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INTERACTIONS OF SURFACE-ACTIVE ALKYLTRIMETHYLAMMONIUM SALTS WITH THE ERYTHROCYTE MEMBRANE

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Abstract—The interactions of a homologue series of surface-active alkyltrimethylammonium salts (C_{10}–C_{20}) with the rat erythrocyte membrane were studied. The surfactants were found to have a biphasic effect on the erythrocyte membrane. At low concentrations they protected or stabilized erythrocytes against hypotonic haemolysis, but at higher concentrations they caused rapid haemolysis. The stabilizing and lytic effect increased with an increase in length of the alkyl chain to maximum activity at about C_{16}. It is suggested that laminar–micellar transitions in the lipid bilayer of the membrane are responsible for the lytic activity of the surfactants. Micellar regions in the lipid bilayer abolish the ability of the membrane to prevent the free exchange of ions, and haemolysis of the cell results from a secondary osmotic effect. The stabilizing effect, on the other hand, is proposed to stem from an expansion of the membrane caused by a fluidizing effect of the surfactants on the lipid bilayer. Binding studies with the C_{16} homologue revealed that at a concentration causing 50 per cent haemolysis in an isotonic solution there are about 780,000 molecules bound per μm^2 of the erythrocyte membrane. At a concentration giving 50 per cent protection against hypotonic haemolysis, the number of molecules bound per μm^2 of the erythrocyte membrane was estimated to be 190,000.

Cationic surfactants are well known for their lytic properties. There are many studies indicating that the principal point of attack of cationic surfactants is the lipid bilayer of the cell membrane [1–4], and in a previous study [5] it was found that there was no significant binding of cetyltrimethylammonium bromide (CTAB) to the membrane proteins of erythrocytes at lytic concentrations. Despite many studies on the lytic activity of cationic surfactants, there is still much uncertainty regarding the mechanism whereby cationic surfactants alter the permeability properties of the cell membrane. Pethica and Schulman [6] stated that ionic surfactants, by lowering the interfacial tension, cause a collapse of a cholesterol–phospholipid–protein complex in the membrane. It was suggested that a critical collapse pressure (34 dynes/cm) exists for the membrane of erythrocytes. In a theoretical examination of the physical properties of the lipid bilayer of the cell membrane, Haydon and Taylor [7] took the view that ionic surfactants, when adsorbed into the bimolecular leaflet, will tend to break up the leaflet into discrete micelles. Kondo and Tomizawa [8] on the other hand suggested that surface-active cations interact electrostatically with phospholipid anions to liberate them into the surrounding medium. The removal of phospholipids should, according to the authors, cause an alteration in the conformation of the proteins in the membrane which in turn gives rise to haemolysis. The present paper is an attempt to examine the interactions of surface-active alkyltrimethylammonium salts with the erythrocyte membrane and to elucidate the mechanism by which these surface-active agents produce lysis of cells.

MATERIALS AND METHODS

Chemicals. Cetyltrimethylammonium bromide (CTAB, C_{16}), 99 per cent pure, was obtained from E. Merck AG, Darmstadt, Germany. Decyltrimethylammonium bromide (C_{10}), dodecyltrimethylammonium bromide (C_{12}), tetradecyltrimethylammonium bromide (C_{14}) and eicosyltrimethylammonium bromide (C_{20}) were generously supplied by MoDoKemi AB, Stenungsund, Sweden. These compounds had a chemical purity of 94–98 per cent. Trimethyl-[1-^{14}C]cetylammonium bromide ([^{14}C]CTAB) was obtained from the Radiochemical Centre, Amersham, Bucks. The specific activity of the compound was 6.4 mCi/m-mole and the radiochemical purity was 97 per cent. All other reagents used were of analytical grade.

