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# Interaction of Alkyl Ammonium Derivatives with Red Cells: Hemolysis and Sodium Pump Inhibition Studies

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**Summary.** Members of four homologous series of tetra-alkyl ammonium bromides ($R_3\overset{+}{N}(CH_2)_{n-1} \cdot CH_3Br^-$ where $R = H$, $CH_3$ or $C_2H_5$ and $R'\overset{+}{N}H_3Br^-$ where $R'$ represents the isomeric butyl series) have been synthesized and tested as sodium pump inhibitors, measured as ouabain-sensitive $K^+$ influx, and as hemolytic agents on human red cells.

Potency for both effects is presented graphically, plotting the logarithm of the concentration for half maximal effect against alkyl chain length. Both hemolysis and pump inhibition studies yielded a biphasic response consisting of two good straight lines, with effectiveness increasing up to C10–12 and then remaining constant up to C20.

For hemolysis the alkyl ammonium series was most effective. The calculated free-energy change per methylene group was the same for three series of compounds, but the free-energy contribution from the headgroup was lower for the ammonium series.

In contrast, although pump inhibition studies also yielded simple biphasic plots, inhibition occurred at 3- to 50-fold lower concentrations and there were significant differences between the three series, both in the free-energy changes per methylene group and in the headgroup contributions.

We have analyzed these results thermodynamically to take account of hydrophobic interactions and the conformation of the alkyl chains.

Tetra-alkyl ammonium cations (TAA) have been used as $K^+$-like inhibitors in several biological systems, including the $K^+$ channel in squid axons (Tasaki & Hagiwara, 1957; Armstrong & Binstock, 1965) and the sodium pump in red cells (Sachs & Conrad, 1968; Kropp & Sachs, 1977). Although the simple tetraethyl ammonium cation (TEA) was originally used, longer chain TAA derivatives were found to be more effective (Armstrong, 1971; Armstrong & Hille, 1972; Rojas & Rudy, 1976).

TAA compounds can be considered amphipathic, since they contain both a long alkyl side chain and a charged quaternary nitrogen head group. For the sodium pump, there is good evidence that these compounds behave as competitive reversible inhibitors with respect to $K^+$ (Kropp & Sachs, 1977), although for the long alkyl chain derivatives other interactions may be present (Ellory, Simonsen & Klein, 1979). Thus, in an earlier paper (Ellory et al., 1979) we investigated the inhibitory effect of two series of TAA derivatives on the sodium pump. First, using the triethyl-alkyl ammonium series $Et_3\overset{+}{N}(CH_2)_{n-1}CH_3Br^-$ for $n = 2$–20, we showed an increasing inhibitory potency up to C12, beyond which point there was no further enhancement as $n$ was increased. Secondly, we showed an apparently complex relationship between inhibitory effectiveness and headgroup size for the homologous series $CH_3 \cdot (CH_2)_7 \cdot \overset{+}{N}R_3Br^-$ where $R$ varied from H to $C_5H_{11}$.

Because of their amphipathic character, long-chain TAA derivatives can have a detergent action and cause hemolysis. This effect is likely to represent a less specific membrane interaction than $Na^+$-pump inhibition and occurs at much higher TAA concentrations. In the present paper we measure the concentration effective for half-maximal hemolysis and for $Na^+$-pump inhibition, produced by members of four homologous series of TAA derivatives. The specific series considered were $R = H$, $CH_3$, $C_2H_5$ for $R_3\overset{+}{N}(CH_2)_{n-1}CH_3Br^-$ and $R'\overset{+}{N}H_3Br^-$ where $R'$ represents the isomeric butyl series. The results from both hemolysis and pump inhibition experiments are

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analyzed from a thermodynamic viewpoint, giving particular consideration to the nature of the partitioning of the inhibitor as it transfers from an aqueous environment to a membrane environment.

## Materials and Methods

Chemicals were purchased from various commercial sources, including Aldrich Chemical Co., Eastman Kodak, Koch Light, British Drug Houses and Sigma Chemical Co.

Blood was drawn from males aged 25–40 years into heparinized saline and used within 24 hr.

*Long Chain Alkyl Quaternary Ammonium Bromides*

Quaternary ammonium bromides or iodides were prepared by refluxing the appropriate alkyl halide with a slight molar excess of trialkylamine in either absolute methanol or acetone for 3–4hr (Finkelstein, Petersen & Ross, 1959; Grovenstein et al., 1959). Excess solvent and tertiary amine were removed by rotary evaporation, and a large excess of cold diethyl ether was added to precipitate the quaternary ammonium derivative, which was filtered and thoroughly washed with ether. If necessary, precipitation from a small volume of methanol with ether was repeated before the product was recrystallized from mixtures of ethyl acetate, ether, and ethanol and then dried under vacuum over phosphorus pentoxide. Although most alkyl bromides were available from commercial sources, octadecanyl and eicosanyl bromides were prepared from the corresponding alcohols (octadecan-1-ol and eicosan-1-ol), using bromine and red phosphorus at 250 °C as described by Vogel (1959; 1978), followed by purification using distillation under reduced pressure.

