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Inhibition of choline transport in erythrocytes by *n*-alkanols

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The choline transport system of erythrocytes is reversibly inhibited by ethanol, *n*-butanol, *n*-hexanol, *n*-octanol, and *n*-decanol, but not by *n*-dodecanol. Each methylene group in the alkyl chain contributes 560 cal/mol to the free energy of binding at the inhibitory site. Inhibition results from the cooperative binding of two molecules of an alcohol, judging by the Hill coefficient *n* of 1.7–1.9. The mechanism of inhibition is noncompetitive, and the partition of the carrier between inward-facing and outward-facing forms is unaffected by the alcohols; it follows that the four main carrier forms, the inner and outer free carrier, and the inner and outer carrier-substrate complex, are equally susceptible to inhibition. Hexanol and decanol accelerate the reaction of *N*-ethylmaleimide with a thiol group in the inner carrier channel, but ethanol and butanol, at concentrations that inhibit transport by 70%, do not. The disproportionate effects on substrate transport and the *N*-ethylmaleimide reaction are most simply explained as the direct result of binding of alcohol molecules in different regions of the carrier, rather than as the indirect result of a disturbance in the structure of the lipid bilayer induced by the alcohols.

Introduction

Many nonpolar molecules, widely different in size and structure, disrupt the highest function of the nervous system, inducing general anesthesia. In this state, consciousness and the sensibility to pain are lost, though the effect is fully reversible. Anesthesia, in which vital functions are maintained, is related to 'narcosis', which involves a general depression of vital function [1]; anesthesia could also be related to the disruptive effects of other nonpolar substances, including many fungicides, herbicides, and insecticides. Progress in accounting for such behavior may depend on an understanding of the effects of nonpolar molecules on the function of proteins imbedded in the cell membrane, a subject to be explored here.

General anesthetics modify the activity of nerves, probably by affecting the flow of ions across the nerve cell membrane. The ultimate target sites are almost certainly ion channels. Potency is governed by a very simple physical property, solubility in lipid, and is independent of any specific molecular feature. This would appear to implicate the lipid bilayer in their action, suggesting that the anesthetics perturb the structure of the lipid, which, indirectly, disturbs the function of the membrane proteins involved in ion movement. However, an alternative mechanism has not been ruled out, in which the anasthetics act by binding directly to these membrane protein molecules (see Refs. 2–9). The difficulty is that because of their nonpolar character the anesthetics can be expected to interfere with biological function at many levels. They should certainly dissolve in the lipid bilayer, but they should also bind directly to integral membrane proteins and protein molecules in the cytoplasm, altering their structure. Widespread effects are expected because hydrophobic bonding, which plays an essential role in stabilizing the structure of both proteins and lipids [10], is disrupted by adsorption of hydrophobic molecules from solution.

According to the earliest hypothesis for general anesthesia, the primary site of action is the lipid bilayer, which immediately explains the correlation between lipid solubility and anesthetic potency seen with a wide variety of drugs [1,11]. In agreement with the hypothesis, anesthetics do alter the structure of the lipid bilayer: they have been shown to increase membrane fluidity [12–14], to cause the membrane to expand [15], and to lower the temperature of the phase transition from gel to liquid-crystal in pure phospholipid bilayers [16,17]. The observed reversal of anesthesia by pressure agrees

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with a mechanism involving membrane expansion [18–20].

But other observations are inconsistent with the idea that the membrane is the primary site of action. First, the concentrations of general anesthetics required to perturb the structure of the membrane, for example to increase the number of *gauche* rotamers in dimyristoylphosphatidylcholine/cholesterol vesicles, are higher than the pharmacological concentrations [21]. Second, a shift in temperature of one or two degrees, which does not produce anesthesia, can induce changes in lipid order comparable to those seen in the presence of anaesthetics [21]. Third, the structural perturbation induced in the membrane by an anesthetic is not always correlated with the impairment of nerve conduction; for example, compounds having equal potency in anaesthesia sometimes have opposite effects on membrane fluidity [22]. Fourth, increased pressure, in the case of a freshwater shrimp, failed to reverse anaesthesia, which is at odds with a mechanism involving expansion of the lipid bilayer [23].

The alternative hypothesis, that anesthetics interact directly with essential protein components of the membrane, impairing their function, is favored by these observations, and is in accord with the finding that isolated enzymes in solution are sometimes inhibited by anesthetics. With luciferase, the inhibition by a wide variety of drugs was found to be correlated with lipid solubility [24,25], even though no lipid is present. Here, the anaesthetic molecules become bound at the substrate site, understandable considering that the substrate luciferin is a nonpolar molecule. It follows that anesthesia could as well involve direct effects on membrane proteins as primary effects on the lipid bilayer. Thus, the reversal of anesthesia by pressure was explained by Ueda, Kamaya, and Eyring [26] in terms of a protein conformational change, which is induced by adsorption of a molecule of the anesthetic, and which is accompanied by the release of structured water, with an increase in volume. But the effects of pressure can also be explained in terms of the lipid bilayer, the tranfer of a molecule of *n*-heptanol or *n*-octanol into phosphatidylcholine bilayers having been shown to increase the volume of the system [15].