Erythrocytes. Blood was collected from adult male Sprague–Dawley rats. The rats were anaesthetized lightly with ether, the chest was rapidly opened and blood was collected by heart puncture using an EDTA solution as an anticoagulant. The plasma and the buffy coat were removed following centrifuging and the erythrocytes were washed three times in phosphate-buffered saline (PBS: 150 mM NaCl and 10 mM phosphate, pH 7.4) using at least ten vol. of washing solution per vol. of erythrocytes. The erythrocytes were then suspended in PBS to a concentration of 1.6–1.7 $\times$ 10^9 cells per ml. Cell counts were made using a Coulter Counter. All experiments were carried out on the day the blood was collected.

Determination of lytic activity. Aliquots of 1 ml from the erythrocyte suspension were pipetted into glass test-tubes containing 9 ml PBS (warmed to 37°) with various concentrations of the alkyltrimethylammonium surfactants. The contents of the tubes were briefly

mixed with a vortex mixer and the tubes were incubated for 1 hr at 37 ± 0.5° in a shaking thermostat bath. Following incubation the erythrocyte suspensions were centrifuged at 10,000 *g* for 5 min and the haemoglobin and potassium contents of the supernatants were determined. Haemoglobin was determined photometrically as the methaemoglobin–cyanide derivative at 545 nm according to Zade-Oppen [9]. Potassium was determined with an atomic absorption spectrophotometer at 766.5 nm using an air–acetylene flame. The percental haemolysis and the percentage of potassium released were estimated from standard curves prepared by haemolysing a known number of cells in distilled water. The experimental values were corrected for the release of haemoglobin and potassium occurring during incubation in a test-tube without a surfactant.

Protection against hypotonic haemolysis. The ability of the alkyltrimethylammonium salts to protect erythrocytes against hypotonic haemolysis was studied in a solution containing 69 mM NaCl and 4.9 mM phosphate (pH 7.4). This solution gave about 80 per cent haemolysis in a test-tube without surfactant. The experiments were performed as described above.

Measurement of the rate of potassium and haemoglobin release. Erythrocytes were incubated as described above in PBS containing 50 μM CTAB and in PBS containing 50 μM CTAB and 0.3 M sucrose. At certain intervals samples of 0.4 ml were pipetted into ice-chilled tubes and centrifuged in a Beckman Microfuge (about 10,000 *g*). The total centrifuging time was about 50 sec. Aliquots of the supernatants were quickly removed. The haemoglobin and potassium released into the supernatant were determined as described above. The experimental values were corrected for release of haemoglobin and potassium occuring in test-tubes without the surfactant.

Adsorption of [^{14}C]CTAB to erythrocytes. Erythrocytes were incubated as described above in PBS containing 0–50 μM [^{14}C]CTAB–CTAB. The specific activity of the [^{14}C]CTAB–CTAB mixture was 0.35 mCi/m-mole. Following incubation the erythrocyte suspensions were centrifuged in polyethylene tubes at 12,000 *g* for 20 min. Aliquots (0.2–1.0 ml) of the supernatants were taken to determine radioactivity. The samples were counted in a liquid scintillation spectrometer using Aquasol (New England Nuclear Corp., Boston, MA) as the scintillation fluid. The results were corrected for quenching by internal standardization using [^{14}C]toluene (New England Nuclear Corp.) as internal standard.

Treatment of glasswares. Cationic surfactants are readily adsorbed onto glass because glass can be negatively charged in water. The adsorbed surfactants do not readily desorb off the glass surface. This adsorption is troublesome when working with weak solutions of cationic surfactants because a considerable amount of the surfactant molecules can adsorb to the glass surface. Attempts were made to overcome this problem by soaking the glasswares prior to every experiment in weak solutions of the surfactants and then rinsing them several times with distilled water. This treatment was supposed to leave the glass surface covered by a monolayer of surfactant molecules. The polyethylene tubes used in determining the adsorption of [^{14}C]CTAB to the erythrocyte membrane were treated in a similar way.

