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Permeability alterations and antihaemolysis induced by amphiphiles in human erythrocytes

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In an attempt to define the parameters in amphiphilic molecules important for their interaction with the erythrocyte membrane, the effects of cationic, anionic, zwitterionic and nonionic amphiphilic agents (C_{10}–C_{16}) on osmotic fragility and transport of potassium and phosphate in human erythrocytes were studied. (1) All the amphiphiles protected the erythrocytes against hypotonic haemolysis. Half-maximum protection occurred at a concentration which was about 15% of that inducing 50% haemolysis. The concentrations of amphiphiles required to induce protection or haemolysis were related to the length of the alkyl chain in a way indicating that a membrane / aqueous phase partition is the mechanism whereby the amphiphile monomers intercalate into the membrane. (2) At antihaemolytic concentrations all the amphiphiles increased potassium efflux and passive potassium influx. The increase in the fluxes was about the same in both directions through the membrane and there were no clear differences in the effects of the different amphiphilic derivatives at equi-protecting concentrations. (3) Active potassium influx was decreased by cationic, zwitterionic and non-ionic amphiphiles. The ability of the amphiphiles to inhibit the influx was not related to the length of the alkyl chain. Anionic amphiphiles had no or only a weak stimulatory effect on the influx. (4) Phosphate efflux was reduced by all the amphiphiles. The inhibitory potency of the different amphiphiles decreased in the following order; anionic > zwitterionic, non-ionic > cationic. Short-chained amphiphiles were more potent inhibitors than long-chained. (5) The possible participation of non-bilayer phases (mixed inverted micelles) in the intercalation of amphiphiles into the membrane is discussed.

Introduction

Agents with an amphiphilic character are known to interact with the erythrocyte membrane in a biphasic way. At low concentrations they protect erythrocytes against hypotonic lysis or mechanically induced lysis, whereas at higher concentrations they induce lysis. This biphasic phenomenon has been observed in a great variety of chemically unrelated amphiphiles, including simple detergent molecules [1–3] and more complex molecules such as phenothiazines [4,5]. The mechanism underlying the antihaemolytic effect of the amphiphiles is not fully understood. It is thought that, by intercalating into the lipid bilayer of the membrane, the amphiphiles expand the membrane, thereby permitting the cell to swell to a greater volume before it lyses [4].

For many amphiphilic local anaesthetics and tranquillizers it has been shown that the concentrations producing nerve blockage correspond to those resulting in half-maximum protection against hypotonic haemolysis [4]. In addition to the well-documented effects of local anaesthetics on membrane permeability and membrane transport, chemically unrelated amphiphiles have been shown to affect, at sublytic or antihaemolytic concentrations, several membrane-associated func-

tions such as endocytosis [6–8], cell spreading [9], cell adhesion [10], membrane protein mobility [11,12], cell agglutination [13], and cell aggregation [14,15].

There are findings in a number of studies indicating that amphiphiles with a distinct hydrophobic and polar part are intercalated in the lipid bilayer of membranes so that the polar group is located at the polar-apolar interface of the membrane and the hydrophobic part in the hydrocarbon region of the bilayer [16–19]. This intercalation is apparently the primary step which triggers alterations in membrane associated functions. Some events, such as protection against hypotonic haemolysis, seem to be a result of a nonspecific interaction between the bilayer and the perturbant, while other functions, such as memrbane ion transport, are presumably more sensitive to particular features of the perturbant. The association between amphiphilic character and ability to affect membrane functions in a wide variety of compounds is hardly merely coincidental. It appears that the membrane-perturbing ability is an intrinsic consequence of the amphiphilic character. This calls attention to the possibility that there is a common site and common consequences of membrane perturbation by amphiphiles. However, the details of the interaction of amphiphiles with membranes are still very unclear. It is not known how and to what extent particular features of the perturbant determine or influence the consequences of perturbation. Our approach to this question was to select chemically simple amphiphiles with a distinct amphiphilic character (surfactants) and to study the effect of these agents on membrane permeability at concentrations where they stabilized erythrocytes against hypotonic haemolysis. By varying the length of the alkyl chain and the nature of the polar head group in these agents we wanted to define the roles of the polar and apolar parts of the perturbant and find out to what extent these two parameters influence the consequences of perturbation.

Materials and Methods

Erythrocytes

Blood was drawn from healthy donors by vein puncture into heparinized tubes. The erythrocytes were washed three times in a medium containing 145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM glucose (pH 7.4). The erythrocytes were then suspended in the medium at a concentration of $(1.6–1.7) \cdot 10^9$ cells/ml and kept cold until they were used. All experiments were carried out within 48 h after the blood was drawn.

Protection against hypotonic haemolysis

These experiments were carried out in the medium diluted to such a tonicity that about 80% of untreated erythrocytes were haemolysed. The erythrocytes were added to the diluted medium containing various concentrations of the amphiphiles. The final erythrocyte concentration was $(1.6–1.7) \cdot 10^8$ cells/ml and incubation was carried out in polyethylene tubes in a shaking thermostat bath at 37°C for 60 min. Following incubation, the tubes were centrifuged and the percentage of haemolysis was determined by comparing the absorbance (545 nm) of appropriate dilutions of the supernatants with that of control samples totally haemolysed by dilution to 20 vol. with distilled water. Six to ten different concentrations of each amphiphile were used in each experiment and the concentrations resulting in maximum protection, 75, 50 and 25% of the maximum protection were estimated from dose-response curves. For every amphiphile four to six dose-response curves were made with blood from two or more different donors.

Haemolysis induced by the amphiphilic agents

Erythrocytes were added to the medium containing various concentrations of the amphiphiles. The erythrocyte concentration was $(1.6–1.7) \cdot 10^8$ cells/ml and incubation was carried out in a shaking thermostat bath at 37°C for 60 min. Following incubation the samples were centrifuged and the percentage of haemolysis was determined as described above. The concentrations resulting in 50% haemolysis were determined from dose-response curves. For every amphiphile, three to six dose-response curves with blood from two or more different donors were made.

Efflux studies

Erythrocytes ($(1.6–1.7) \cdot 10^9$ cells/ml) were in-

cubated for about 2 h in the medium containing $^{32}PO_4^{3-}$ or ^{43}K (2–30 μCi/ml) at 37°C in a shaking thermostat bath. Following incubation the erythrocytes were rapidly washed five times with cold (0°C) medium and the cell concentration was readjusted to $(1.6–1.7) \cdot 10^9$ cells/ml. The efflux studies were started by pipetting 1 vol. of the erythrocyte suspension into 9 vol. medium containing the amphiphiles in concentrations previously shown to give maximum protection against hypotonic haemolysis, and 75, 50 and 25% of maximum protection. The incubations were carried out in Eppendorf or polyethylene tubes at 37°C in a shaking thermostat bath. At suitable intervals the erythrocytes were separated from the medium by centrifugation at $15\,000 \times g$ for 50 s and aliquots of the supernatants were taken for determination of radioactivity. The total content of radioactivity in the erythrocytes was determined by lysing a known amount of washed erythrocytes in distilled water and the results are expressed as the percentage of tracer released. Immediatley before the efflux studies were started, supernatant samples of the washed erythrocyte suspension were collected by centrifugation in order to determine extracellular radioactivity. The extracellular radioactivity was subtracted from the experimental values when calculating the percentage of tracer released.