For an understanding of the molecular mechanisms underlying anesthesia, then, the simplest elements of the system should be examined — the lipid bilayer, carriers and channels in membranes, and even isolated protein molecules in solution. The erythrocyte membrane, whose structure has been widely studied, should be a useful model system, particularly as transport across the membrane is known to be inhibited by anesthetics. Glucose transport, for example, is blocked by halothane, ether [27], *n*-alkanols [28,29], and local anesthetics [30], choline transport by benzyl alcohol [31], and anion exchange by halothane and *n*-alkanols [32]. Again, it has not been established whether the inhibition is a direct result of the interaction of the anesthetic molecule with the carrier protein, or whether it is indirect, through absorption of the anaesthetic into the lipid bilayer. Here we describe the effects of a series of *n*-alkanols on the choline carrier of erythrocytes. As anesthesia results from a disturbance in the function of ion channels in nerve membranes, the choline carrier, with its cationic substrate, may be an appropriate model, and it lends itself to such an investigation because the translocation mechanism is understood in some detail. The mechanism is cyclic, corresponding to the carrier model, and involves two interconvertible carrier conformations, one inward-facing and the other outward-facing [33]. Our aim in this first stage of the study is to determine the effects of alcohols on the two conformations of the carrier and carrier-substrate complex and on individual steps in the transport cycle.

Materials and Methods

1. Chemicals

n-Alkanols (ethanol, *n*-butanol, *n*-hexanol, *n*-octanol, *n*-decanol) were purchased from Aldrich Chemical Co. (Wisconsin, U.S.A), decamethonium and *N*-ethylmaleimide (NEM) from Sigma, and labeled [*Me*-^{14}C]choline chloride (50 mCi/mmol) from New England Nuclear. Other chemicals were of commercial reagent grade.

2. Preparation of cells

Human blood was obtained fresh from donors, with heparin as an anticoagulant. Cells (2.5% suspension) were washed free of endogenous choline by incubation for approx. 14 h at 25 °C with 5 mM sodium phosphate buffer (pH 6.8), containing 154 mM NaCl. The same buffer was used in all the experiments. When long incubations were carried out, chloramphenicol (0.02%) was included.

3. Exit assay

(a) Loading with labeled choline. Cells that had been washed free of choline were incubated at 50% hematocrit for approx. 6 h at 37 °C in buffer containing [Me-^{14}C] choline and 0.02% chloramphenicol. The concentration of choline was varied, depending on the type of experiment.

(b) Measurement of transport rates. Aliquots of loaded cells were added at 10% hematocrit to the incubation buffer. The temperature was held at 37 °C. Samples were withdrawn at intervals, up to 10 min, and rapidly centrifuged in tubes containing dibutyl phthalate. The cells sedimented below the organic layer, which separated them from the aqueous radioactive solution [34]. The volume of the extracellular solution in the pellet was about 4% of the total, as shown with radioactive

inulin, which does not enter the cell. The radioactivity in the supernatant was determined by scintillation counting, and rates were calculated from the initial linear relationship between counts and time.

4. Entry assay

(a) Loading with unlabeled choline. In some experiments, the uptake of radioactive choline was followed into cells containing a high concentration of unlabeled choline. Here, the cells (20% hematocrit) had previously been incubated for a period of 15 h at 25 °C in a buffered solution of 20 mM cold choline, with isotonicity maintained by reduction of the NaCl concentration. In order to estimate the final internal choline concentration, a separate suspension was incubated with 20 mM labeled choline over the same period of time, and the radioactivity in the cells was determined; the internal concentration was found to be 1 to 1.5 mM.

(b) Measurement of transport rates. Entry was followed in cells (10% hematocrit) added at time zero to a solution of radioactive choline. Samples of the suspension were withdrawn at intervals and placed in tubes containing dibutyl phthalate. The aqueous layer was taken off by aspiration and the walls of the tubes were thoroughly washed to eliminate contaminating radioactivity. After removal of the dibutyl phatalate, the cells were precipitated by addition of 5% trichloroacetic acid. The suspension was centrifuged, and the radioactivity in the supernatant was determined by scintillation counting.

5. Inactivation of transport activity by N-ethylmaleimide

A 2.5% suspension of cells was treated with 1 mM NEM at 37 °C with or without an alcohol in the solution. The reaction was started by adding 2 ml of packed cells to 80 ml of the medium (154 mM NaCl, 5 mM sodium phosphate buffer at pH 6.8, 1 mM NEM, with or without one of the alcohols at a given concentration; as the NEM stock solution was aqueous, no other organic solvent was present). Samples were withdrawn at intervals and placed in tubes containing mercaptoethanol at a final concentration of 10.6 mM. The treated cells were separated and washed, and their transport activity was measured as in 4(b), above. The detailed method has already been described [33].