RESULTS

Lytic activity of the alkyltrimethylammonium salts. The haemolytic activity of the surfactants is shown in Fig. 1, where percental haemolysis is plotted against

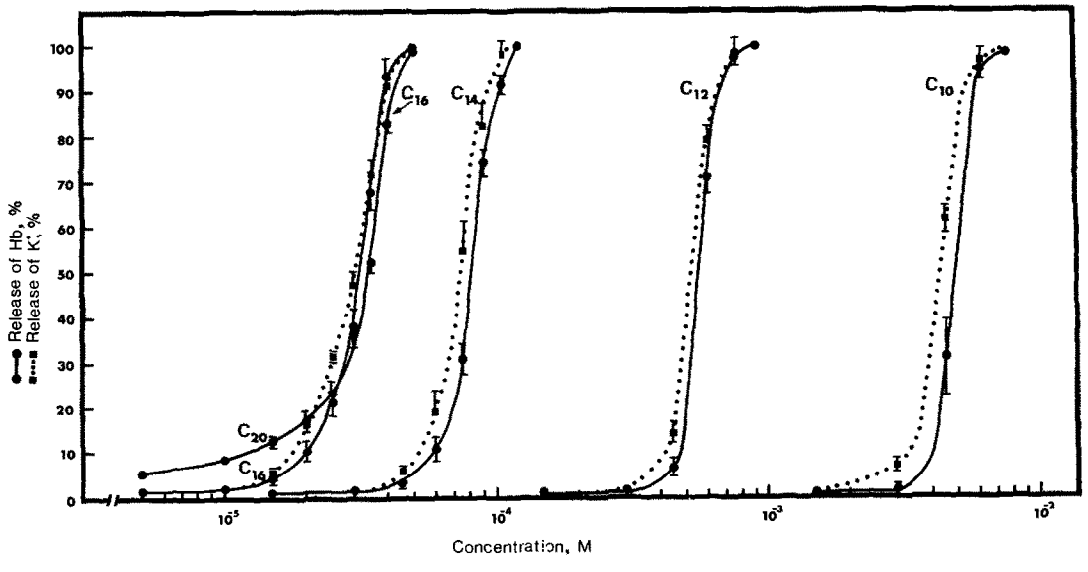

Fig. 1. The lytic activity of alkyltrimethylammonium salts in rat erythrocytes. C_{10}, decyltrimethylammonium bromide; C_{12}, dodecyltrimethylammonium bromide; C_{14}, tetradecyltrimethylammonium bromide; C_{16}, cetyltrimethylammonium bromide; C_{20}, eicosyltrimethylammonium bromide. Release of Hb, ●——●. Release of K^+, ■ · · · · ■. The release of K^+ caused by the C_{20} homologue has for sake of clarity been omitted from the figure. Erythrocyte concentration was 1.6–1.7 × 10^8 cells per ml and incubation temperature 37 ± 0.5°. Each point represents the mean of 3–6 separate experiments. Vertical bars indicate S.E.M.

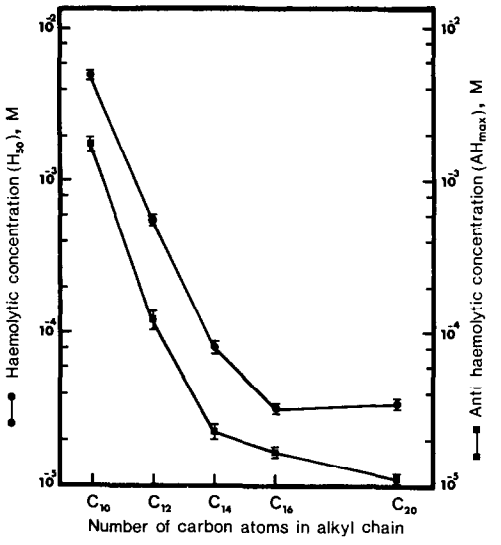

Fig. 2. The haemolytic and stabilizing effects of alkyltrimethylammonium salts on rat erythrocytes. C_{10}, decyltrimethylammonium bromide; C_{12}, dodecyltrimethylammonium bromide; C_{14}, tetradecyltrimethylammonium bromide; C_{16}, cetyltrimethylammonium bromide; C_{20}, eicosyltrimethylammonium bromide. Concentration giving 50 per cent haemolysis (H_{50}) in isotonic PBS, ●——●. Concentration giving maximum protection (AH_{max}) against haemolysis in a hypotonic solution (69 mM NaCl and 4.9 mM phosphate), ■——■. Erythrocyte concentration was 1.6–1.7 × 10^8 cells per ml and incubation temperature 37 ± 0.5°. Each point represents the mean of 3–6 separate experiments. Vertical bars indicate S.E.M.