*Long-Chain Alkyl Ammonium Bromides*

The long-chain alkyl ammonium bromides were prepared by adding slightly in excess of the theoretical amount (105 %) of 48 % hydrogen bromide to the well-cooled alkylamine kept on ice with efficient mixing. The concentrated aqueous solution was then shell-frozen and freeze dried. The crude alkyl ammonium bromides were then recrystallized two to three times from appropriate mixtures of ethanol, ethyl acetate and diethyl ether, and then dried *in vacuo* over phosphorus pentoxide.

Tetradecylamine, hexadecylamine and octadecylamine were obtained from the Aldrich Chemical Company. These materials were of technical grade purity (75–90 %) and were, therefore, further purified before use to remove homologous primary amines and any secondary or tertiary amines present as impurities. Purification was achieved by fractional vacuum distillation using a heated 50-cm Vigreux column with a high reflux to distillation ratio. The distilled alkylamines were very pale yellow to colorless liquids which solidified on cooling with boiling points under reduced pressure as follows:

tetradecylamine 146–152 °C/4–5 mm Hg
(liter · 291 °C/760 mm; 162 °C/15 mm).

hexadecylamine 166–169 °C/4–5 mm Hg
(liter · 322 °C/760 mm; 144 °C/2 mm).

octadecylamine 182–184 °C/2–3 mm Hg
(liter · 232 °C/32 mm).

*Active $K^+$ and $Rb^+$ Uptake*

Washed red cells were incubated at 2 % hematocrit in 1 ml of a solution containing (in mM): 150, choline Cl; 1, NaCl; 5, glucose; 10, tris; pH 7.5 at 37 °C and the appropriate TAA inhibitor. A parallel series, with 0.1 mM ouabain added, was also included. Following 5 min preincubation at 37 °C, the reaction was started by adding KCl (containing tracer amounts of $^{42}K^+$ or $^{86}Rb^+$) solution to give a final $K^+$ concentration of 0.2 mM. After 30 min the samples were cooled in an ice bath (5 min) and the cells washed by centrifugation in isotonic (107 mM) $MgCl_2$ solution containing 10 mM tris, pH 7.6 at 4 °C, using an Eppendorf microcentrifuge. The final pellet was lysed in 0.1 % Triton X-100 solution (0.5 ml) and trichloracetic acid added to a final concentration of 5 %. The protein precipitate was removed by centrifugation and the supernatant assayed for Cerenkov radiation in a $\beta$-scintillation spectrometer. Samples of the original supernatant were counted as standard and assayed for K by flame photometry to guard against hemolytic effects on external K concentration. Samples were run in duplicate (and $\pm$ ouabain) for 5 inhibitor concentrations and a control. From preliminary experiments, TAA concentrations were adjusted to give 15–75 % inhibition. The calculated values for ouabain-sensitive $K^+$ influx were used in Dixon plots ($1/v$ *vs.* $I$) and the concentration for half-maximal pump inhibition ($I_{50}$) was determined (Ellory et al., 1979). Each TAA derivative was tested at least twice, and usually 3 times.

*Hemolysis*

Washed red cells were incubated under identical conditions to the flux tubes in the absence of isotope and at higher TAA concentrations. After 30 min at 37 °C the samples were centrifuged and aliquots of the supernatant assayed spectrophotometrically for Hb at 541 nm and compared with cell suspensions lysed with Triton X-100. Again, preliminary experiments established the appropriate range over which to assay the TAA derivatives.

## Results

### *Hemolysis*

Figure 1*a–c* presents data for the concentration of TAA derivative giving 50 % hemolysis after 30 min at 37 °C (2 % haematocrit) for members of the three series, alkylammonium, alkyltrimethylammonium and alkyltriethylammonium bromides. For the lower members of the series, very high concentrations were necessary to produce lysis, so data are only presented for $n_c > 6$ ($RNH_3^+Br^-$) or $n_c > 7$ ($RTMA^+$, $RTEA^+Br^-$). All three types of compound produced clearly biphasic plots for log $C_{50}$ *vs.* chain length. The TMA and TEA derivatives gave virtually identical results, with the data from $C_8$–$C_{14}$ fitting well for a straight line of negative slope, values for compounds of chain length $n_c > 14$ being parallel to the $x$ axis. The $RNH_3^+$ derivatives proved more effective hemolytic agents than the TMA and TEA series. In general, the data for $R\overset{+}{N}H_3$ compounds were similar to the other two series, but the initial part of the curve was

