the alkyl chain. Binding constants, K_{T_0}, are calculated from apparent constants, $\bar{K}_{T_0}$, on the basis of Eqn. 3 and calculated f_3/f_{-1} ratios (Table III), with the assumption that $f_1 = f_{-1}$. For $n > 3$, relative values are independent of these assumptions (see text). Hatched and solid bars represent dimethyl and diethyl derivatives, respectively.

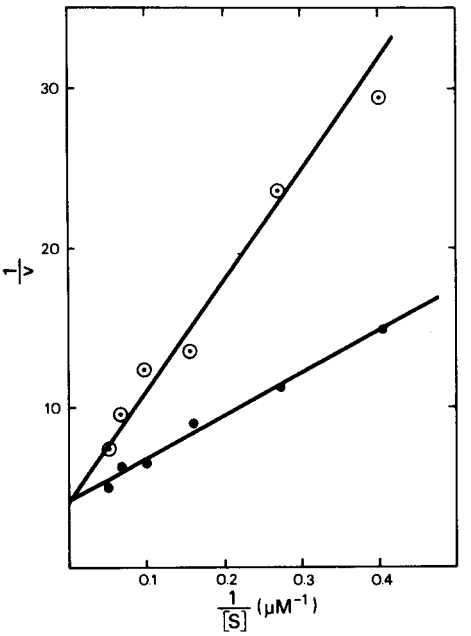

Fig. 6. The inhibition of [^{14}C]choline entry by dimethyl-*n*-pentyl(2-hydroxyethyl)ammonium iodide (12.9 μM). Points on the upper and lower lines represent rates in the presence and absence of the inhibitor respectively. Units of influx rates: μmoles per litre of packed cells per min. The lines are drawn from a least-squares analysis of the data.

may be concluded that neither the diethyl-*n*-decyl nor dimethyl-*n*-pentyl analog enters the cell, either by transport via the carrier, or by passive diffusion through the membrane.

(V) Effect of iodide ion on choline transport

Since some of the analogs were prepared as the iodide, the possible effect of this ion on transport was determined. Rates of choline exit were measured at varying concentrations of NaI in a medium containing a total concentration of NaI and NaCl of 154 mM together with 5 mM sodium phosphate buffer. Iodide

TABLE IV

EFFECTS OF DETERGENT AND CHOLINE ANALOGS ON THE GLUCOSE AND THE CHOLINE TRANSPORT SYSTEMS OF HUMAN ERYTHROCYTES

Assays of transport rates: uptake of [^{14}C]choline (3.7 μM, 24 Ci/mol) measured by the method described in the text; exit of D-glucose (110 mM) determined by the light-scattering method [9].

Inhibitor	Concentration	Percent inhibition	
		Glucose system	Choline system
Triton X-100	1.33 mg/100 ml	83.3	12
Cetyldimethyl benzyl ammonium chloride	$7 \cdot 10^{-3}$ mM	47	19.8
Di-*n*-butylamino ethanol	1.4 mM	2	56
Diethyl *n*-decyl (2-hydroxyethyl) ammonium bromide	$4.5 \cdot 10^{-2}$ mM	2	95.8
Dimethyl *n*-pentyl (2-hydroxyethyl) ammonium bromide	$1.16 \cdot 10^{-2}$ mM	0	61

ion was without effect, as the following figures show: rates in the presence of either 0, 1, 3, 10 or 30 mM NaI were 2.81 ± 0.04, 2.84 ± 0.18, 2.78 ± 0.05, 2.87 ± 0.05 and 3.08 ± 0.16, in units of 10^{-2} μmol/l of cells per min.