6. Alcohol solutions

Saturated solutions of the longer alcohols were prepared by shaking a weighed quantity in the buffer for several hours at 25–30 °C. The final aqueous concentration in an experiment may be diminished through absorption of the alcohol by the cell. Partition into the cell membrane reduces the concentration according to the formula:

$$C_w = \frac{C_{w0}}{1 + P_m(V_m/V_w)} = \frac{C_{w0}}{1 + (P_m/999)} \quad (1)$$

where C_w is the final aqueous concentration, C_{w0} the initial aqueous concentration, and P_m the partition of the alcohol between the membrane and the surounding medium; V_w is the relative aqueous volume, 0.999, and V_m the relative volume of the membrane, 0.001, both calculated for 10% hematocrit. From the experimental values of P_m [2], the correction is insignificant with the smaller alcohols — less than 1.5% with ethanol, butanol, and hexanol — but is larger with octanol and decanol, whose concentrations should be reduced by factors of 1.15 and 2.22, respectively.

Hemoglobin would complicate the problem if it too absorbs alcohols, as it may, considering that ethanol and butanol, at concentrations comparable to those inhibiting transport, induce a local conformational change in this protein [35]. No correction for any cell constituent except the membrane appears to be required, though. First, the inhibition by hexanol was independent of the concentration of cells in the assay medium, being the same at 5%, 10%, and 20% hematocrit, just as expected if only the membrane absorbs the alcohol. Second, declining inhibition by decanol at rising cell concentrations is observed, but can be accounted for by absorption into the membrane. At 5%, 10%, and 20% hematocrit, a 0.24 mM decanol solution inhibited by 59%, 46%, and 28%, respectively. The inhibition constant is given by $K_i = [I]/((v_0/v) - 1)$, where v_0 is the control and v the inhibited rate; the experimental values of v_0/v were 2.42, 1.86, and 1.38, respectively. In a plot of these values against the hematocrit, an approximate value of 3 is found by rough extrapolation to zero hematocrit, and according to this the true value of K_i is about 0.24/2, or 0.12 mM. But if we take account of absorption by the membrane, we get almost the same result. The calculated alcohol concentration at 10% hematocrit (from Eqn. 1, with P_m = 1222 [2]) is 0.24 mM/(1 + 1.22), or 0.11 mM, and the corrected value of K_i is 0.11/0.86 = 0.126 mM. It is likely, therefore, that any absorption by hemoglobin is too slight to affect the results.

Results

The effects of increasing concentrations of aliphatic alcohols on choline entry are shown in Fig. 1. The carrier is inhibited by ethanol, *n*-butanol, *n*-hexanol, *n*-octanol and *n*-decanol. *n*-Dodecanol produced little or no inhibition even when added at 19 μM, which is close to the limit of its solubility (though as a result of partition into the membrane the final aqueous concentration would have been only 1.2 μM, from Eqn. 1). The inhibition is reversible: when cells preincubated for 5 min in the presence of 10 mM *n*-hexanol were washed three times with twenty volumes of buffer, full activity was recovered.

Several other aspects of the inhibition mechanism

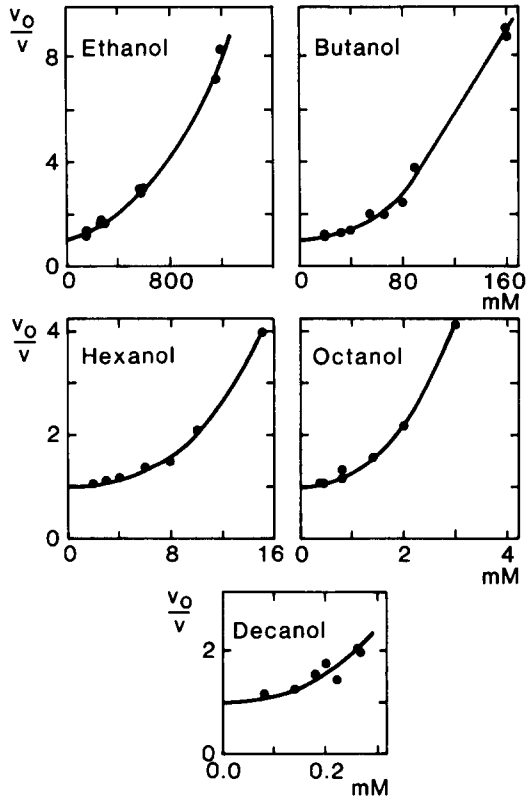

Fig. 1. Inhibition of choline entry by *n*-alkanols under zero trans conditions. In these experiments, the substrate is initially absent from the cell interior. The choline concentration in the external medium is 1–1.8 μM, compared with a half-saturation constant of about 6 μM. The ratio of entry rates in the absence (v_0) and presence of an alcohol (v) is plotted against the alcohol concentration, expressed on the basis of the total volume of the suspension (without correction for partition into the membrane).