the concentration of the surfactants. The curves are sigmoidal with almost identical slopes, although there is a trend toward a steeper initial region of the curve with a decrease in the length of the alkyl chain. Microscopic examinations of erythrocytes incubated with surfactants revealed that the liberation of haemoglobin is a all-or-none phenomenon. Cells which had not undergone haemolysis were cup-shaped or had the shape of invaginated spheres. Haemolytic activity increased with an increase in the length of the alkyl chain to an optimum activity at C_{16}. In Figure 2 the concentration giving 50 per cent haemolysis is plotted against the length of the alkyl chain. As can be seen from the figure there is a progressive bending in the activity vs chain length profile with an increase in chain length. For each surfactant the release of potassium preceded the haemoglobin liberation, over the whole range from 0 to 100 per cent haemolysis (Fig. 1).

Stabilization against hypotonic haemolysis. The alkyltrimethylammonium salts exhibited an ability to stabilize erythrocytes against hypotonic haemolysis (Fig. 3). The C_{20} homologue showed a very poor stabilizing effect but the other homologues reduced the degree of haemolysis to about half of that in the control. The concentration required for maximum protection decreased with the length of the alkyl chain in a way parallel to the change in the lytic activity of the compounds (Fig. 2). Stabilization occurred at concentrations very near those causing haemolysis in an isotonic solution, maximal stabilization occurring at a concentration only about one half to one quarter of that causing 50 per cent haemolysis.

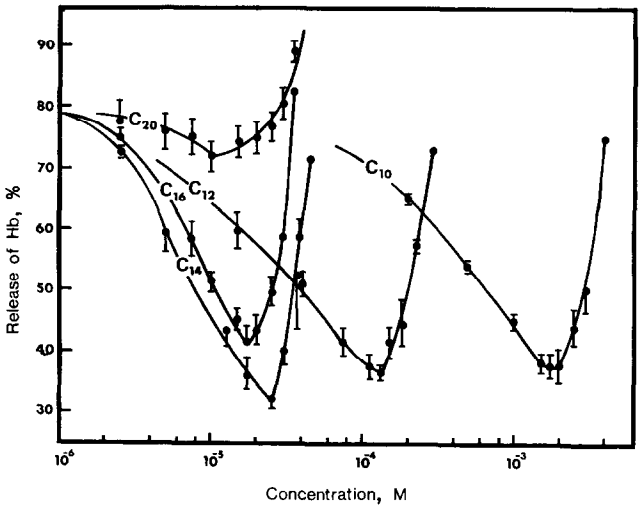

Fig. 3. The stabilizing effects of alkyltrimethylammonium salts against haemolysis in a hypotonic solution (69 mM NaCl and 4.9 mM phosphate). C_{10}, decyltrimethylammonium bromide; C_{12}, dodecyltrimethylammonium bromide; C_{14}, tetradecyltrimethylammonium bromide; C_{16}, cetyltrimethylammonium bromide; C_{20}, eicosyltrimethylammonium bromide. Erythrocyte concentration was 1.6–1.7 × 10^8 cells per ml and incubation temperature 37 ± 0.5°. Each point represents the mean of 3–5 separate experiments. Vertical bars indicate S.E.M.