Radioactivity was measured in a liquid scintillation spectrometer. For every amphiphile four separate experiments were performed with blood from two or more different donors.

K^+-influx studies

K^+ influx was measured in the presence and absence of ouabain ($1 \cdot 10^{-4}$ M) and the samples were run in parallel. Incubation was carried out in Eppendorf tubes in the medium previously described. Ouabain was first added to the samples and then the amphiphiles. The time lapse between additions was 2 min and the samples were agitated following the additions. The amphiphiles were used in concentrations corresponding to maximum protection against hypotonic haemolysis, and to 75, 50 and 25% of maximum protection. 2 min after the addition of the amphiphiles $^{43}K^+$ was added (about 10 μCi/ml). The final erythrocyte concentration was $(1.6–1.7) \cdot 10^8$ cells/ml and incubation was carried out in a shaking thermostat bath at 37°C for 30 min. Following incubation 0.3 ml of the samples were gently pipetted into Eppendorf tubes containing 1.0 ml phthalate (di-*n*-butylphthalate/dinonoyl phthalate; 2:1, v/v). The samples were centrifuged at $15\,000 \times g$ for 1 min. Following centrifugation the tubes were frozen (−70°C) and the tips of the tubes, containing the cell pellet, were cut off and placed in scintillation vials. 400 μl of a solubilizer (Luma Solve, Lumac B.V.) was added and the samples were vigorously shaken. Following solubilization of the samples, a toluene-based scintillation fluid was added and radioactivity was measured in a liquid-scintillation spectrometer.

The amount of extracellular radioactivity in the cell pellet due to the trapped medium was measured as follows. A cold (0°C) erythrocyte suspension was added to cold medium containing $^{43}K^+$ in the same concentration as in the influx experiments. Aliquots of this sample were then immediately centrifuged through cold phthalate as described above. The radioactivity of these pellets was considered to represent extracellular radioactivity and it was subtracted from the experimental values. It corresponded to less than 18% of the radioactivity in pellets of control samples after 30 min incubation at 37°C.

The difference between the total K^+-influx (without ouabain) and the influx in the presence of ouabain was taken to represent the active potassium influx (K^+-Na^+-pump activity) and the influx in the presence of ouabain was taken to represent the passive K^+-influx. Four or five separate experiments with blood from three different donors were made with each amphiphile.

Chemicals

Alkyltrimethylammonium bromides (C_{10}, C_{12}, C_{14}, C_{16}) and sodium laurate were purchased from Sigma Chemical Co. Zwittergents (3-(alkyldimethylammonio)-1-propanesulfonates) (C_{10}, C_{12}, C_{14}, C_{16}) and decyl β-D-glucopyranoside were obtained from Calbiochem-Behring, sodium alkyl sulphates (C_{10}, C_{12}, C_{14}, C_{16}) from E. Merck AG., hexadecylpyridinium chloride from FLUKA and dodecylamine hydrochloride from Eastman Kodak Co. All the amphiphilic agents used, as well as all other chemicals, were of reagent grade.

Appropriate stock solutions of the amphiphiles were made by dissolving them in the medium or in the hypotonic medium (haemolysis-protection experiments). Sodium laurate was dissolved in a medium from which Ca^{2+} and Mg^{2+} were omitted (to avoid precipitation of the amphiphile) and in all experiments with sodium laurate Ca^{2+} and Mg^{2+} were omitted from the medium. $^{32}PO_4^{3-}$ with a radiochemical purity of 99% was obtained from New England Nuclear. $^{43}K^+$ (γ, β^-, $t_{1/2}$ 22.4 h), with a radionuclidic purity exceeding 99%, was produced in the Accelerator Laboratory of Åbo Akademi.

Statistical analysis

Treated samples were compared with control samples by using Student's *t*-test for paired cases. Probability values less than 0.05 were taken to indicate statistically significant differences.

Results

Membrane stabilization and haemolysis

All the amphiphiles studied protected the erythrocytes against hypotonic haemolysis. The concentrations resulting in maximum protection (AH_{max}) and in 75, 50 and 25% of maximum protection (AH_{75}, AH_{50} and AH_{25}) are listed in Table I. Half-maximum protection occurred at concentrations which were about 15% of these resulting in 50% haemolysis (H_{50}) in an isotonic medium (Table I). In this respect, the zwittergents differed slightly from the other amphiphiles. They induced half-maximum protection at a signifi-

TABLE I

ANTIHAEMOLYTIC AND HAEMOLYTIC CONCENTRATIONS OF AMPHIPHILES IN HUMAN ERYTHROCYTES

The erythrocyte concentration was $(1.6–1.7)\cdot10^8$ cells/ml and the erythorcytes were incubated with the amphiphiles for 1 h at 37°C. Other experimental details as given in Materials and Methods. The ratio AH_{50}/H_{50} has been calculated only for experiments where AH_{50} and H_{50} were determined in parallel.

Amphiphile	Concentration (μM)					
	AH_{25}	AH_{50}	AH_{75}	AH_{max}	H_{50}	AH_{50}/H_{50}
Sodium alkyl sulphates						
C_{10}	75	153	253	708	1150	0.13
C_{12}	10	20.8	30.5	50	119	0.18
C_{14}	5	8	12	23.3	49.3	0.15
C_{16}	9	18	24	40	70	0.26
Alkyltrimethylammonium bromides						
C_{10}	360	720	1300	3300	7300	0.12
C_{12}	38	76.7	123	300	647	0.12
C_{14}	7	15.3	25.3	40	79.3	0.19
C_{16}	4	7.5	10.2	14.2	40.3	0.19
3-(Alkyldimethylammonio)-1-propanesulfonates (zwittergents)						
C_{10}	700	1430	2180	3630	12100	0.11
C_{12}	50	103	158	263	1040	0.10
C_{14}	4	8	16	27.5	110	0.08
C_{16}	2.5	5	8.6	13.8	41.3	0.12
Decyl β-D-glucopyranoside	85	170	258	740	988	0.16
Sodium laurate	70	135	190	425	760	0.18
Dodecylamine hydrochloride	4	7.5	13.8	26.8	110	0.08
Cetylpyridinium chloride	4	9	17.7	32.7	64	0.16

cantly lower fraction ($X = 10\%$) of the concentration for H_{50} than the other amphiphiles ($X = 16\%$). Dodecylamine hydrochloride and sodium cetyl sulphate also differed from the other amphiphiles. In these cases, however, there were anomalies in the dose-response curves for haemolysis probably due to the occurrence of liquid crystalline phases of the amphiphiles in the incubation medium at lytic concentrations (indicated by a somewhat cloudy solution). For this reason the results obtained with these two amphiphiles are uncertain.