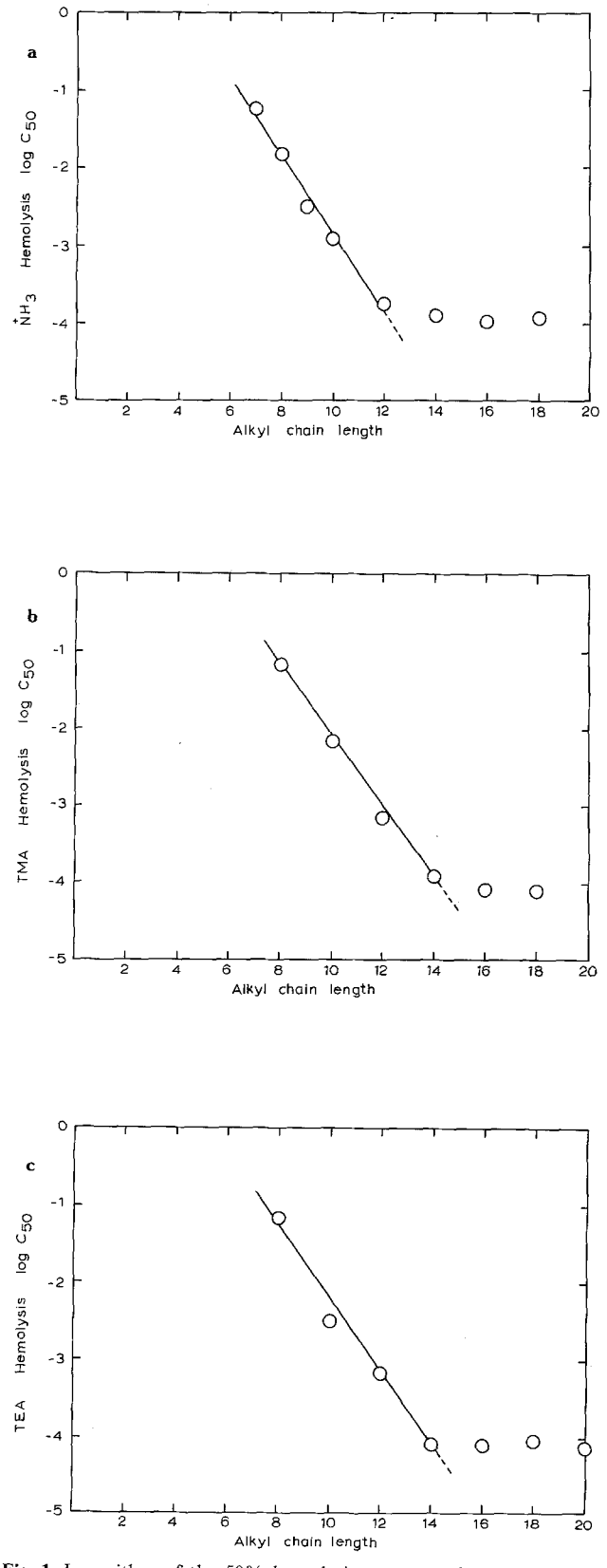


**Fig. 1.** Logarithm of the 50% hemolytic concentration *vs.* carbon atoms in the alkyl chain for (*a*) *n*-alkylammonium bromides, (*b*) *n*-alkyl trimethylammonium bromides and (*c*) *n*-alkyl triethylammonium bromides.

shifted to the left so that the curve breaks around $C_{12}$ rather than $C_{14}$. Linear regression analysis of the hemolysis data for the straight-line portion of the curves below $C_{12-14}$ gave the following equations:

ammonium

$$\log C_{50} = -0.501(\pm 0.034)n_c + 2.17(\pm 0.13)$$

trimethylammonium

$$\log C_{50} = -0.459(\pm 0.024)n_c + 2.45(\pm 0.11)$$

triethylammonium

$$\log C_{50} = -0.473(\pm 0.047)n_c + 2.50(\pm 0.21)$$

where $n_c$ is the number of carbon atoms in the alkyl chain.

The data are consistent in that each compound was tested 2 or 3 times and gave the same $C_{50}$ value. The concentrations for significant hemolysis are between 3- and 50-fold greater than the values at which the K influx was significantly inhibited (*see* below).

### *$K^+$-Influx Inhibition*

Figure 2*a–c* presents data for the concentration of TAA derivatives to produce 50% inhibition of the ouabain-sensitive $K^+$ influx in human red cells in choline medium. As for the hemolysis results, $\log C_{50}$ is plotted against chain length, $n_c$, for members of the three series $R\overset{+}{N}H_3$, $RTMA^+$ and $RTEA^+$. Here, inhibition occurs at relatively low concentrations compared with hemolysis, so it is possible to obtain results for even-numbered members of the $RTEA^+$ series from $C_2$–$C_{20}$, $C_4$–$C_{18}$ ($RTMA^+$) and $C_2$–$C_{18}$ ($R\overset{+}{N}H_3$ and including $C_7$ and $C_9$). Again, for all three series the curves clearly consist of two components, one of which having a negative slope and covering the region to $C_{11}$–$C_{13}$, whilst beyond this chain length the inhibitory concentration remains approximately constant, settling around a $C_{50}$ of 15–40 μM. For the earlier component, linear regression analysis of the K influx inhibition data gave the following equations:

ammonium

$$\log C_{50} = -0.352(\pm 0.012)n_c - 0.808(\pm 0.086)$$

trimethylammonium

$$\log C_{50} = -0.402(\pm 0.022)n_c + 1.03(\pm 0.18)$$

triethylammonium

$$\log C_{50} = -0.294(\pm 0.016)n_c - 0.72\ \pm 0.13).$$

For pump inhibition, in contrast to hemolysis, there are clear differences between the behavior of the three series. Alkylammonium compounds are still the most