Discussion

(I) The relationship between apparent and true values of affinity and rate constants

The interpretation of trends in affinities for series of related substrates and inhibitors is complicated by discrepancies between true and apparent values, discrepancies which are greater the higher the rate at which the substrate is transported. This is evident upon inspection of Eqn. 3. With rapidly transported substrates such as choline, for which $f_3 >> f_{-1}$, saturation may occur at much lower concentrations than is expected from the value of the true dissociation constant K_{T_0}, for in this case $\overline{K}_{T_0} << K_{T_0}$ (assuming that $f_1 \approx f_{-1}$) *. In the case of inhibitors (where the transport rate, and f_3, are equal to zero) true and apparent affinities are related by the factor $(1 + f_1/f_{-1})$, which depends only on the partition of the carrier in the membrane in the absence of substrates or inhibitors, and is therefore a constant. For this reason, the relative affinities of different inhibitors are directly seen.

It is also true that the rates of reorientation of the carrier-substrate complex for good substrates are not directly apparent in zero *trans* flux experiments, because of the rate-limiting return of the unloaded carrier. Fortunately, the more useful ratio of f_3/f_{-1} may be estimated from $(\tilde{v}^T/\bar{v})S_{i\to 0}$ values if the ratio f_1/f_{-1} is known. Later, we shall present evidence to show that this ratio is approximately unity, and accordingly, from Eqn. 2,

$$(\tilde{v}^T/\bar{v})_{S_i\to 0} = 2/(1 + f_3/f_{-1}) . \qquad (4)$$

Once values for f_3/f_{-1} have been assigned, the apparent affinities may be corrected. The calculated values of both f_3/f_{-1} and K_{T_0} are included in Table III, where it is seen that uncertainty about the value of f_3/f_{-1} is large only in the case of the best substrate, choline itself, where the ratio f_3/f_{-1} is at least 17 and could be much larger. Correspondingly, the true affinity of choline must be smaller than the observed value by a factor of 10 or more. Owing to the uncertainty in this measurement, we shall avoid basing any conclusions on these particular values of the constants for choline.

(II) Evidence for specific interaction between choline analogs and the carrier

The largest choline analogs, such as dimethyl- and diethyl-*n*-decyl(2-hydroxy-

* On an intuitive level, the increase in apparent affinity of a good substrate may be understood as a consequence of the cyclic carrier movement involved in transport. With such a substrate, ($f_3 >> f_{-1}$), the loaded carrier moves through the membrane faster than the free carrier returns, and carrier on the *trans* side of the membrane accumulates. The latter is unavailable to bind substrate and therefore adds to the total concentration which, in effect, has formed a complex with the substrate. The substrate concentration required to 'saturate' the carrier is therefore lowered.

In the case of inhibitors, saturation occurs when the carrier is entirely in the form of a complex. However, since the inhibitor is restricted to the outside medium, only a fraction of the carrier can form a complex directly; the rest must migrate across the membrane, and only then can it be trapped by the inhibitor, whose concentration must therefore be higher than otherwise. In a sense, the inhibitor competes with the *trans* side of the membrane for the carrier.

ethyl)ammonium, may superficially resemble detergents. Their potency against the choline system, however, cannot depend on any property of this kind, as the following observations prove. (1) The glucose transport system of the same cells is highly sensitive to inhibition by anionic, cationic and neutral detergents, as well as non-polar substances generally [10], but is not inhibited by the choline analogs at concentrations that greatly depress choline transport (Table IV). (2) The choline system is relatively insensitive to detergents. Even the cationic detergent cetyldimethylbenzylammonium chloride, which resembles the larger choline analogs, is more effective against the glucose than the choline system (Table IV). (3) The concentration dependence of inhibition of choline transport conforms to a simple saturation process, in which one inhibitor molecule binds to one carrier site. Detergents should probably exhibit a higher than first order dependence on concentration, without apparent saturation. (4) The dimethylpentyl analog was shown to inhibit choline uptake in a purely competitive fashion (Fig. 6), whereas unspecific detergent effects might be expected to be non-competitive. (5) There is a constant difference between the affinities of corresponding dimethyl and diethyl analogs (having the same substituent R), which extends throughout the series and includes both substrates and inhibitors (Fig. 5). This constancy is indicative of a highly specific binding site, and shows that all the analogs become bound at this site. (6) Affinities are not simply determined by the hydrophobic nature of the analogs. Thus, inhibitors containing about the same number of methylene groups may have widely different affinities: e.g. the dimethylpentyl, diethylbutyl and tripropyl analogs have binding constants of 3.7, 105 and 630 μM, respectively (Table III). (7) In experiments to be reported later, we show that when present in the external solution, inhibitors with both long and short chains protect the system against inactivation by *N*-ethylmaleimide. They do so by holding the carrier in the form in which the binding site is exposed on the external surface of the membrane. By contrast, Triton X-100, even at concentrations that visibly alter membrane morphology, fails to alter the distribution of the carrier in the membrane and so has no effect on inactivation by the reagent.