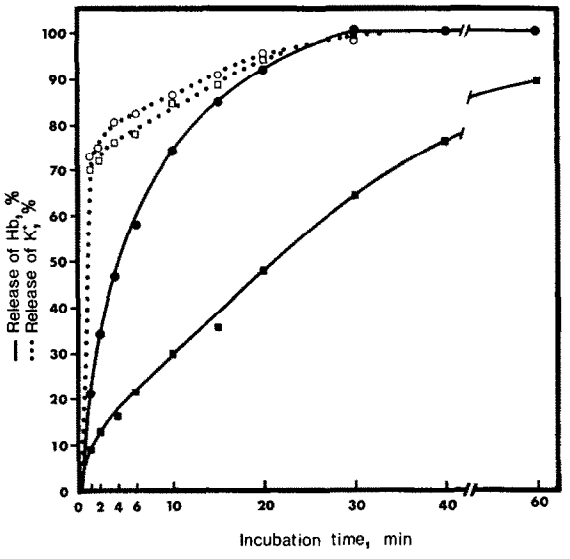

Fig. 4. The rate of Hb and K^+ release from rat erythrocytes incubated in PBS containing 50 μmoles/l of CTAB. Release of Hb, ●——●. Release of Hb in the presence of 0.3 M sucrose, ■——■. Release of K+, ○ · · · · ○. Release of K^+ in the presence of 0.3 M sucrose, □ · · · · · □. Erythrocyte concentration was 1.6–1.7 × 10^8 cells per ml and incubation temperature 37 ± 0.5°. Each point represents the mean of two separate experiments.

Effect of sucrose on release of K^+ *and Hb.* The rate of release of potassium and haemoglobin was studied with the C_{16} homologue (CTAB). At a concentration of 50 μM this homologue caused a rapid release of potassium and haemoglobin. About 80 per cent of the potassium and about 60 per cent of the haemoglobin was released within 6 min of incubation (Fig. 4). Sucrose (0.3 M) produced a marked inhibition on the release of haemoglobin. No essential alteration in the release of potassium was noted (Fig. 4). These findings, together with the fact that potassium release precedes haemoglobin liberation, indicate that the haemolysis produced by the surfactant involves a "colloid osmotic" mechanism [10].

The adsorption of [^{14}C]*CTAB to erythrocytes.* The adsorption isotherm for [^{14}C]CTAB is presented in Fig. 5. The isotherm corresponds to the L2 type in the classification system of Giles *et al.* [11] and is characterized by a progressive decrease in the proportion adsorbed with an increase in the added amount of CTAB. A Scatchard plot of the binding data is shown in the inset of Fig. 5. The straight line obtained is consistent with a single set of adsorption sites of uniform affinity. From the Scatchard plot the number of "binding sites" per erythrocyte was estimated to be 1.7 × 10^8. The number of CTAB molecules adsorbed per erythrocyte at a concentration corresponding to 50 per cent haemolysis was determined as 9.4 × 10^7 from the adsorption data. This is equivalent to about 780,000 molecules per μm^2, assuming the mean area of rat erythrocytes to be 121 μm^2 [12]. Assuming the fraction of CTAB bound under hypotonic conditions to be the same as under isotonic conditions, the number of molecules bound per μm^2 at a concentration giving 50 per cent protection against hypotonic haemolysis (AH_{50}) is about 190,000. This number is quite close to that (250,000) calculated by Seeman [13] for various lipid-soluble anaesthetics and tranquilizers at AH_{50}.

In some experiments the erythrocytes were washed several times with PBS following incubation with [^{14}C]CTAB. This washing with buffer removed only small amounts of radioactivity, showing that CTAB does not readily desorb off the erythrocyte membrane.

DISCUSSION

The surface-active alkyltrimethylammonium salts studied were found to have a biphasic effect on the erythrocyte membrane. Such a biphasic effect of stabilization and lysis is a common effect of many lipid-soluble anaesthetics [13]. The lytic activity of surface-active agents has been attributed by many authors to their ability to penetrate into the lipid bilayer of the membrane [3, 4, 6, 7]. In a previous work [5] it was found that there were no significant interactions between CTAB and membrane proteins at lytic concentrations. The increase in lytic effectiveness and in stabilizing effect with increased length of the alkyl chain, established in the present work, clearly demonstrates the hydrophobic nature of the interaction between the alkyltrimethylammonium surfactants and the erythrocyte membrane.