were studied: (i) the number of alcohol molecules involved in blocking transport, (ii) the dependence of the affinity on the chain length of an alcohol, (iii) the interaction between an alcohol and the substrate choline,

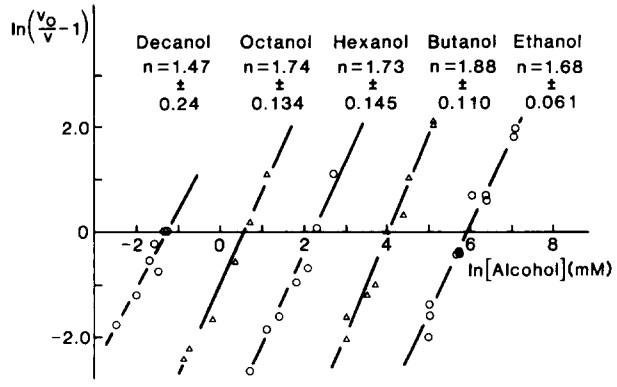

Fig. 2. Inhibition data from Fig. 1 plotted according to the Hill equation; v_0 is the uninhibited rate and v the rate in the presence of varying alcohol concentrations (uncorrected for partition into the membrane). The value of the slope n, with its standard deviation, is recorded. I_{50}, the inhibitor concentration giving 50% inhibition, is found from the point at which $\ln((v_0/v)-1)=0$ (where $v/v_0=1/2$).

(iv) the sidedness of the effects, that is, the vulnerability of inward-facing compared with outward-facing carrier forms, and (v) the effect of alcohols on the reactivity of an essential thiol group on the inner surface of the membrane. With respect to the first of these, a non-linear relationship — upward curvature — is found between the reciprocal of the rate and the concentration of alcohol (Fig. 1), indicating that more than one molecule participates in the inhibition. Hill plots of the data (Fig. 2) have slopes, n, of 1.7 to 1.9, where n depends on the number of inhibitor molecules producing the inhibition and the degree of cooperativity in their binding.

Regarding chain length, the logarithm of the alcohol concentration for 50% inhibition (log I_{50}), which is given by the intercept on the x-axis in the plot in Fig. 2, is inversely related to the number of carbon atoms in the molecule (Fig. 3), and it follows that each methylene group makes an equal contribution to binding. From

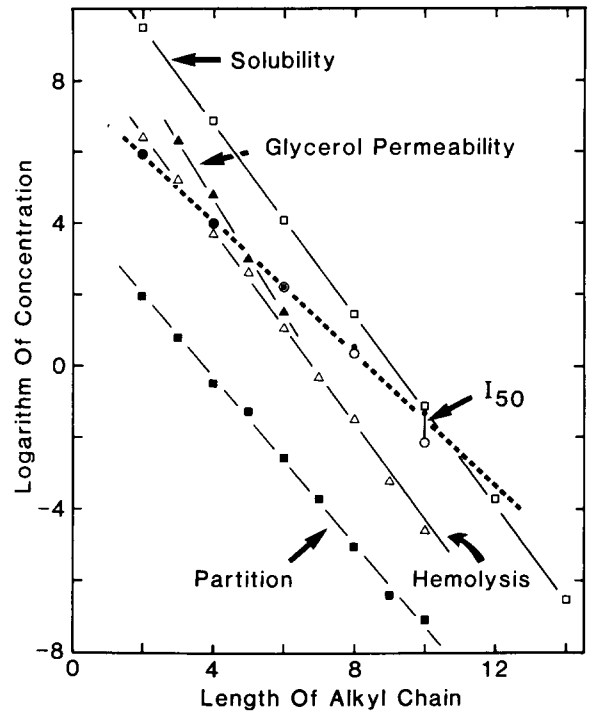

Fig. 3. The natural logarithm of I_{50}, the alcohol concentration required to reduce the rate of choline entry by 50%, plotted as a function of the number of carbon atoms in the alcohol molecule (filled circles). Except in the case of decanol, these uncorrected values of I_{50} do not differ significantly from values corrected for depletion of the alcohol concentration through partition into the membrane (Eqn. 1; open circles). Two other effects of the alcohols on the red cell membrane are shown for comparison: protection against osmotic lysis [2] and acceleration of the rate of glycerol permeation (measured in the presence of 0.1 mM Cu, which inhibits the carrier-mediated transport of glycerol) [42]. Also plotted, on the same numerical scale, are the saturating concentrations of the alcohols in aqueous solution and the negative logarithm of the partition coefficients between water and the red cell membrane [2,43]. The corrected I_{50} values (mM) are: ethanol, 384; *n*-butanol, 56.4; *n*-hexanol, 9.5; *n*-octanol, 1.49; *n*-decanol, 0.12.