The stabilizing ability of the amphiphiles correlated positively with the size of the hydrophobic moiety, i.e., the length of the alkyl chain. This apparently reflects the increase in the membrane/medium partition coefficient with an increase in the length of the alkyl chain. When the concentrations resulting in AH_{50} for the amphiphiles within the three homologue series (alkyltrimethylammonium bromides, sodium alkyl sulphates and zwittergents) are plotted against the chain length of the homologues, it can be seen that there is an approximately linear relationship up to a chain length of 14 carbon atoms (Fig. 1). Above this chain length the activity versus chain length line levels off. This is a phenomenon frequently observed with aliphatic amphiphiles [20–23] and it seems to indicate complications in the partition of long-chained amphiphiles in the membrane.

The degree of protection induced by the amphiphiles varied. The C_{10}, C_{12} and C_{14} homologoues of the zwittergents (Fig. 2a), decyl β-D-glucopyranoside (Fig. 6) and sodium laurate (not shown) reduced the degree of haemolysis from about 80% in the control to less than 10% at AH_{max}, whereas the degree of protection induced by the alkyltrimethylammonium bromides (Fig. 2b) and sodium alkyl sulphates (not shown) was considerably less (reduction to 30–50%). No explanation for the variation in the degree of protection could be deduced from the molecular configuration of the amphiphiles. It was simply related neither to the length of the alkyl chain nor to the nature of the polar head of the molecules. In case of the zwittergents the degree of protection decreased with the length of the alkyl chain (Fig. 2a), but with the alkyltrimethylammonium bromides (Fig. 2b) and sodium alkyl sulphates no such relationship was seen.

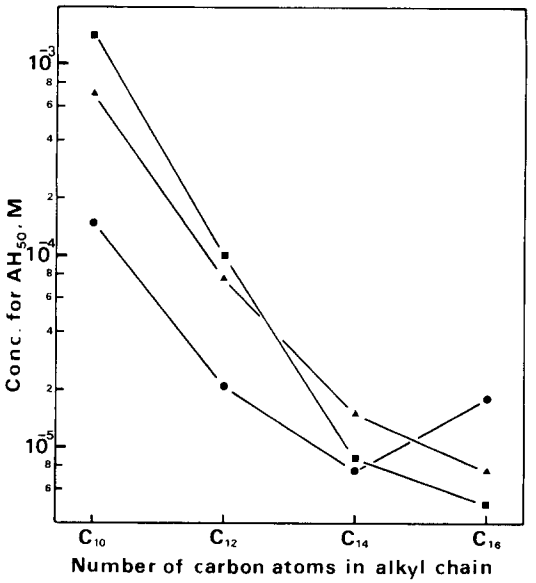

Fig. 1. The concentrations for half-maximal protection against hypotonic haemolysis (AH_{50}) plotted against the length of the alkyl chain of the amphiphiles. (■), zwittergents; (▲), alkyltrimethylammonium bromides; (●), sodium alkyl sulphates. The erythrocyte concentration was $(1.6–1.7)\cdot10^8$ cells/ml and the incubation temperature 37°C. Each point represents the mean of four to six separate experiments.

The dose-response curves for haemolysis showed a characteristic change with the length of the alkyl chain. As the alkyl chain increased, the initial region of the dose-response curves became more and more extended (Fig. 2). This did not alter the concentration range between AH_{50} and H_{50}. The concentration range between AH_{max} or AH_{50} and the onset of haemolysis, however, changed. For agents with an alkyl chain length of 16 carbon atoms, and also with 14 carbon atoms for the sodium alkyl sulphates, the concentration corresponding to AH_{max} coincided with the onset of haemolysis (Fig. 2). This urges caution when using long-chained amphiphiles at concentrations corresponding to maximum stabilization.

Phosphate efflux

These studies and the following efflux and influx studies were carried out at concentrations corresponding to AH_{max}, AH_{75}, AH_{50} and AH_{25}. All the amphiphiles studied reduced the efflux of phosphate from the erythrocytes. The dose-re-