It is generally assumed that equivalent biological responses mean equivalent numbers of molecules at the site of action for each member of a congeneric set. For several lipid-soluble anaesthetics it has been shown that the concentration of anaesthetics in the membrane phase at equinarcotic effects is about the same [13–15]. The increase in lytic effectiveness and in stabilizing ability with increasing length of the alkyl chain thus solely reflects the increase in the membrane/buffer partition coefficient with an increased chain length. At equivalent biological response (H_{50} or AH_{50}) there should be about the same concentration of the alkyltri-

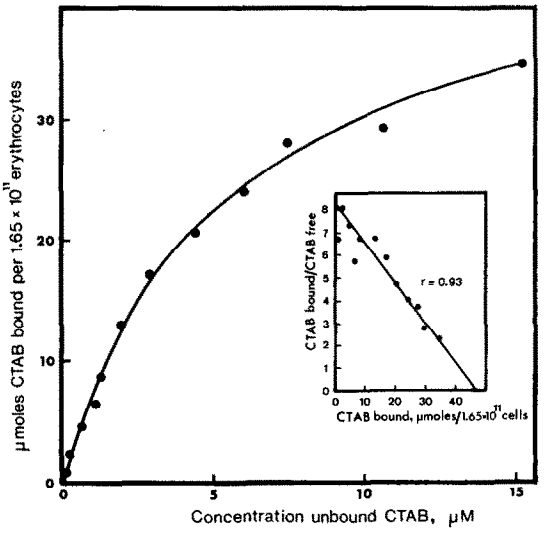

Fig. 5. The adsorption of CTAB (C_{16}) to rat erythrocytes at 37°. The inset shows a Scatchard plot of the binding data. Erythrocyte concentration was 1.65 × 10^8 cells per ml. Each point represents the mean of four separate experiments.

methylammonium surfactants in the membrane. The number of CTAB molecules bound at 50 per cent haemolysis was calculated to be 780,000 per μm^2, which corresponds to about one CTAB molecule per three phospholipid molecules. The number of molecules bound at a concentration giving 50 per cent protection against hypotonic haemolysis was estimated to be 1900,000 per μm^2, which is about the same number as calculated by Seeman [13] for various lipid-soluble anaesthetics and tranquilizers at AH_{50}.

In the present investigation it was found that there is a progressive bending in the activity versus chain length profile with an increase in chain length (Fig. 2). A cut-off in the lytic activity of cationic surfactants at a chain length of about 16 carbon atoms has been reported by several authors [1, 2, 4]. Such a plateau in activity with increasing chain length is generally ascribed to reduced solubility in water or to micelle formation. Micelle formation cannot be the reason for the cut-off in activity of the alkyltrimethylammonium salts. The CMC for CTAB in the buffer solution used should be about 0.1 mM [16], which is far above the concentration of unbound CTAB at 50 per cent haemolysis (about 0.006 mM). If a hydrocarbon/water partition were the only factor governing the adsorption of the surface-active alkyltrimethylammonium salts into the lipid bilayer of the membrane, there should be a linear relationship between the length of the alkyl chain and lytic activity. However, the fact that there is a pronounced orientation into a hydrophobic and hydrophilic region in the bilayer of the membrane makes the bilayer quite different from a non-polar hydrocarbon phase. The hydrophilic region of the bilayer, built up of the polar heads of the amphiphilic lipids, should offer resistance to penetration by molecules having a long hydrocarbon chain. The resistance offered should increase with increasing chain length of the penetrating molecule, thus giving rise to a progressive bending in the activity versus chain length profile with an increase in chain length.