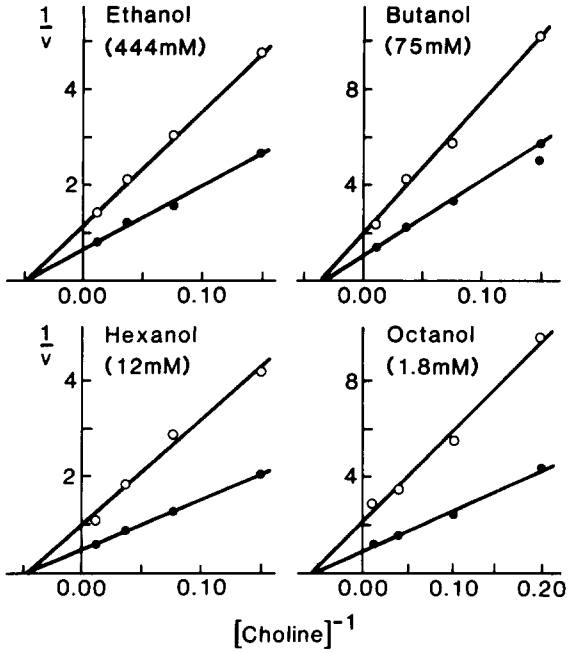

Fig. 4. The effect of the substrate concentration on the inhibition of choline entry by alcohols, in infinite-trans experiments. The concentration of radioactive choline in the external medium was varied; the cells contained unlabeled choline at a concentration of 1–1.5 mM. The upper and lower lines correspond to the reciprocal of the relative rates in the presence and absence of the inhibitor, respectively.

the slope of the plot the binding energy per methylene is 557 ± 6 cal/mol (slope $= \Delta G/RT$).

To decide whether the mechanism of inhibition is competitive, noncompetitive, or uncompetitive, rates of entry of [*Me*-^{14}C]choline were measured in cells containing a saturating level (1–1.5 mM) of non-radioactive choline. This arrangement is known as 'infinite trans entry', and is required because the alcohols, which rapidly diffuse through the lipid bilayer, are unavoidably present inside the cell when transport is measured. An inhibitor inside, even if bound at the substrate site, would give rise to noncompetitive behavior in the absence of substrate inside the cell (that is, in zero-trans entry experiments) because the labeled substrate in the external solution, whose movement is followed, is unable to displace an inhibitor in the opposite compartment. In infinite trans entry experiments, the high concentration of unlabeled substrate inside the cell prevents a competitive inhibitor inside from binding to the carrier, allowing the interaction of the external substrate and external inhibitor to be studied without interference [36]. Lineweaver-Burk plots of the experimental results are shown in Fig. 4. The mechanism is noncompetitive, and it is therefore clear that if the alcohols bind to the carrier, they do so outside the substrate site.

Though the alcohols will be present on both sides of the membrane, it cannot be assumed that the effects on the inner and outer carrier forms are the same, because carrier proteins, including the choline carrier, are not necessarily symmetrical in structure. For example certain choline analogs, such as *N*,*N*-dipropylaminoethanol, are exclusively bound to the inward-facing carrier site [37]. A simple method for determining the sidedness of action of noncompetitive inhibitors, which depends on the effect of an inhibitor on the flux ratio in substrate transport, was described in the preceding paper [38]. The flux ratio (for exit) is the ratio of the rates of exit of a low concentration of labeled substrate into a medium containing unlabeled substrate at a saturating concentration and into a medium free of substrate (infinite-trans and zero-trans experiments, respectively). If the inhibitor reduces one rate more than the other, the flux ratio changes. The direction and magnitude of the change depend on the symmetry of binding of the inhibitor, which can therefore be inferred. The rationale is as follows. The high concentration of choline outside the cell under infinite trans conditions shifts the equilibrium distribution of the carrier inward. It does so because the loaded carrier reorientates faster than the free carrier, the evidence [33] being that the infinite-trans rate is higher than the zero-trans rate ($f_2 > f_1$; see Fig. 5). In the choline system the flux ratio is approximately two, and it follows from this and other observations that while the carrier is about equally distributed between its inner and outer forms in the absence of the substrate, it is present almost entirely in the inward-facing form under infinite trans conditions. As a result, an inhibitor bound predominantly inside finds more of the carrier available in an infinite-trans experiment, and therefore, regardless of whether the mechanism is competitive or noncompetitive, it inhibits the infinite-trans more than the zero-trans rate; consequently it reduces the flux ratio. On the other hand, an inhibitor bound outside increases the flux ratio, because substrate in the external medium lowers the steady-state concentration of the outward-facing carrier, reducing the effectiveness of the inhibitor. Understandably, an inhibitor bound equally on the two sides leaves the flux ratio unchanged. In the experiments, none of the alcohols altered the flux ratio (Table I), showing that they have identical effects on the inner and outer carrier forms.