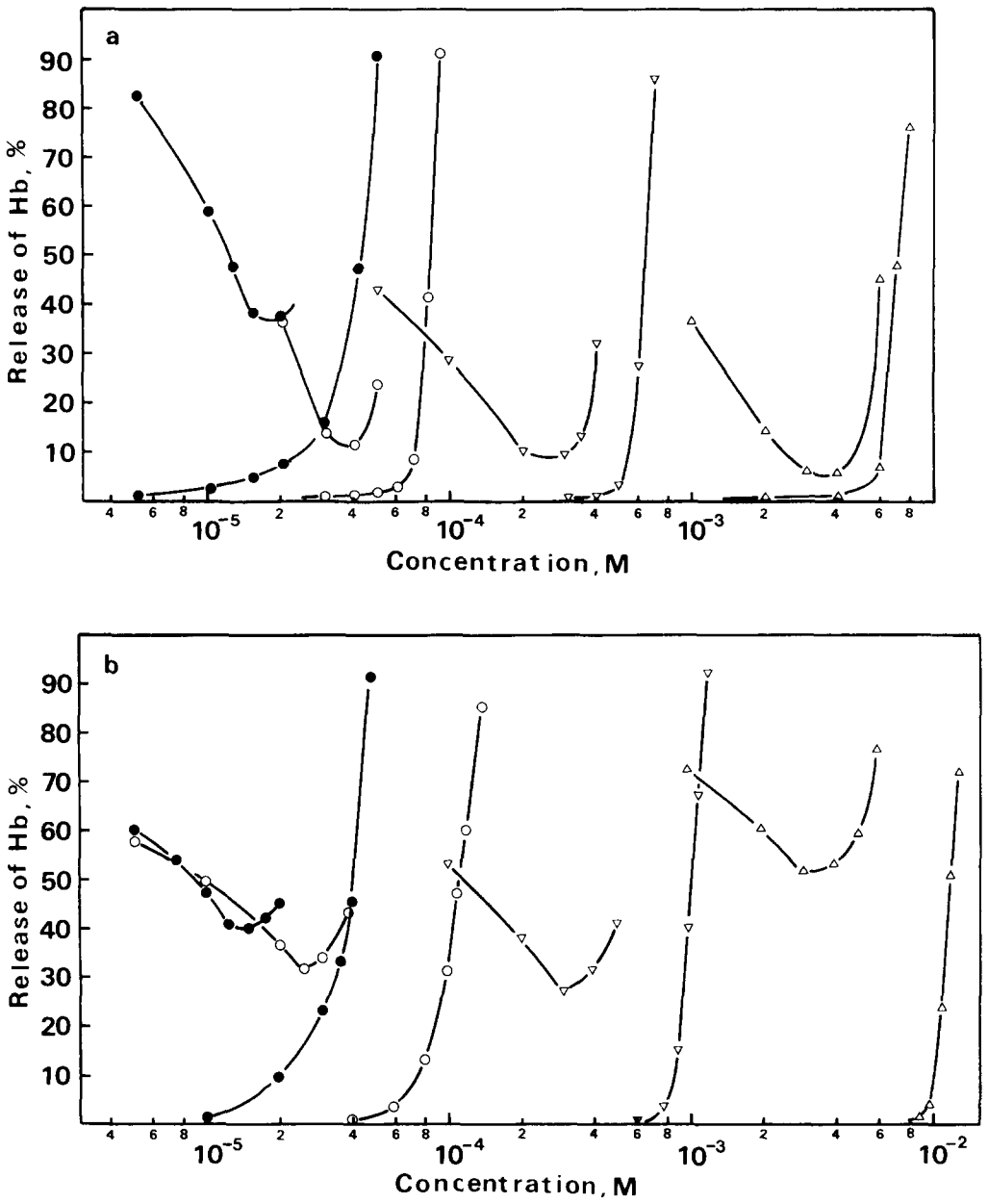

Fig. 2. The antihaemolytic and haemolytic activity of (a) zwittergents and (b) alkyltrimethylammonium bromides. (●), C_{16}; (○), C_{14}; (▽), C_{12}; (△), C_{10}. The determination of the antihaemolytic activity was carried out in a medium of such a tonicity that about 80% of untreated erythrocytes were haemolysed. Cell concentration and incubation temperature as in Fig. 1. Each point represents the mean of three to six separate experiments.

sponse curves for the different amphiphiles had essentially the same appearance as that of sodium decyl sulphate (Fig. 3), and efflux kinetics were linear for at least 60 min (not shown). The comparison between the amphiphiles is based on the percentage of tracer released following 30 min incubation. The results are summarized in Fig. 4. For the sake of clarity, phosphate release is plotted against equivalent concentrations in respect to stabilization.

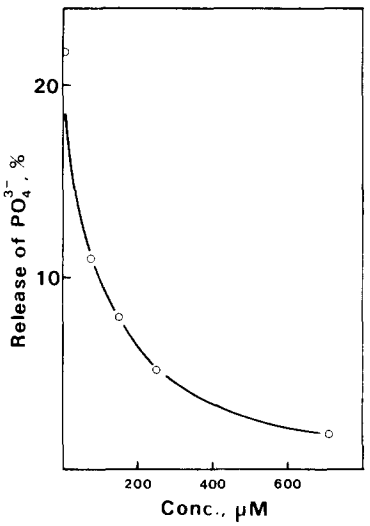

Fig. 3. Dose-response curve for phosphate release in erythrocytes incubated with sodium decyl sulphate for 30 min. Phosphate release was determined as described in Materials and Methods. Cell concentration and incubation temperature as in Fig. 1. Each point represents the mean of four separate experiments.

For almost all the amphiphiles phosphate efflux was significantly reduced even at concentrations corresponding to AH_{25}. Several clear differences in inhibitory potency between the different amphiphiles can be seen. Comparing the different homologues within the three homologue series, it is evident that the inhibiting ability is related to the length of the alkyl chain. The C_{10} and C_{12} homologues reduced the efflux significantly more than the C_{14} and C_{16} homologues. The nature of the polar head also had a clear influence on the inhibitory potency of the amphiphiles. Amphiphiles with a negatively charged polar head were more efficient than amphiphiles with a positively charged, neutral or zwitterionic head. If the amphiphiles are compared at equal length of the alkyl chain they fall, with respect to inhibitory potency, in the following order; $R\text{-}COO^- > R\text{-}SO_4^-$ > zwittergents, R-glucopyranoside > $R\text{-}N^+(CH_3)_3$, $R\text{-}NH_3^+$, R-pyridinium. The C_{16} derivatives and the C_{14} derivative of the sodium alkyl sulphates induced a slight increase in phosphate efflux at concentrations corresponding to AH_{max}. This is apparently due to a release of phosphate from lysed cells, because with these derivatives the concentration for maximum stabilization coincided with the onset of haemolysis.

Potassium efflux

All the amphiphiles increased potassium efflux at the stabilizing concentrations. The dose relationship was linear and the efflux kinetics were linear for at least 60 min. Potassium efflux was measured following 30 min incubation. The results are summarized in Fig. 5. The differences in efficiency between the different amphiphiles were small. The alkyltrimethylammonium derivatives were slightly more effective than the other amphiphiles. They increased significantly the release of potassium at concentrations corresponding to AH_{25}, whereas the sodium alkyl sulphates increased the release significantly only at concentrations exceeding those corresponding to AH_{50}. The zwittergents were slightly more effective than the sodium alkyl sulphates but less effective than the alkyltrimethylammonium bromides.

At concentrations exceeding that of AH_{75} the non-ionic derivative (decyl β-D-glucopyranoside) differed markedly from the other amphiphiles. Above this concentration it caused a marked concentration-dependent increase in potassium efflux (Fig. 6). At a concentration corresponding to AH_{max} about 45% of the tracer was released and it was further raised to nearly 60% without any sign of haemolysis. No marked alteration in phosphate efflux occurred at these concentrations (Fig. 6), excluding nonspecific membrane damage as a cause of the increase in potassium efflux. It seems unlikely that a similar marked increase in efflux also occurred for other cations, because such an increase in overall cation permeability should apparently have led to lysis of the erythrocytes. It thus appears that this amphiphile affects a pathway specific to potassium.

Potassium influx

Influx of potassium was measured following 30 min incubation in the absence and presence of ouabain (run in parallel). Influx in the presence of ouabain represents passive potassium influx, and the difference in influx between samples incubated with and without ouabain are taken to represent