(III) Structure-activity relations

(1) Affinity. Our observations are readily understood if we consider the cation binding site to have subsites for the three methyl substituents on the quaternary nitrogen atom in choline. At two of these positions the restrictions upon size are severe, at the third mild. Presumably when a choline analog enters the substrate site, its hydroxyethyl moiety and positive charge become fixed in position, but the trialkyl ammonium group is free to rotate so that the two smallest substituents occupy the most restricted methyl sites, while the largest occupies the third position and may project upwards, out of the binding site. In all members of the dimethyl series, the two methyls would therefore occupy the restricted sites, while the alkyl chain extends upwards; in all members of the diethyl series with the exception of the first (the diethylmethyl analog), two ethyl groups should occupy these sites and the third, the alkyl chain, would project upwards. With the diethyl-methyl analog, a methyl and an ethyl group are likely to occupy the restricted sites, rather than two ethyls. The

affinity of this compound is therefore higher than would be expected from its position in the diethyl series.

That enlargement at two of the methyl subsites is severely limited is shown by the observations that one methyl and one ethyl group are tolerated (compare the dimethylethyl and diethylmethyl analogs, for which K_{T_0} values are 59 and 57 μM, respectively), whereas two ethyl groups are squeezed out (the dimethylethyl and diethylmethyl analogs, above, are to be compared with the triethyl, for which K_{T_0} is 367 μM).

The relative constancy of the calculated K_{T_0} values in the first members of the dimethyl and diethyl series (excepting, as noted above, the diethylmethyl analog) suggests that the third, and least restrictive, methyl site accommodates an alkyl chain as long as propyl without crowding and without any positive contribution to binding. The introduction of a branched chain at this site does, however, result in crowding, judging by the fall in affinity of the dimethylisopropyl analog.

Lengthening of the alkyl chain beyond propyl leads to steadily increasing affinities in both the dimethyl and diethyl series, and this suggests the existence of non-polar structures of considerable size in the region immediately adjacent to the choline binding site, in agreement with observations made by Martin on a series of trimethyl-*n*-alkylammonium ions [11].

When corresponding analogs (with the same R substituents) in the dimethyl and diethyl series are compared, it is found that in every case (except R = 1) the diethyl analog has the lower affinity by a constant factor of 6.5, as is illustrated in Fig. 5. The constancy of this effect is of interest for several reasons. First, it depends on the increment in binding strength with each added methylene group being very similar in both series. The increment is in fact relatively constant throughout, even with the addition of 12 carbon atoms (Fig. 3). The conclusion from this must be that a rather extensive region is available immediately adjacent to the transport site, where nonspecific, probably hydrophobic bonding occurs. Second, it confirms the conclusion reached above regarding severe crowding at the point in the substrate site where two of the methyl groups are bound. Third, the unusual degree of specificity for the dimethyl(2-hydroxyethyl)ammonium ion indicates that in all members of both series the quaternary group is held at the same place in the carrier, which must be the choline binding site. The competitive inhibition seen with the dimethylpentyl analog (Fig. 6) serves to confirm this conclusion. Fourth, the simplicity of the relationship suggests that estimates based on calculated K_{T_0} values rather than experimental constants, $\overline{K}_{T_0}$, are essentially correct. If based on $\overline{K}_{T_0}$, constancy is still seen for those members of the series that are inhibitors but not for substrates, since $\overline{K}_{T_0}$ and K_{T_0} values for inhibitors differ by a constant factor $(1 + f_1/f_{-1})$.