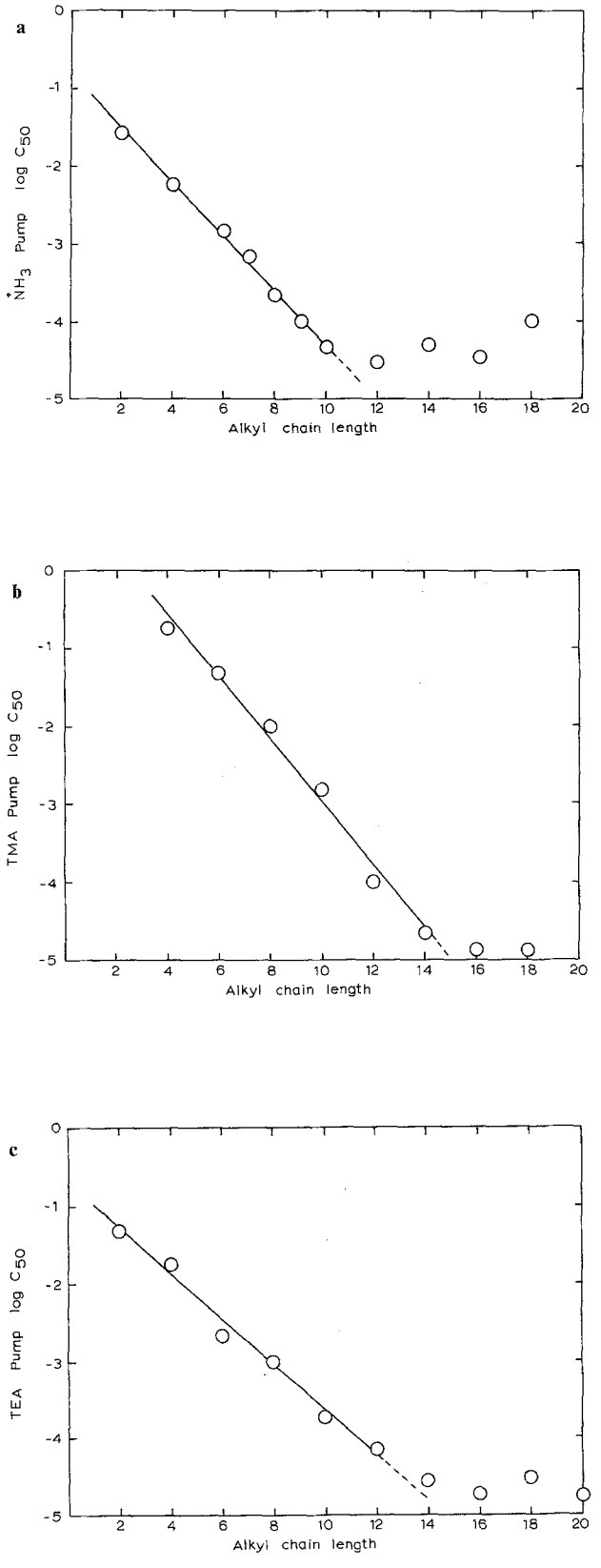


**Fig. 2.** Logarithm of the 50% inhibitory concentration for the sodium pump *vs.* carbon atoms in the alkyl chain for (*a*) *n*-alkyl ammonium bromides, (*b*) *n*-alkyl trimethylammonium bromides, and (*c*) *n*-alkyl triethylammonium bromides.

**Table 1.** Inhibition of ouabain-sensitive K influx by isomeric butylammonium bromides

| | 50% inhibitory conc. (mM) |
|---|---|
| *n*-butylammonium bromide | 6.0 |
| *iso*-butylammonium bromide | 8.8 |
| *sec*-butylammonium bromide | 20.7 |
| *tert*-butylammonium bromide | 49 |

effective and show a negative slope comparable to the TMA compounds for the initial part of the curve. The TEA series, although acting almost as effectively as the $N\overset{+}{H}_3$ compounds at $C_2$, showed a shallower negative slope, with the $C_{10}$ compound being markedly less inhibitory than $C_{10}N\overset{+}{H}_3$. Finally, for the TMA derivatives the slope of the initial component was almost comparable with the $RN\overset{+}{H}_3$ series, although at every concentration these derivatives are less effective inhibitors than the alkylammonium compounds.

### *Butyl Ammonium Bromide Isomers*

The four isomeric members of the series $C_4H_9N\overset{+}{H}_3Br$ were tested for their inhibitory potency on ouabain-sensitive $K^+$ influx in red cells. The results are summarized in Table 1. It can be seen that the straight-chain *n*-butylammonium cation is the most effective inhibitor, with increased branching leading to a decrease in inhibitory potency. The data are also compared graphically with the relative retention times for the butanol series by GLC on squalene or dodecanol (Fig. 3*a*). There is a correlation between the relative retention time and effective inhibition by these compounds such that the straight-chain butylammonium bromide, which is retained for the longest period on the GLC column, is also the most effective inhibitor of membrane transport.

## Discussion

Both the hemolysis data and the results for inhibition of the sodium pump by the various alkyl ammonium compounds may be analyzed thermodynamically. In our analysis we have made the tacit assumptions that the charged headgroup is located in the predominantly hydrophilic interfacial region, and that the alkyl chain extends from this region into the hydrophobic core of the membrane.