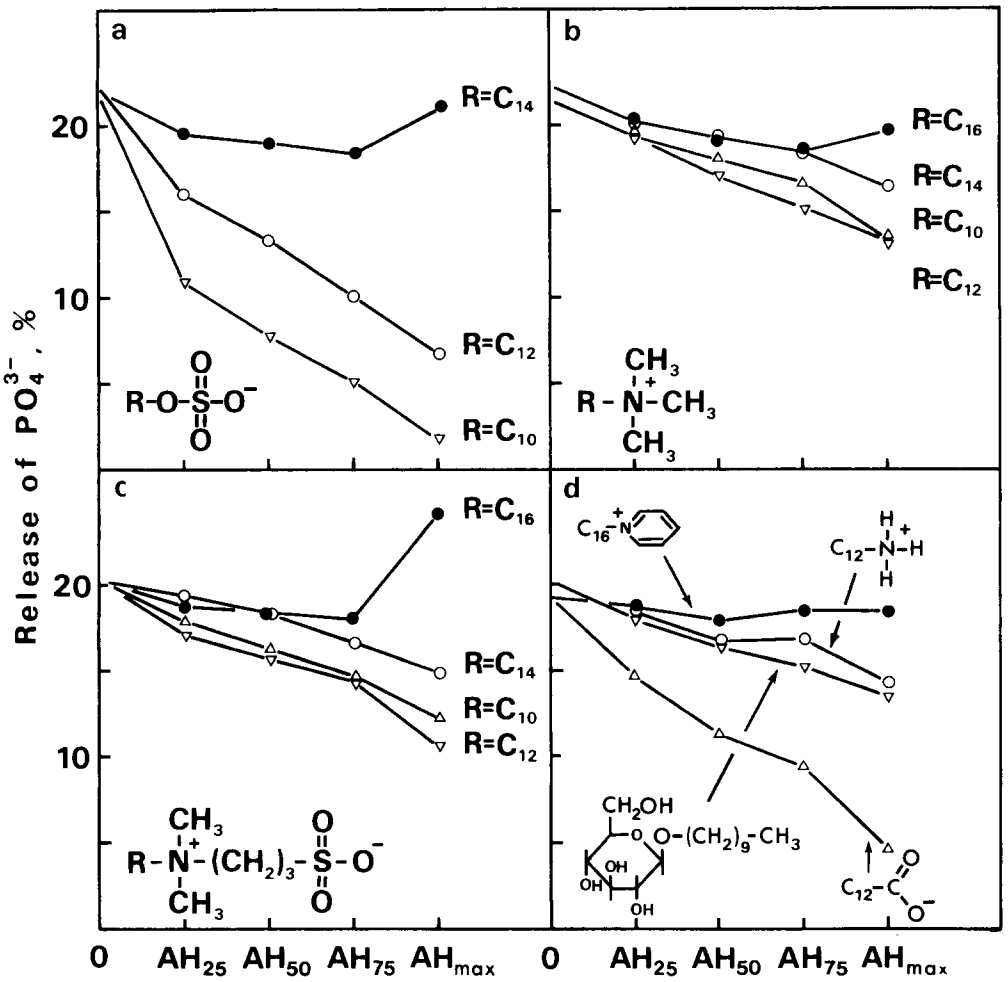

Fig. 4. Release of phosphate from erythrocytes following 30 min incubation in the presence of the amphiphiles. The release of phosphate is plotted against equi-protecting concentrations (the concentrations resulting in maximum protection, 75, 50 and 25% of maximum protection against hypotonic haemolysis). The molar concentrations of the amphiphiles at these protecting effects are given in Table I. Phosphate release was measured as described in Materials and Methods. Cell concentration and incubation temperature as in Fig. 1. Each point represents the mean of four separate experiments.

active potassium influx ((K^+-Na^+)-pump activity). No influx studies were made with sodium tetradecyl sulphate, cetylpyridinium chloride and the C_{16} homologue of the zwittergents.

The passive potassium influx was increased by all the amphiphiles (Fig. 7) to about the same extent as potassium efflux. For most of the amphiphiles the influx was increased about 2-fold at concentrations corresponding to those for AH_{75}–AH_{max}. Dodecylamine hydrochloride and sodium laurate were slightly less effective and decyl β-D-glucopyranoside slightly more effective than the other derivatives at lower concentrations. At concentrations corresponding to those for AH_{max}, dodecylamine hydrochloride and decyl β-D-glucopyranoside induced considerably higher influxes than the other amphiphiles. No other clear differences between the different amphiphiles were seen.

In the case of active potassium influx there were more clear-cut differences between the different types of amphiphile (Fig. 8). The alkyltri-

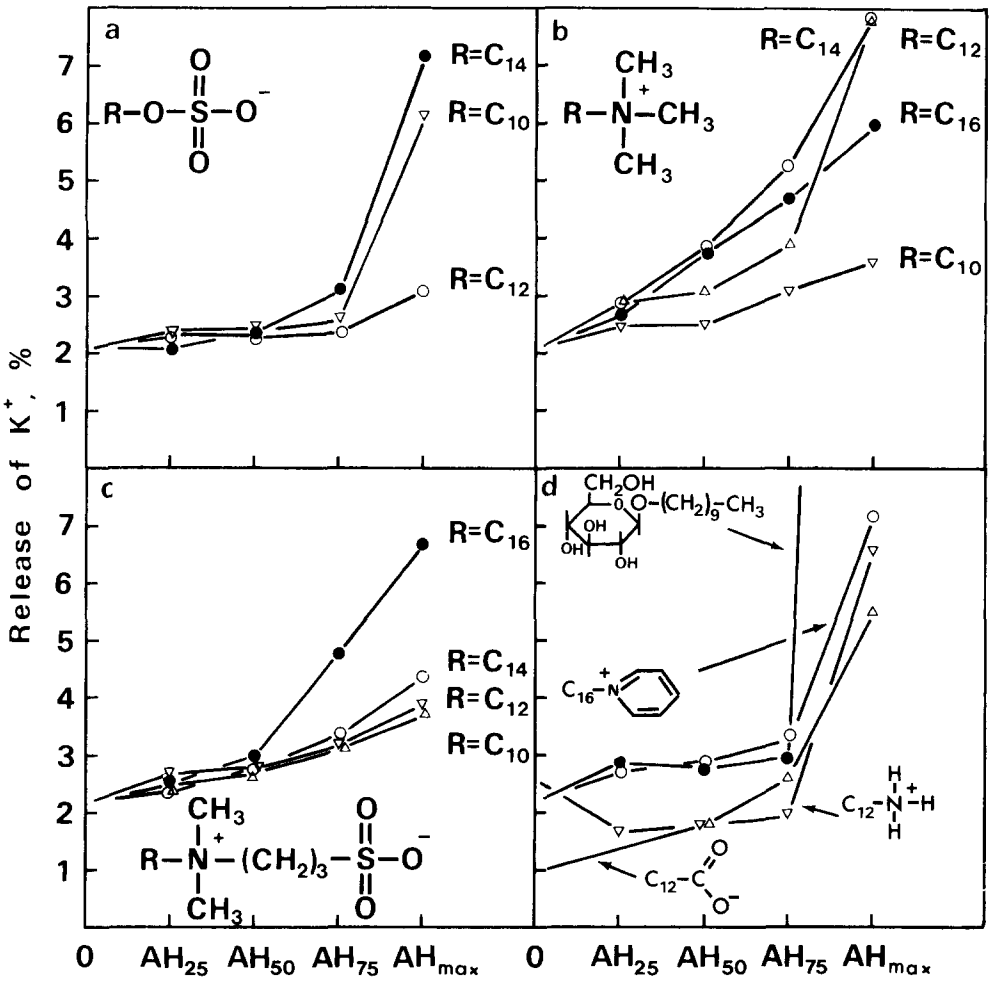

Fig. 5. Release of potassium from erythrocytes following 30 min incubation in the presence of the amphiphiles. The release of potassium is plotted against equi-protecting concentrations as in Fig. 4. Potassium release was measured as described in Materials and Methods. Cell concentration and incubation temperature as in Fig. 1. Each point represents the mean of four separate experiments.