Concerning the mechanism whereby surface-active agents cause lysis of cells, several authors have suggested that surface-active agents can induce the formation of micelles in the lipid bilayer of the membrane [7, 17, 18]. Haydon and Taylor [7] suggested that the adsorption of long-chained ionic surface-active molecules into the lipid bilayer can cause, due to electrostatic repulsion between the polar heads of the surfactants, the bilayer to break up into discrete micelles. The surfactants studied in the present work contain a bulky polar group carrying a net positive charge. The molecules should orientate in the lipid bilayer in such a way that the alkyl chains are situated amongst the hydrocarbon chains and the polar groups at the lipid–water interface. As the adsorption proceeds the repulsive interactions between the positive charges should tend to break up the bilayer according to the mechanism suggested by Haydon and Taylor [7]. Furthermore, the alkyltrimethylammonium surfactants with their charged polar groups do not readily diffuse across the lipid bilayer and should therefore be bound preferentially to the outer half of the bilayer. This should result in an expansion of the outer half relative to the inner half and exert an additional disruptive effect upon the bilayer. In a recent study on the effects of certain compounds upon the endothermic phase transitions in phosphatidylcholine model biomembranes Eliasz *et al.* [19] obtained results consistent with the alkyltrimethylammonium salts (C_8–C_{16}) initially penetrating the bilayer and then subsequently disrupting the bilayer to give mixed micelles. The formation of micellar regions in the lipid bilayer would markedly alter the permeability of the membrane and abolish the ability of the membrane to prevent a free exchange of ions. Although the transmembrane ion concentration gradients are thus dissipated, water will enter the cell because of the higher intracellular concentrations of non-diffusible macromolecules. This kind of process leading to lysis has been called "colloid osmotic swelling" by Wilbrandt [20]. If the osmotic pressure of the external medium is increased by addition of non-penetrating molecules like sucrose and dextran, swelling can be prevented and haemoglobin does not escape from the cell. In the present investigation considerable delay was observed in the escape of haemoglobin from CTAB-treated erythrocytes in presence of 0.3 M sucrose. The presence of sucrose did not significantly alter the release of potassium from the erythrocytes. This clearly indicates that the lytic effects of surface-active alkyltrimethylammonium salts are due to an increased ion permeability and that lysis results from secondary osmotic effects.

The alkyltrimethylammonium surfactants were found to protect, like many lipid-soluble anaesthetics, erythrocytes from hypotonic haemolysis. There are several studies indicating that protection from hypotonic haemolysis is associated with an expansion of the membrane [21–24]. The expansion of the membrane increases the critical haemolytic volume and the cell haemolysis in a solution of lower osmotic pressure. In the case of the surface-active alkyltrimethylammonium salts the concentration required for protection decreased with the length of the alkyl-chain in a way parallel to the change in lytic activity of the compounds. The surfactants altered the shape of the erythrocytes to a cup form or to invaginated spheres, indicating an expansion of the membrane. The expansion of the membrane caused by lipid-soluble anaesthetics is much greater than can be explained by the volume occupied by anaesthetic molecules in the membranes [23]. It has been found in several studies [19, 25–28] that both charged and neutral anaesthetics decrease the transition temperature of phospholipid model membranes and it has been suggested that the expansion of the membrane stems primarily from a fluidizing effect of the anaesthetics on the lipid bilayer [13]. It has also been suggested that the cause of anaesthesia is through an increase in the "fluidity" of the lipid bilayer [13, 25, 28]. For many lipid-soluble anaesthetics it has been shown that the concentration which gives 50 per cent protection against hypotonic haemolysis is about the same as the concentration blocking the nerve impulse of the sciatic nerve in the frog [13]. Surface-active alkyltrimethylammonium salts have been found to lower initially the transition temperature of phospholipid model membranes [19]. It is tempting to attribute the protection from hypotonic haemolysis by the alkyltrimethylammonium salts to an expansion of the membrane caused by an increase in "fluidity" of the lipid bilayer. The surfactants should thus, when they become intercalated in the lipid bilayer, initially increase the "fluidity" of the membrane by lowering the transition temperature of the bilayer. In-

creasing the concentration of the surfactants should, due mainly to electrostatic repulsion, favour a break-up of the bilayer into discrete micelles leading to subsequent lysis of the cell.

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