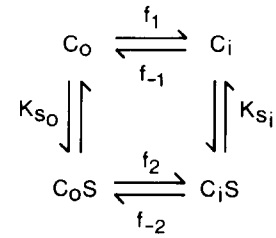

Fig. 5. The carrier model, in which the carrier exists in inward-facing and outward-facing forms, C_0 and C_i, respectively. Substrate in the external compartment forms a complex with C_0, substrate in the internal compartment with C_i.

TABLE I

Effect of n-alkanols on the flux ratio for the substrate

The flux ratio, $(\vec{v}^S/\bar{v})_{S_i \to 0}$, is the ratio of the rates of exit of radioactive choline (3–4 μM) into a suspending medium containing cold choline at a saturating concentration (1 mM) and into a medium free of choline. The aqueous concentration of an alcohol is found from Eqn. 1, which corrects for partition into the membrane; the partition coefficients are plotted as a function of chain length in Fig. 3.

Alcohol	Concn. (mM)	Flux ratio	
		control	+alcohol
Ethanol	433	2.08±0.12	2.31±0.15
Butanol	75	2.08±0.12	2.32±0.21
Hexanol	10.9	1.91±0.13	1.79±0.09
Octanol	1.39	2.08±0.12	1.92±0.18

Two of the alcohols have an effect on the rate of inactivation of the carrier by *N*-ethylmaleimide (NEM), and two do not. NEM irreversibly inhibits choline transport by reacting with an SH group in the inner gated channel of the carrier [39,40]. The SH group is exposed in the inward-facing but not the outward-facing carrier; its rate of reaction is therefore dependent on the carrier partition, as well as on intrinsic reactivity. In the experiments, *n*-hexanol and *n*-decanol accelerated the rate of inactivation, but ethanol and *n*-butanol left the rate unchanged (Fig. 6). As the alcohols do not appear to shift the carrier partition, they probably increase the reactivity of the thiol group. In agreement, the addition of a high concentration of choline to the external medium, which shifts the carrier inward, did not alter the effect of hexanol; with choline (1 mM), the rate of inactivation was 0.52 ± 0.021 min^{-1} in the presence of 14.5 mM hexanol and 0.31 ± 0.015 min^{-1} in the absence of alcohol, while without choline the corresponding rates were 0.24 ± 0.023 and 0.16 ± 0.008 min^{-1} (Fig. 6).

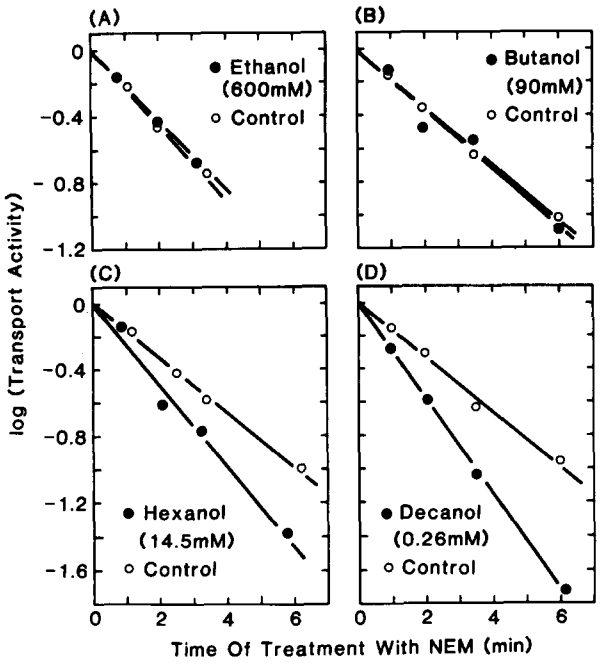

Fig. 6. Irreversible inhibition of choline transport by *N*-ethylmaleimide (NEM), in the presence or absence of an alcohol. The residual activity is plotted as a function of the time of treatment with 1 mM NEM. The pseudo-first order inactivation rate constants (k, in units of min^{-1}) calculated from the slopes of the lines are as follows: Ethanol (600 mM): control, 0.22±0.020; plus ethanol, 0.21±0.012. Butanol (90 mM): control, 0.17±0.007; plus butanol, 0.18±0.027. Hexanol (14.5 mM): control, 0.16±0.008; plus hexanol, 0.24±0.023. Decanol (0.22 mM, uncorrected): control, 0.16±0.013; plus decanol, 0.29±0.005.