Following the solvophobic theory of solute-solvent interactions as advanced by Sinanoglu (1968)

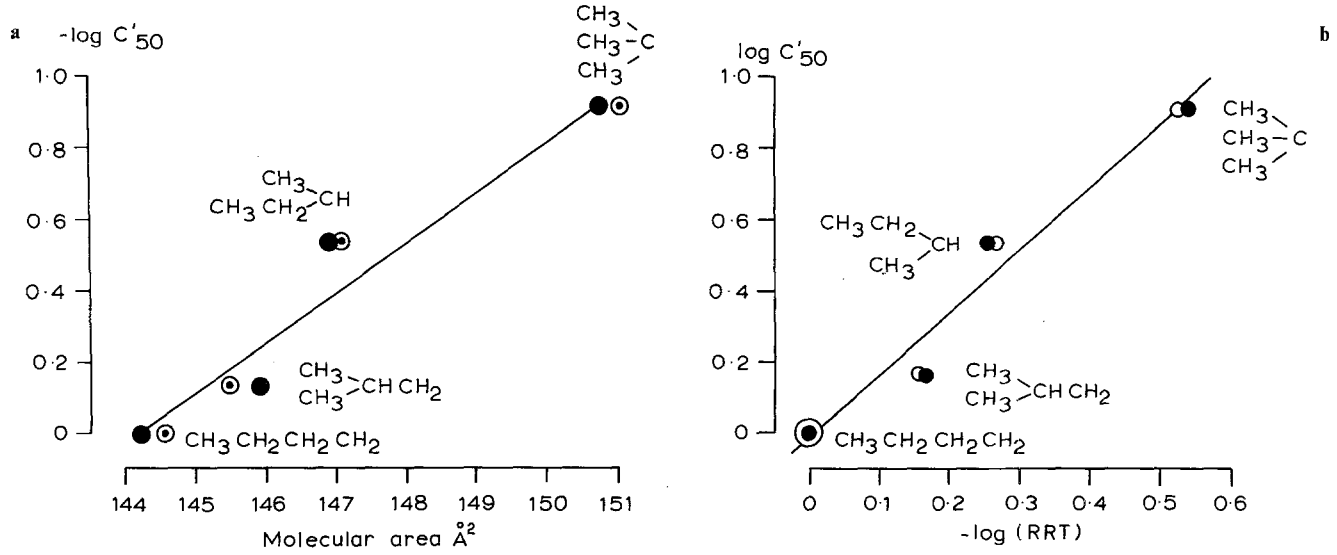


**Fig. 3.** (**a**): Estimate of alkyl chain dimension perpendicular to interface *vs.* the logarithm of the relative inhibitory concentration: n.b. where two conformational extremes are possible, both these are shown (**b**): Logarithm of the relative retention time for the GLC of isomeric butanols on dodecan-l-ol (○) or squalene (●) at 80 °C *vs.* the logarithm of the relative inhibitory concentration.

and extended recently by Horvath and coworkers to a consideration of liquid chromatographic systems (Sinanoglu & Abdulnur, 1965; Halicioglu & Sinanoglu, 1969; Horvath, Melander & Molnar, 1977; Horvath & Melander, 1978), the free energy of interaction for a solute in solvent I can be written

$$\Delta F^I = \Delta F^I_{CH_2} + \Delta F^I_{\text{headgroup}} = \Delta F^I_{\text{cav}} + \Delta F^I_{\text{vdw}} + \Delta F^I_{\text{es}} + \Delta F^I_{\text{solv}} + \Delta F^I_{\text{conf}} \quad (1)$$

where the terms $\Delta F_{CH_2}$ and $\Delta F_{\text{headgroup}}$ indicate the free energy of interaction for the alkyl chain methylene groups and for the charged headgroup, respectively. $\Delta F_{\text{cav}}$, $\Delta F_{\text{vdw}}$, $\Delta F_{\text{es}}$, and $\Delta F_{\text{solv}}$ indicate the free energies of cavity formation, Van der Waals, electrostatic, and solvation interactions. $\Delta F_{\text{conf}}$ allows for conformational free-energy changes due to rotational isomerism of the alkyl chain. This expression (1) may be generalized to include the case of the transfer of a solute species from solvent I to solvent II, as arises in the transfer of a molecule from aqueous medium to the membrane or *vice versa*. Sinanoglu (1968) gives an approximate expression for the free energy of association in a solvent which ignores conformational effects

$$\Delta F_o = a - b\Delta\left(\frac{\mu_i^2}{v}\right) + c\Delta(v_i^{2/3})\gamma_i \quad (2)$$

where the change in the inverse effective volume or solvation term, $\Delta\left(\frac{\mu_i^2}{v}\right)$, and in the effective surface area or cavity term, $\Delta(v_i^{2/3})\gamma_i$ are the solvophobic forces driving the equilibrium. For small groups solvation forces may predominate, but with larger groups the cavity term usually assumes greater significance. Horvath and Melander (1978) have indicated that the cavitation term may be the predominant factor affecting separations of hydrophobic materials by chromatography.