methylammonium bromides, zwittergents, decyl β-D-glucopyranoside and dodecylamine hydrochloride inhibited the active potassium influx. The alkyltrimethylammonium bromides were the most potent inhibitors. They caused about 50% inhibition at a concentration corresponding to AH_{50}. No relationship between the length of the alkyl chain and inhibiting ability was seen. Sodium alkyl sulphates, on the other hand, had no effect, while sodium laurate increased the active potassium influx slightly. With the amphiphiles used in this study there were consequently clear differences due to the nature of the polar head. Positively charged, neutral or zwitterionic amphiphiles inhibited active potassium influx, whereas negatively charged amphiphiles had no effect or produced a slight increase in the influx.

Discussion

The intercalation of the amphiphilic molecules into the membrane

The approximate linear relationship between the length of the alkyl chain and the concentration for AH_{50} or H_{50} strongly suggests a membrane/aqueous phase partition as the mechanism whereby

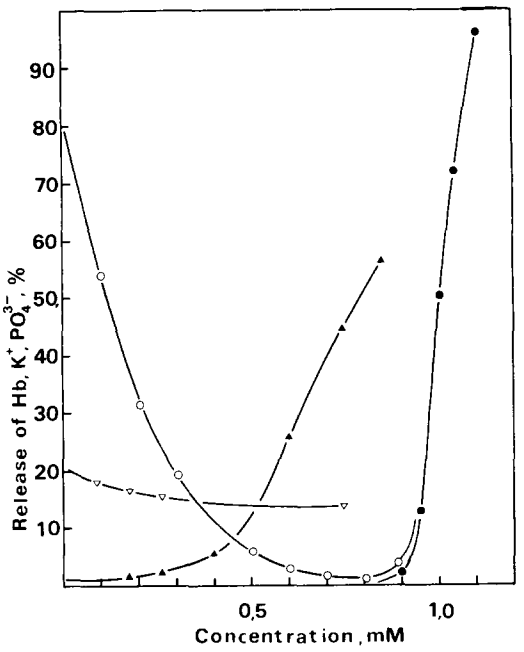

Fig. 6. Release of (▲) potassium and (▽) phosphate from erythrocytes incubated for 30 min in antihaemolytic concentrations of decyl β-D-glucopyranoside. (○), release of haemoglobin in a hypotonic medium; (●), degree of haemolysis in an isotonic medium. Cell concentration and incubation temperature as in Fig. 1. Each point represents the mean of two to four separate experiments.

amphiphilic molecules intercalate into the membrane and that hydrophobic interactions govern the intercalation. This is only what might be expected, since many studies have shown that the lipid bilayer is the main incorporation site for amphiphilic molecules [4,16–19]. However, the levelling off in the activity vs. chain-length profile (Fig. 1) is puzzling. If hydrophobic character were the only factor governing the intercalation of the amphiphiles into the bilayer, a linear relationship between activity and chain length is to be expected. A levelling off in activity or partition versus chain-length profile at chain lengths of 14 to 16 carbon atoms has frequently been observed with amphiphilic molecules [17,20–23], but it has not been satisfactorily explained. It is not due to micelle formation in the aqueous solution because the cmc values reported for the amphiphiles are well above the concentrations corresponding to AH_{max} [24–26]. The levelling off seems to indicate complications in the intercalation of long-chained homologues into the bilayer. By having a polar and an apolar region the bilayer is quite different from a non-polar hydrocarbon phase. Due to this the partition kinetics may be complex and it is possible that the partition of the members in a homologue series is not merely related to the length of the alkyl chain.

In the discussion above concerning the partition of the amphiphiles in the membrane it is assumed that an equal effect (AH_{50} or H_{50}) means an equal amount of amphiphilic molecules in the membrane. This assumption is based on the extensive data presented by Seeman [4], which show that the membrane concentration for a wide variety of lipid-soluble molecules and amphiphiles at AH_{50} is the same, despite several-fold variations in the oil/water partition coefficients. Studies made with lysophosphatidylcholines (C_{10}–C_{22}) have shown that the amount of homologues bound at H_{50} is approximately the same [21], indicating that the concept of equal effects at equal membrane concentrations is also valid in the case of haemolysis. It seems justified, without overextending the concept, to use the concentrations resulting in AH_{50} as an approximation of the membrane/buffer partition coefficients. Comparison of the three homologue series (Fig. 1, Table I) shows that the negatively charged alkyl sulphates have a greater partition coefficient at chain lengths below 16 carbon atoms than the zwittergents and the positively charged alkyltrimethylammonium compounds.

Alterations in transport functions induced by the amphiphiles

It should first be pointed out that, since these studies were carried out at equi-stabilizing concentrations (AH_{25}, AH_{50}, AH_{75} and AH_{max}), the effects are studied at approximately equal membrane concentrations of the amphiphiles. With this approach the qualitative and quantitative differences found between the agents should be attributable to differences in the molecular configuration of the amphiphiles.

The common effects shared by all the amphiphiles studied was a decrease in phosphate efflux, an increase in potassium efflux and an increase in passive potassium influx. The increase