(2) The rate of transport. Even the slightest enlargement in the structure of the substrate results in a severe reduction in transport activity. This is to be contrasted with the effect on binding strength, since increased size may either have little effect on affinity, may weaken binding, or more often may have the opposite effect and elevate affinity, as we have seen. The smallest additions to the choline structure, which fail to affect affinity, sharply diminish the carrier-reorientation rate. For example, with the dimethylpropyl and diethylmethyl

analogs, the rate of the carrier reorientation step f_3 is at least four times smaller than that for the dimethyl analog or choline. A reduction in affinity, which is a reflection of crowding at the binding site, is always accompanied by a severe reduction in the transport rate. For example, in the transition from the diethylmethyl to the triethyl analog, affinity falls by a factor of 6 to 7, but the rate of the carrier reorientation step is depressed by a factor of at least 30, virtually abolishing transport. Positive contributions to binding by the long alkyl substituents are also accompanied by a loss in transport activity, as is seen in the comparison of the *n*-butyl and *n*-propyl derivatives in the dimethyl series: the affinity is seen to rise by a factor of 4, while f_3 falls by a factor of 30, again reducing the transport rate to a barely detectable level.

Whereas enlargement of the substrate molecule lowers the rate of transport, diminishing its size has no measurable effect on the rate, even where affinity declines. This is shown by the example of tetramethylammonium, bound 30 times less strongly than choline, but transported at approximately the same rate.

We conclude from this that the requirements for binding at the carrier site on the surface of the membrane and for the subsequent step of translocation through the membrane are distinctly different.

Earlier, a series of trimethyl-*n*-alkylammonium ions was investigated by Martin [11], and it was found that as the alkyl chain was lengthened an abrupt transition occurred from a substrate transported approximately as well as choline (trimethyl-*n*-propylammonium ion) to an inhibitor which was not transported at all (trimethyl-*n*-butylammonium ion). At the transition point, a drop occurred in apparent affinity, rather than the regular increments seen elsewhere in the series.

In view of our present understanding of interactions in the binding site, this transition may be understood as a displacement of the alkyl chain from the 2-hydroxyethyl site to the non-polar region outside the cation binding locus. So long as the alkyl chain lies in the former site, transport is permitted, but when it protrudes outwards to form an attachment in the adjoining non-polar region, movement is prohibited. Clearly, severe spatial limitations must be encountered around both the quaternary nitrogen atom and the alcoholic hydroxyl group of choline.

(IV) The mechanism of transport

As seen above, the speed of the translocation step is sharply reduced by even the smallest increment in the substrate size, whereas binding becomes stronger when the substrate molecule is large. It appears to follow that restraints upon occupancy of the carrier site become far more severe when the site leaves the surface of the membrane and penetrates into the interior. This is understandable if we envisage the substrate bound at the surface site as being only partly enclosed by the carrier and free to protrude out of the site; and the substrate within the membrane, as being completely surrounded by structures in the carrier that match its shape at every point. Two half sites complementary to the substrate may therefore co-operate in the process of transport, one presented to the aqueous medium, the other capping the exposed surface of the substrate in the course of the carrier reorientation step (see Fig. 7).