The linear relationship commonly observed between alkyl chain length and the logarithm of the equilibrium constant in chromatographic and other partition processes arises because of the often extremely good linear dependence of the incremental surface energy term, $\Delta(v_i^{2/3})\gamma_i$, on alkyl chain length. Average molecular surface area has been calculated for the homologous *n*-alkylamines considered either as spheres or as cylinders, since this represents the extreme conformations possible. Calculations were made using literature values for the density of the liquid, suggested as a reasonable approximation by Moelwyn-Hughes (1947), and from molecular models using a value for the cross-sectional area of an extended hydrocarbon chain of 20.5–20.7 Å$^2$ (Glasstone, 1960; Davies & Rideal, 1961; Adam, 1941). Regression analysis of these calculated areas (*see* Fig. 4) indicated the constancy of the area increase per methylene group: 14.3 Å$^2$ per $CH_2$ as a sphere and 27.6 Å$^2$ as a cylinder.

Although solvophobic theory is useful in highlighting qualitative features of solute behavior (Horvath & Melander, 1977), great care must be exercised in attributing physical meaning to macroscopic concepts such as average molecular area and the formation of molecular cavities. Attempts to calculate the quantitative effect of microscopic or molecular

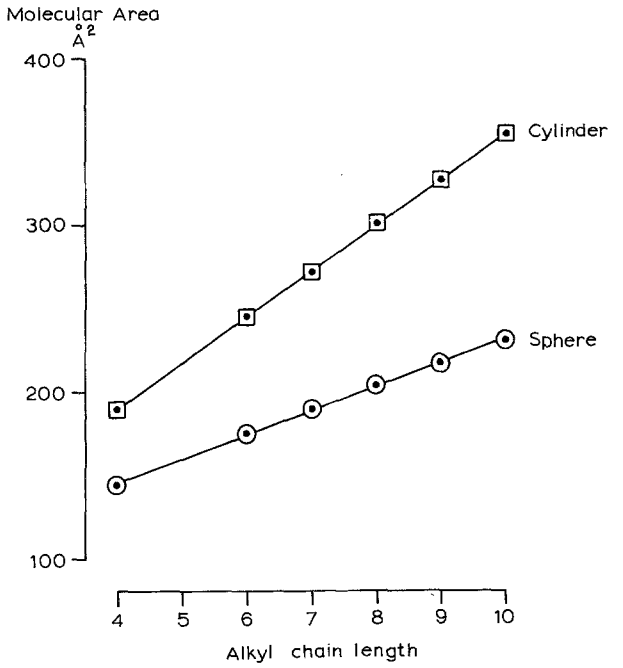


**Fig. 4.** Molecular surface area for the homologous *n*-alkylamines estimated from molecular models and density data as either spheres (⊙) or cylinders (⊡).

forces using macroscopic values of parameters such as surface tension or molecular area usually need correction factors for which there is little physical basis. However, as we shall show later, it is possible to use experimentally determined values of the free-energy change per methylene group in conjunction with a conformational free-energy term obtained using statistical mechanics to analyze rotational isomerism in the alkyl chains.

The data for hemolysis and for inhibition of the sodium pump both show a linear dependence of the 50 % effective concentration upon chain length below C12±2. Above this region the curves become more or less horizontal and, as shown in a previous paper (Ellory et al., 1979), the kinetics of inhibition change. If one considers only that portion of the curve below C12, one obtains the following free-energy terms for hemolysis by alkylammonium, trimethylammonium and triethylammonium bromides.

The thermodynamic parameters for hemolysis by the three different homologous series shown in Table 2 are probably not significantly different given experimental error. We chose hemolysis as a measure of the nonspecific hydrophobic interaction of our derivatives with the lipid of the erythrocyte membrane, and the experimental results support the hypothesis that this interaction is relatively nonspecific. On the other hand, similar thermodynamic parameters for inhibition of the sodium pump show very great differences between the homologous series, suggesting

**Table 2.** Free energies of hemolysis

| | $F_{CH_2}$ | $F_{headgroup}$ |
|---|---|---|
| Ammonium | −2.98±0.20 kJ | 12.86±0.78 kJ |
| Trimethylammonium | −2.72±0.14 kJ | 14.52±0.64 kJ |
| Triethylammonium | −2.81±0.28 kJ | 14.82±1.24 kJ |

**Table 3.** Free energies of $K^+$-inhibition

| | $F_{CH_2}$ | $F_{headgroup}$ |
|---|---|---|
| Ammonium | −2.09±0.07 kJ | −4.80±0.51 kJ |
| Trimethylammonium | −2.39±0.13 kJ | +6.10±1.09 kJ |
| Triethylammonium | −1.67±0.08 kJ | −4.71±0.86 kJ |

a more specific effect (Table 3). It is also worth noting that inhibition of the sodium pump occurs at much lower concentrations than hemolysis (approximately 3- to 50-fold difference in concentration depending upon the derivative - *see* Figs. 1 and 2), again indicating a more specific effect for inhibition of the sodium pump.