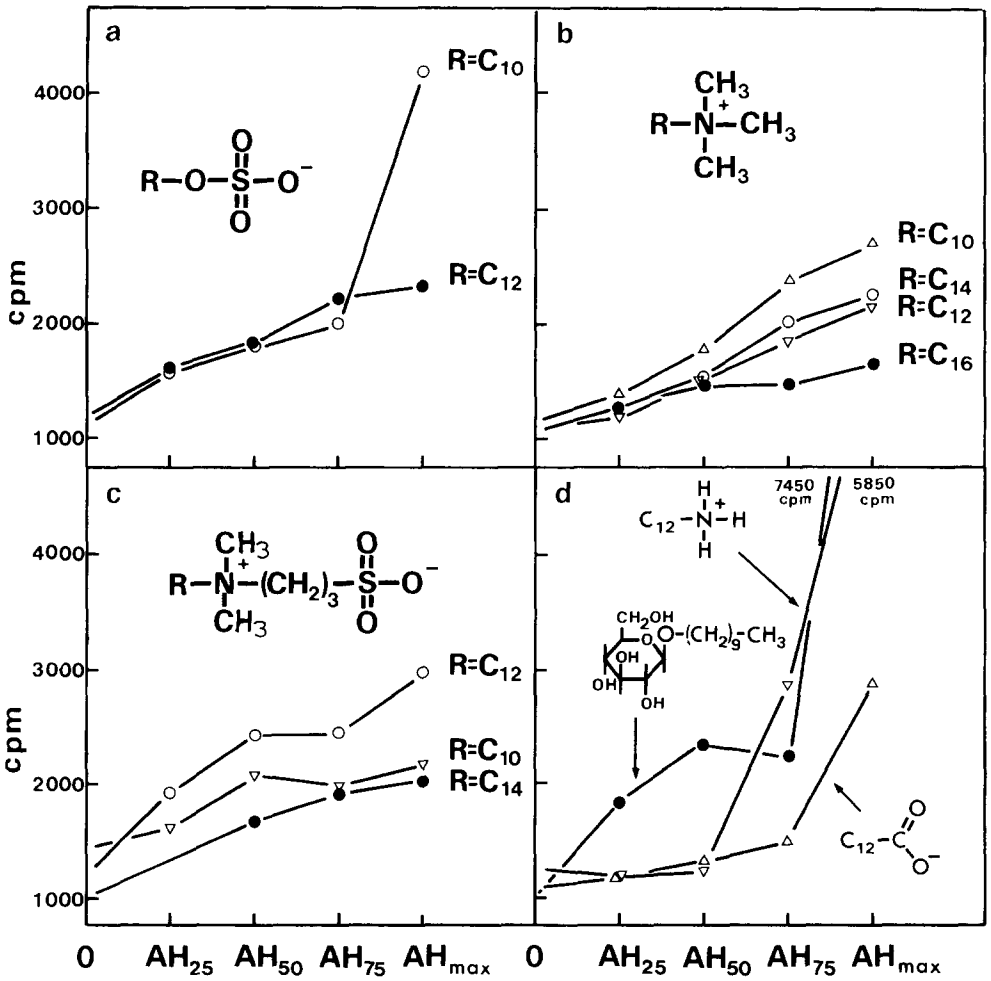

Fig. 7. Passive potassium influx in erythrocytes (ouabain-treated) following 30 min incubation in the presence of the amphiphiles. Intracellular radioactivity following incubation with the tracer (^{43}K) was measured as described in Materials and Methods and influx is expressed as cpm in the erythrocyte pellet. Influx is plotted against equi-protecting concentrations as in Fig. 4. Cell concentration and incubation temperature as in Fig. 1. Each point represents the mean of four or five separate experiments.

in the potassium flux induced by the amphiphiles was quite similar in both directions through the membrane and there were, with one exception (decyl β-D-glucopyranoside), no distinct differences between the different amphiphiles. Since the increase in fluxes was relatively independent of the nature of the polar head and the length of alkyl chain of the amphiphiles, it must be due to nonspecific interactions. The most likely explanation is that the amphiphiles, by affecting the dynamics of the lipid bilayer, increase the ground permeability of the bilayer to potassium (possibly also to other ions). This view is supported by several studies in which it has been shown that amphiphiles increase the permeability of bilayers [27–30]. Many amphiphiles, including surfactants, have been shown to decrease the transition temperature of lipid bilayers [28,31–33]. It is therefore tempting to assume that the amphiphiles, by decreasing the order or packing of the hydrocarbon chains of the lipids in the bilayer, increase the permeability of the bilayer to small ions. If this is the case, there should also have been an increase in phosphate efflux. However, our experimental

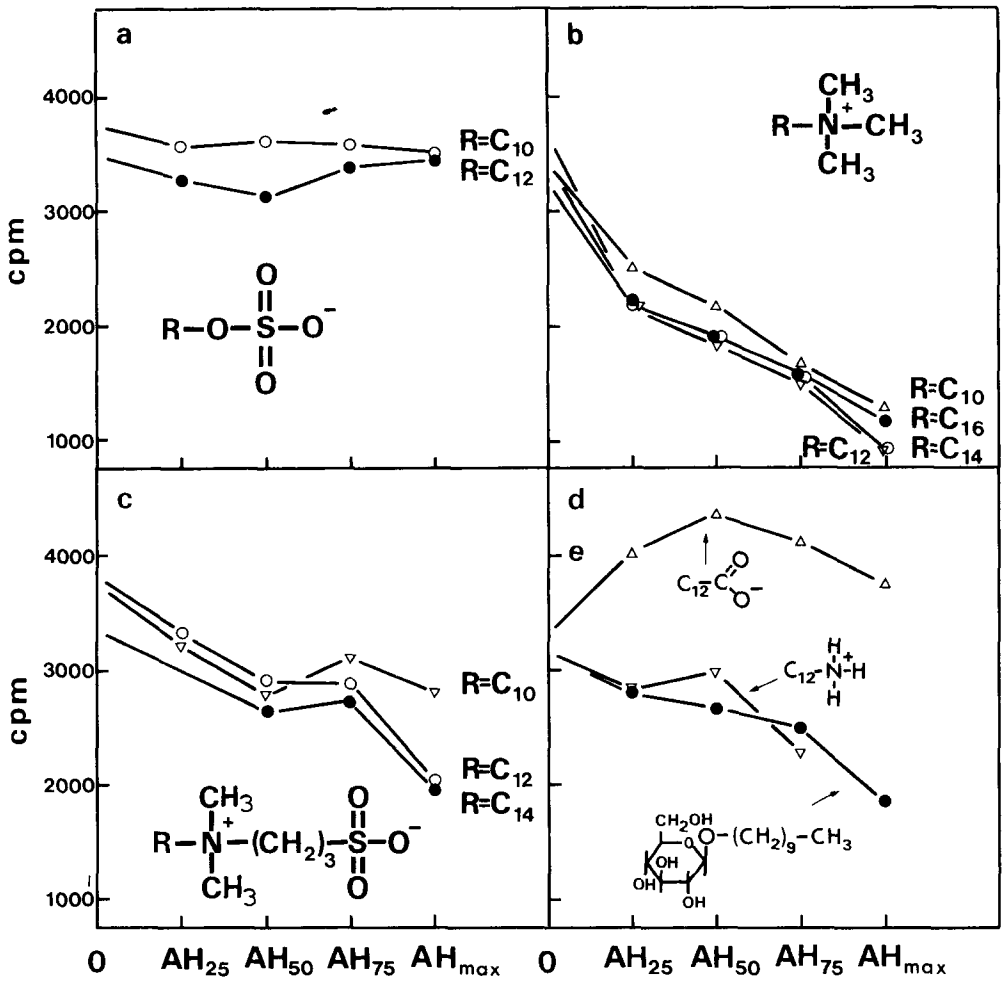

Fig. 8. Active potassium influx in erythrocytes following 30 min incubation in the presence of the amphiphiles. Intracellular radioactivity following incubation with the tracer (^{43}K) was measured as described in Materials and Methods and the active potassium influx is expressed as cpm in the erythrocyte pellet. Active influx is plotted against equi-protecting concentrations as in Fig. 4. Cell concentration and incubation temperature as in Fig. 1. Each point represents the mean of four or five separate experiments.

design did not permit detection of a possible increase in the permeability of the bilayer to phosphate. It may have occurred but have been camouflaged by a stronger inhibitory effect of the amphiphiles on other pathways.