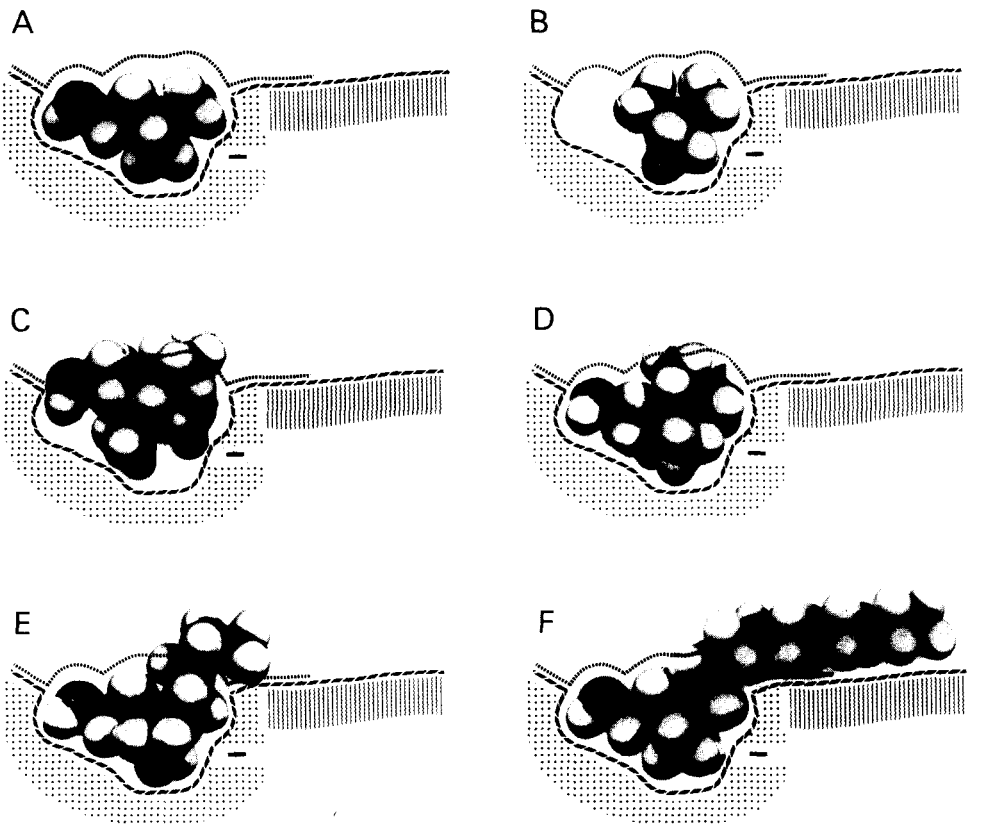

Fig. 7. A diagrammatic representation of the proposed carrier site with various substrate analogs bound. The lower section shows a profile of the site exposed on the external surface of the cell membrane. This site is closely complementary to one half of the choline molecule, and is open above. It contains a negative charge, as indicated in the drawing, adjacent to which is an extensive non-polar region capable of adsorbing long alkyl chains, as in F. The dotted profile above this external site represents a complementary site which, as a prerequisite for carrier reorientation must move into place, thus completely enclosing the substrate molecule. Substrates too large to fit into the space created by the apposition of the two half-sites fail to undergo translocation in the membrane but may be strongly bound to the lower half-site (F). Substrates that are bulkier than choline at all three *N*-methyl positions fail to make close contact with the lower site and are therefore both weakly bound and nontransported (C). Substrates small enough to be enclosed in the double site without crowding are well transported, even though weakly bound (B). Substrate analogs are as follows: A, choline; B, tetramethylammonium; C, triethyl(2-hydroxyethyl)ammonium; D, dimethylisopropyl(2-hydroxyethyl)ammonium; E, dimethyl-*n*-butyl(2-hydroxyethyl)ammonium; F, dimethyl-*n*-decyl(2-hydroxyethyl)ammonium ion.

That the binding of substrate could induce a conformational change in the carrier, facilitating the reorientation steps in transport, may be suggested by the observation that the complex with choline traverses the membrane far more rapidly than free carrier ($f_3/f_{-1} > 17$). This hypothesis, however, fails to account for the rapid transport of tetramethylammonium ion, which is equal to that of choline, despite the fact that its affinity is 30 times lower. Clearly, the hydroxymethylene group makes an important contribution to choline binding, but does not facilitate the translocation step.

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