Two features of the results in Table 3 distinguish them from those for hemolysis (Table 2). The first is that the free energy of the headgroup interaction separates the trimethylammonium derivatives from the ammonium and triethylammonium compounds, being of opposite sign. If the free energy of headgroup interaction depended on size, one might expect the order to be

$$\overset{+}{N}H_3 : \overset{+}{N}(CH_3)_3 : \overset{+}{N}(C_2H_5)_3.$$

On the other hand, a plausible explanation of the results might be that solvation renders the ammonium group more of an equal size with the triethylammonium group. The second feature which Table 3 highlights is that the slope or free-energy change per methylene group is significantly different for the three series of compounds. Moreover, these values for $\Delta F_{CH_2}$ are all much lower than the equivalent values for hemolysis.

In view of the implications of solvophobic theory as advanced by Sinanoglu and certain conformational terms to be discussed later, it seems to us both unreasonable and simplistic to attribute the value of this free-energy change per $CH_2$ group solely to the hydrophobicity of the membrane as has been done recently by Sallee (1978). This term has little quantitative meaning, and it has been argued that its use may lead to a misunderstanding of the effects involved (Hildebrand, 1979) and the basis for the interaction between hydrocarbons and water (Tanford, 1979).

The driving force for a molecule moving from the aqueous phase to the membrane hydrocarbon phase includes a cavity surface free-energy term and conformational terms. The change in the cavity term on passing from water to the membrane has been given by Horvath et al. (1977) as

$$\Delta F_{cav}^{CH_2} = N((k_j^{e'} A_j^{H_2O} \gamma^{H_2O}(1-W_j')) - (k_j^{e''} A_j^{memb} \gamma^{memb}(1-W_j'))) \quad (3)$$

where $N$ is Avogadro's number, $A_j$ the molecular surface area of the solute, $\gamma$ the solvent surface tension, and $W_j$ and $k_j^e$ correction terms accounting for enthalpic and entropic contributions to changing from macroscopic to microscopic dimensions. As indicated earlier, there are difficulties in applying macroscopic concepts such as interfacial surface area or surface tension to individual molecules without the use of unacceptable correction factors (Tanford, 1979).

Rather than using doubtful macroscopic numerical values for the parameters in this equation (3), it would seem more appropriate to utilize experimentally determined values of $\Delta F_{CH_2}$ for long-chain fatty acids, alcohols, or alkanes derived from data for their solubility in water. Such values take account experimentally of both the cavity surface free energy and Van der Waals free-energy terms, but do not allow for ordering of the alkyl chains as is known to occur in biological membranes. Values for *n*-alkanes, fatty acids, and alcohols (in kJ) are available in Tanford (1973): *n*-alkanes, $-3.70\,kJ/CH_2$; fatty acids, $-3.45\,kJ/CH_2$; alcohols, $-3.44\,kJ/CH_2$.

The situation in which the substituted alkylammonium compounds partition between the aqueous environment and the membrane lipid is, however, significantly different in one respect. Compared to liquid hydrocarbon, the membrane alkyl chains are considerably ordered particularly in the presence of high cholesterol concentrations as found in erythrocyte membranes (Marsh & Smith, 1973; Shimsick & McConnell, 1973; Nelson, 1967*a*, *b*). Because of this, there will be a significant conformational free-energy term related to the probability of *trans* to *gauche* interconversions in the alkyl chain as solute molecules pass from one environment to the other. One can assume that the molecules in aqueous solution are monomers with their alkyl chains in considerable rotational disorder – the concentrations involved are well below the critical micelle concentrations (Tanford, 1973). On passage into the relatively ordered hydrocarbon core, the alkyl chain will extend to give a cylinder; as indicated in Fig. 4 this would be expected to involve a considerable change in contact surface area. To calculate the unkinking energy of the alkyl chains, we make use of the treatment of Flory (1969) for polymethylene chains, to whom reference should be made for a more detailed account.

The energy of a *gauche* rotational state relative to the *trans* state is defined as $E_g$ with a value of $500\,cal\,mole^{-1}$ (2.09 kJ/mole) deduced from Raman spectra and entropic analysis of *n*-alkanes (Mizushima & Okazaki, 1949; Sheppard & Szasz, 1949; Person & Pimentel, 1953). The energy for a $g^{\pm}g^{\mp}$ pair in excess of the calculated energy for a $g^{\pm}g^{\pm}$ pair, which approximates the sum of the energies for the two first order interactions, is defined as $E_w$ and has a value of approximately $2200\,cal\,mole^{-1}$ (9.21 kJ/mole) (Abe, Jernigan & Flory, 1966). The statistical weights are then given by

$$\sigma = \exp(-E_g/RT) = 0.4441 \quad (4)$$

$$\omega = \exp(-E_w/RT) = 0.0281 \quad (5)$$

both at 37 °C.