With regard to the phosphate efflux, there were clear differences in inhibitory potency between the different derivatives. Their inhibitory potency was dependent on both the nature of the polar head and the length of the alkyl chain. The anionic amphiphiles were the most potent inhibitors, and short-chained homologues were more potent than long-chained ones. Several different types of amphiphile have previously been found to inhibit the transport of phosphate and other anions through the erythrocyte membrane [34]. The most likely explanation to the inhibitory effect of the amphiphiles is that they interfere with the function of the anion-exchange protein. The main anion-exchange protein in the erythrocyte membrane is band 3 [35], and it has been proposed that the transport function of this protein is sensitive to

the dynamics of the lipid bilayer [36,37]. Since it is quite well established that amphiphilic molecules increase the fluidity of bilayers [16,31–33] and cell membranes [38–40], it is reasonable to assume that the amphiphiles, by increasing the fluidity of the bilayer, affect the conformation of the anion-exchange protein and thereby reduce the transport of phosphate. The differences in potency between the different derivatives could be due to differences in their ability to affect the state of the bilayer. Alternatively, it might be hypothesized that, due to differences in the nature of the polar head, they intercalate into different lipid domains in the plane of the bilayer and thereby diverge in their effects on membrane proteins. It is even conceivable that the amphiphiles, having a shape that differs from that of the bulk lipids in the bilayer (the cross-sectional area of the polar head is greater than that of the alkyl chain), may have a preference for lipid-protein interfaces in the membrane. Such an orientation has been suggested for some local anaesthetics [41,42] and it seems likely that a location adjacent to the anion-exchange protein could give the polar head of the amphiphile an opportunity to influence the functional state of the anion-exchange protein.

The mechanisms suggested above as possible reasons for the inhibition of phosphate transport may also be valid in explaining the inhibition of active potassium influx induced by the amphiphiles. Several authors have reported inhibitory effects of amphiphiles on membrane (Na^+-K^+)-ATPases [38,43,44] and other membrane-bound enzymes [45,46]. This inhibitory effect has been proposed to be due to alterations in the state of the membrane proteins induced through an alteration of the dynamics of the bilayer [38,45,46]. In the present study, the nature of the polar head proved to be a determining factor in the inhibition of the (Na^+-K^+)-pump, since cationic, zwitterionic and neutral derivatives were inhibitory, whereas anionic derivatives had no effect or were slightly stimulatory.

Are non-bilayer structures involved in the intercalation of amphiphiles into the membrane?

It has been proposed that lipid-soluble and amphiphilic agents protect erythrocytes against hypotonic haemolysis by expanding the membrane and thereby increasing the critical haemolytic volume of the cell [4]. This concept may well apply to lipid-soluble agents able to rapidly equilibrate between the two leaflets of the bilayer. However, amphiphiles such as the alkyltrimethylammonium derivatives having a bulky quaternary ammonium head which is charged at physiological pH values are considered by many authors to be unable, at least in a short time (the antihaemolytic effect is dependent on events occurring within seconds), to penetrate the bilayer [47–49]. Such amphiphiles are kinetically trapped in the outer leaflet of the bilayer. In a previous study with rat erythrocytes [3] it was found that about $2.3 \cdot 10^7$ molecules of the C_{16} homologue of the alkyltrimethylammonium bromides (CTAB) were bound per erythrocyte at a concentration resulting in AH_{50}. This number is quite close to that calculated by Seeman [4] for a variety of lipid-soluble and amphiphilic agents at AH_{50}. Assuming the area of the alkyltrimethylammonium group in a monolayer to be 40 $Å^2$ [50] the total area of the intercalated molecules at AH_{50} will be 9.2 μm^2. This is 6.6% of the area of the erythrocyte (140 μm^2) and at a concentration corresponding to AH_{max}, it would be about 13%. It has been shown that an expansion of the outer leaflet of the bilayer by 0.4–1.3% is sufficient to transform biconcave-shaped erythrocytes to echinocytes [51–53]. In the light of these findings, the expansion calculated above seems quite extnesive, and the question is whether such an extensive expansion of the outer leaflet is at all possible without a collapse of the bilayer structure.

Furthermore, it has been pointed out that the dynamic shape of the molecules is important for the stability of the bilayer. Phospholipid molecules with a cylindrical shape are most easily accommodated in the bilayer structure, whereas molecules with the shape of cones and inverted cones are thought to combine in a complementary fashion to arrive at a net bilayer structure [54,55]. In a human erythrocyte the number of phospholipid molecules is $1.9 \cdot 10^8$ [56]. Assuming that $2.3 \cdot 10^7$ CTAB molecules are bound to the outer monolayer at AH_{50} [3], the ratio between phospholipids and CTAB in the leaflet would be about 4 : 1. At a concentration corresponding to AH_{max} it would be about 2 : 1. It seems very unlikely that the outer

leaflet can be loaded with such a quantity of wedge-shaped molecules (inverted cones) and still survive as a monolayer. In the case of molecules unable to rapidly penetrate the bilayer, it thus seems difficult to explain the intercalation process without assuming the participation of some mechanism able to correct the imbalance between the two leaflets, and possibly also an imbalance in the outer leaflet due to an unsuitable shape of the intercalated molecule.

The works of Cullis and De Kruijff (for review see Ref. 57) have shown that non-bilayer structures may occur in bilayers. These non-bilayer structures have been interpreted to be inverted micelles or H_{II} phases located between the two leaflets of the bilayer and they are thought to be involved in membrane events such as fusion and exocytosis [57]. The point we wish to make here is that such inverted micelles or H_{II} phases may be involved in the membrane incorporation of amphiphiles which, due to the charge of the polar head, are unable to rapidly penetrate the bilayer. If mixed inverted micelles, consisting of some of the intercalated molecules and lipid species from the bilayer, were formed between the two leaflets of the bilayer, the imbalance between the two leaflets and the imbalance within the outer leaflet could be coped with and the bilayer could maintain its stability. Furthermore, such a mechanism could possibly also stabilize the bilayer by equilibrating the non-penetrating amphiphiles between the two leaflets of the bilayer. The energy barrier to the transition between the bilayer and the non-bilayer configuration is proposed to be small [57], so it may be assumed that the non-bilayer structures may be only transient. However, the capacity of the stabilizing mechanism is apparently limited. If the number of amphiphilic molecules in the bilayer exceeds a certain level, the bilayer collapses and the cell will be lysed.

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