Discussion

A striking feature of the inhibition of the choline carrier by aliphatic alcohols is the simplicity and regularity of the behavior. All the alcohols inhibit the carrier noncompetitively and symmetrically. It follows that they interact equally with the main carrier forms: the free carrier and the carrier-substrate complex in both the inward-facing and outward-facing states (see Fig. 5). Moreover, each methylene group in the alkyl chains contributes equally to the free energy of binding (560 cal/mol).

The observation of purely noncompetitive inhibiton implies that the alcohols do not interfere with the binding of the substrate and therefore, that they block the translocation step. This step, involving a change in carrier conformation, with interconversion of the outward-facing and inward-facing forms, is probably dependent on the simultaneous opening and closing of gated channels connecting the substrate site with the medium on either side of the membrane [41].

The choline system exhibits the cut-off phenomenon often considered an important characteristic of the behavior of anesthetics in nerves. The potency of decanol as an inhibitor tallies with the free energy change for an alkyl chain of its length (Fig. 3), but that of dodecanol does not: extending the chain by two additional methylene groups appears to abolish rather than enhance affinity. Nevertheless, such behavior is understandable, as Franks and Lieb [8] have explained. Because the individual trends in affinity and solubility (Fig. 3) intersect at C_{10} (decanol), the predicted I_{50} value for dodecanol is higher than the concentration of a saturated solution: from C_2 to C_8 (ethanol to octanol) the saturating concentration in aqueous solution is higher than the I_{50}, at C_{10} the two concentrations are about equal, and above C_{10} the saturating concentration falls below the predicted I_{50}. Moreover, the concentration of dodecanol in the experiment is even lower than the saturating concentration, owing to partition into the membrane: the I_{50} for dodecanol extrapolated from the other values is about 30 μM, and the aqueous concentration, as

calculated from Eqn. 1, is 1.5 μM (based on a solubility in water of 25g μM and a partition coefficient in the membrane, by extrapolation, of $1.55 \cdot 10^4$). Hence the sudden loss of observable activity with dodecanol is probably not due to an abrupt fall in affinity.

Inhibition of choline transport is less sensitive to the length of the alkyl chain than are two other effects of the alcohols, those on the resistance of the cell to osmotic lys is [2] and on the permeability of the membrane to glycerol [42] (Fig. 3). Each added methylene group, which strengthens inhibition by 2.5-fold, strengthens the effect on hemolysis by 3.9-fold and on permeability by 4.9-fold; at the same time the partition into the membrane is increased by 3.1-fold [43]. Different mechanisms are clearly involved here. The disproportionate effects of the smaller alcohols on the rates of transport and of carrier inactivation by *N*-ethylmaleimide (Fig. 6) also suggests different mechanisms, in this case distinct effects on different regions of the carrier. Ethanol and butanol, at concentrations that inhibit transport by approx. 70%, have no effect on inactivation; by contrast, 14.5 mM hexanol increases the inactivation rate by 50%, somewhat less than the effect on transport, and 0.22 mM decanol increases the inactivation rate by 75%, about the same as the predicted effect on transport (68% inhibition). If substrate transport and the reactivity of the thiol group were responding to a change in carrier conformation induced by a disturbance in the lipid bilayer, the various alcohols might be expected to have similar effects on inactivation since they have similar effects on transport; and though the effects on the membrane itself, phase transitions [44] and membrane order [45] for example, can be different, the simplest interpretation of the results is that the alcohols interact directly with two different regions of the carrier, one affecting mainly transport, and another, reaction of the thiol group in the inner gated channel.

It is of interest that local interactions with alcohols have been demonstrated in the case of hemoglobin in solution. Ethanol and butanol, at concentrations comparable to those that inhibit choline transport, induce local unfolding of the hemoglobin molecule, exposing a thiol group and increasing its reactivity; the conformation of the protein as a whole is not altered, judging by the absorption spectrum and optical rotatory dispersion [35]. The reactive thiol group, a cysteine residue, probably lies near the surface of the molecule [46] and plays a structural role in protein folding through hydrophobic interaction with other nonpolar amino acid side chains. Alkanes have been shown to bind at a single site in β-lactoglobulin; this site, probably a cleft closed to the solvent, can hold two molecules of butane, one as well as the other, or two molecules of pentane, unequally, or one molecule of iodobutane [47]. By binding to nonpolar surfaces [48], small molecules like these could stabilize the separated side chains, preventing refolding. Alcohols could have similar local effects on the choline carrier.