For long chains the configurational partition function

$$Z \cong (\lambda_1^{(2)})^{n/2} \quad (6)$$

where $\lambda_1^{(2)}$ is the largest eigenvalue of the statistical weight matrix $U^{(2)}$ for a chain of $n$ bonds

$$\lambda_{1,2}^{(2)} = \tfrac{1}{2}\{(1+4\sigma+\sigma^2\alpha\beta) \pm \sqrt{((1-\sigma^2\alpha\beta^2)+8\sigma(1+\sigma\alpha)(1+\sigma\beta))}\} \quad (7)$$

where

$$\alpha = \psi_a + \omega_a \qquad \beta = \psi_b + \omega_b \quad (8)$$

with $\psi_a \cong \psi_b \cong 1$ and $\omega_b \cong 0$, giving

$$\lambda_1^{(2)} = \tfrac{1}{2}(2.97915 + \sqrt{8.10851}) = 2.91335$$

therefore

$$Z = (2.91335)^{n/2}. \quad (9)$$

The free energy difference between the alkyl chains in the substantially ordered bilayer and the relatively rotationally disordered situation in the aqueous phase is

$$Z_{bilayer} = 1 \qquad \Delta F = -RT\ln(Z_{bilayer}/Z_{H_2O}) = +RT\,n/2\ln(2.91335) = 329.3\,cal\,mole^{-1} = 1.379\,kJ\,mole^{-1}. \quad (10)$$

This free-energy term of 1.38 kJ (mole $CH_2$ group)$^{-1}$ for the kinking energy of the alkyl chains reduces the

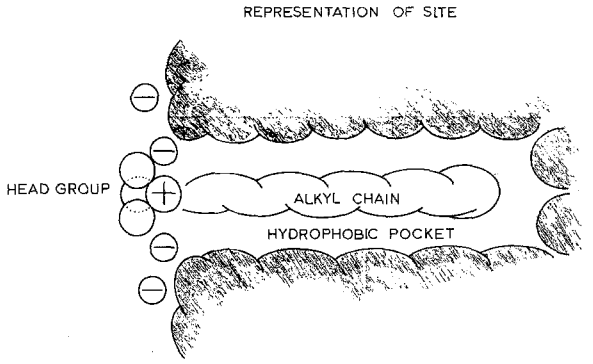


**Fig. 5.** Representation of the binding site for long-chain ammonium compounds in the vicinity of the sodium pump.

expected slope of the $K^+$-inhibition curves (Table 3) to approximately 2.07 kJ (mole $CH_2$ group)$^{-1}$, which agrees well with the experimental values. There still remains, however, the need to explain the differences in slope for the three series of compounds with a range of 0.72 kJ (mole $CH_2$ group)$^{-1}$. A possible explanation of these differences involves small changes in the separation of the alkyl chains of the ammonium derivatives and those of the membrane lipids, with a change in the London-Van der Waals dispersion forces. Such small changes in near-neighbor separation of the alkyl chains could also affect the degree of unkinking necessary for chain insertion into the bilayer and be determined by the headgroup. London-Van der Waals dispersion energies are determined by a $r^{-5}$ dependence (Salem, 1962), and, assuming an alkyl chain separation of approximately 5 Å, differences of the order of $\pm 5\%$ would be sufficient to account for the differences of slope observed experimentally. One can conceive of such small changes in the hydrophobic packing of the binding site occuring with changes in the headgroup if the inhibitor interacts with both an anionic site and a hydrophobic pocket as indicated diagrammatically in Fig. 5 [*compare* the binding of fatty acids by serum albumin (Spector, 1975)].

The importance of the surface free-energy term in determining the effectiveness of inhibitors of the sodium pump is further illustrated for the isomeric butylammonium cations (Fig. 3a). Molecular areas and hence the $\Delta F_{cav}$ term are also seen to be related to the gas chromatographic retention times, taken from Littlewood (1970), for the isomeric butanols (Fig. 3*b*) for similar reasons.

The effect of temperature on hemolysis and inhibition of the sodium pump were quite different. Hemolysis by *n*-alkylammonium bromides showed no temperature dependence between 20 and 37 °C for chain lengths of seven to twelve carbon atoms. The totally entropic nature of hemolysis within this range is typical of many partition-based phenomena (Klein, 1979). Above $n_c = 12$ a complex effect was observed in which the relationship between $\log c_{50}$ and chain length showed a minimum, with longer chain lengths becoming less effective as the temperature was decreased.

Inhibition of the $Na^+$-pump by tetradecylammonium bromide did not change significantly between 20 and 37 °C. In contrast the *n*-heptyl derivative was markedly less inhibitory at 20 than at 37 °C.

We admit to finding it difficult to explain satisfactorily the flattening off in the relationships shown in Figs. 1*a–c* and 2*a–c*, for chain lengths greater than C12–14. One possible explanation is that there is a physical limit to the length of alkyl chain, in the *trans* conformation, that could be accommodated within the ordered structure of the erythrocyte membrane without disrupting it. The kinking of very long alkyl chains ($t \rightarrow g$) which would be necessary, would impose a thermodynamic disadvantage which could offset the advantage of increasing chain length observed below $n_c = 12$. This limit could be expected to lie between chain lengths of 14 to 18 carbon atoms, i.e., half the bilayer thickness. Chain lengths greater than the limit might be expected to show a relative or absolute reduction of inhibitory potency based on this hypothesis. Unfortunately, derivatives with very long alkyl chains (greater than C20) were not available.

Some form of nonspecific uptake or binding to the erythrocyte membrane should be considered as another possible reason for this phenomenon. Although we have demonstrated binding/uptake of alkylammonium derivatives by red cells, this seems rather unlikely as an explanation for the abrupt change in slope since the concentration at which this occurs differs for hemolysis ($10^{-4}$ M) and inhibition of the sodium pump ($1\text{–}3 \times 10^{-5}$ M), suggesting a more specific effect.

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