Such a mechanism, in which nonpolar molecules in solution bind to nonpolar amino acid side chains in a protein, could also explain the observed cooperativity in the unspecific binding of the alcohols to the carrier, with a Hill coefficient slightly less than 2. A possible mechanism is illustrated in Fig. 7. Two alcohol molecules are shown adding in a vulnerable region of the carrier protein, which exists as an equilibrium mixture of folded and unfolded conformations. The alcohols cannot bind to the folded state, but unfolding exposes two previously hidden nonpolar surfaces, to each of which an alcohol molecule can be adsorbed. Consequently the unfolded conformation is stabilized by addition of an alcohol molecule, with the result that addition of the first molecule facilitates addition of the second. According to this hypothesis, there are no specific allosteric sites for the alcohols. As the following analysis of the scheme in Fig. 7 shows, the degree of cooperativity depends mainly on the relative stability of the folded and unfolded forms in the absence of the alcohol. If the folded form C is active in transport and the unfolded form C′ inactive, the transport rate can be shown to have the following dependence on the inhibitor concentration:

$$\frac{v_0}{v} = 1 + \frac{2[I]}{(1+(1/K))K_1}\left\{1 + \frac{[I]}{2 \cdot K_2}\right\} \qquad (2)$$

where $K = [C']/[C]$, and v and v_0 are rates in the presence and absence of the inhibitor. The apparent dissociation constants for the first and second inhibitor molecules are $K_1(1 + (1/K))/2$ and $2 \cdot K_2$, respectively. The statistical factor of 2 enters for the following reasons: there are two ways of adding a molecule of the inhibitor to C′ to form the first complex C′I but only one way of removing it; conversely, there is only one way of adding the inhibitor to C′I to form the second

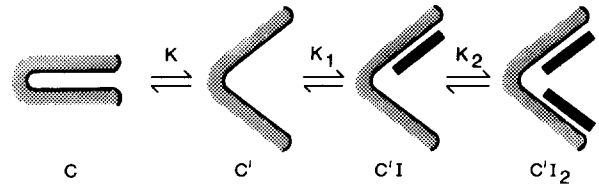

Fig. 7. A mechanism for the cooperative binding of alcohols to a protein molecule. In the folded state, C, two regions of the protein, composed of nonpolar amino acid side chains, are held together by hydrophobic bonding; in the unfolded state, the two regions are separated. An inhibitor molecule I binds to an exposed side chain, stabilizing the unfolded conformation, in which a second nonpolar surface is exposed. As a result, addition of the first inhibitor molecule facilitates addition of the second. Kinetic analysis of this scheme shows that the degree of cooperativity depends on the equilibrium of folded and unfolded forms (K) and on the relative affinity of the inhibitor for the two exposed surfaces (see Eqn. 2).

complex $C'I_2$, but two ways of removing an inhibitor molecule. The half-saturation constant for the first complex therefore involves a division by two, and that for the second complex a multiplication by two. The ratio of the apparent half-saturation constants for the first and second inhibitor molecules is equal to $K_1(1 + (1/K))/(4K_2)$. If in the absence of inhibitor the folded form predominates ($K \ll 1$), and if the intrinsic affinity of the two sites is the same ($K_1 = K_2$), this ratio equals $1/(4K)$, which is large. The affinity for the second inhibitor molecule then appears to be far higher than for the first, and the system exhibits positive cooperativity. The equation can account for a Hill coefficient just under 2, provided K is about $1/200$. Further, while the value of K depends only on the carrier protein, the observed cooperativity in binding also depends on the intrinsic affinity of an alcohol at the two sites: if K_1 and K_2 are unequal, the Hill coefficient n declines. The variation in n with different alcohols can be explained in this way.

An inhibition mechanism involving the unfolding of a small but essential part of the carrier protein might be inferred from the Hill coefficients themselves, so much lower than the values commonly seen in protein denaturation [49]. In enzymes, catalytic activity can be lost long before the protein has completely unfolded; thus creatine kinase becomes inactive at urea concentrations much lower than those producing a detectable change in conformation [50]. Here, local unfolding at a site essential for catalysis probably precedes global unfolding, and the effects on carriers could be similar.

The properties of the choline system appear to make it a suitable model with which to investigate the action of nonpolar molecules on membrane function. The ultimate targets in anesthesia are believed to be integral membrane proteins forming ion-conducting channels in nerves, and the choline carrier is itself a membrane protein that transports an ion across the cell membrane. The anesthetics bear no structural resemblance to the ions being translocated and are unlikely to interfere with channel function by competing with them. In choline transport, where the inhibition mechanism is noncompetitive, the alcohols disrupt a region of the carrier required in the translocation step rather than the region that directly interacts with the substrate; it is carrier reorientation that is blocked, the step in which the substrate site shifts from one side of the membrane to the other and which probably involves the opening and closing of gated channels on either side of the membrane [40]. Anesthesia may result from a similar lesion, the anesthetics somehow interfering with the opening and closing of gates in trans-membrane channels. It may be significant that the concentrations of the alcohols required to inhibit choline transport in erythrocytes are close to those that block conduction in nerves [2]: with ethanol the I_{50} values for the two processes are 384 and 500 mM, respectively; with butanol, 56 and 68 mM; with hexanol, 9.5 and 6 mM; and with octanol,1.5 and 0.7 mM.

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