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Shape transformations induced by amphiphiles in erythrocytes

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Shape alterations induced in human erythrocytes by cationic, anionic, zwitterionic and nonionic amphiphiles (C_{10}–C_{16}) at antihaemolytic concentrations (CAH_{50} and CAH_{max}) and at a slightly lytic concentration (2–10% haemolysis) were studied. Anionic (sodium alkyl sulphates) and zwitterionic amphiphiles (3-(alkyldimethylammonio)-1-propanesulfonates) proved to be potent echinocytogenic agents. Among the nonionic amphiphiles there were potent stomatocytogenicagents (octaethyleneglycol alkyl ethers, pentaethyleneglycol dodecyl ether), one potent echinocytogenic agent (dodecyl D-maltoside) and one weak echinocytogenic agent (decyl β-D-glucopyranoside). Shape alterations induced by cationic amphiphiles (alkyltrimethylammonium bromides, cetylpyridinium chloride and dodecylamine hydrochloride) showed a strong time-dependence. These amphiphiles immediately induced strongly crenated erythrocytes which during incubation shifted to less crenated erythrocytes or to stomatocytes. All of the echinocytogenic amphiphiles induced echinocytes immediately, and there were only small alterations of the induced shape during incubation. Among the stomatocytogenic amphiphiles there were some that induced stomatocytes immediately or after a short lag time while others first passed the erythrocytes through echinocytic stages before stomatocytic shapes were attained. Erythrocytes treated with amphiphiles did not recover their normal discoid shape following repeated washing and reincubation for 1 h in amphiphile-free medium. Our study shows that shape alterations induced by amphiphiles in erythrocytes cannot be explained solely by assuming a selective intercalation of differently charged amphiphiles into the monolayers of the lipid bilayer as suggested in the bilayer couple hypothesis (Sheetz, M.P. and Singer, S.J. (1976) J. Cell Biol. 70, 247–251). We suggest that amphiphiles, when intercalated into the lipid bilayer, trigger a rapid formation of intrabilayer non-bilayer phases which protect the bilayer against a collapse and bring about a transbilayer redistribution of intercalated amphiphiles as well as of bilayer lipids.

Introduction

Numerous studies have shown that erythrocytes respond to various treatments by altering their shape. ATP-depletion [1,2], Ca^{2+}-loading [3], pH changes [4,5], and a variety of amphiphilic agents [4,6,7] have been shown to induce a transformation of the discoid shape to spiculated (echinocytes) or cupped (stomatocytes) forms. Echinocytes and stomatocytes are thought to be induced by opposite forces [8]; stomatocytes by forces tending to produce invaginations and echinocytes by forces tending to produce evaginations.

To explain shape alterations induced by amphiphiles Sheetz and Singer [9,10] formulated the bilayer couple hypothesis. According to this hypothesis shape alterations arise from a differential expansion of the two monolayers of the lipid bilayer. Echinocytogenic amphiphiles are thought

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to be kinetically trapped or to be equilibrated mainly in the outer monolayer thereby expanding it relative to the inner, whereas stomatocytogenic amphiphiles are thought to equilibrate mainly in the inner monolayer expanding it relative to the outer. At equilibrium anionic amphiphiles are stated to be echinocytogenic and cationic ones stomatocytogenic [4,9,10] and this has been rationalized to mean that anionic amphiphiles intercalate into the outer monolayer whereas cationic ones intercalate into the inner. Cationic amphiphiles which can be discharged, like the tertiary amine chlorpromazine, are assumed to diffuse rapidly across the bilayer as the neutral species and then be trapped in their charged form in the inner leaflet [9]. Permanently cationic amphiphiles, like quaternary amines, on the other hand, are thought to slowly 'flip' to the inner leaflet [10]. Erythrocytes treated with such amphiphiles are first echinocytes because the amphiphiles are located mainly in the outer monolayer. With time, however, more and more of the amphiphilic molecules are thought to accumulate in the inner monolayer and at equilibrium they are mainly located in the inner monolayer and the erythrocytes are consequently stomatocytes [6,10]. The difference in location of differently charged amphiphiles within the bilayer is attributed mainly to energetically favourable interactions of cationic amphiphiles with acid phospholipids of the inner monolayer [9,10]. However, it was early suggested that the cytoskeletal network participates in the control of the erythrocyte shape. Crenation following ATP-depletion was suggested to be due to spectrin dephosphorylation [1,2]. The dephosphorylation in turn was thought to induce structural changes of the spectrin complex which contracted the inner monolayer and crenated the erythrocytes. Convincing evidence for an active role of the cytoskeletal network in shape regulation is still lacking and more recent studies suggest a passive role for the cytoskeletal network [11–14]. The cytoskeletal network is thought to be able to assume and preserve the shape of the membrane but unable to initiate shape alterations [11]. In recent studies crenation following both ATP-depletion and Ca^{2+}-loading has been explained in the context of the bilayer couple hypothesis without invoking alterations of the cytoskeletal network [12,15]. Crenation is thought to arise from a bilayer imbalance due to degradation of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate in the inner monolayer which decreases the area of the monolayer.

The current opinion is that shape alterations in erythrocytes following treatment with various amphiphiles are best explained by the bilayer couple hypothesis. Although objections to this hypothesis have been raised [16–18], it appears to successfully explain shape alterations induced by a variety of amphiphiles [6]. However, there are still some unsolved questions in the bilayer couple hypothesis. The molecular events in the process of translocating cationic amphiphiles to the inner monolayer have not yet been worked out. According to the bilayer couple hypothesis it is supposed that amphiphiles are translocated from one monolayer to the other in a relatively short time. However, it seems very difficult to explain a translocation of a charged amphiphile from one monolayer to another without assuming some temporal rearrangement of the bilayer structure including some redistribution of the lipids in the bilayer. If such a rearrangement occurs it would undoubtly complicate the explanation offered by the bilayer couple hypothesis. Furthermore, the cup-forming amphiphilic agents most frequently used in shape transformation studies are chlorpromazine and structurally related compounds. The molecular structure of these compounds is quite different from that of the phospholipids of the bilayer and they may for that reason be less suitable as model molecules in attempts to test the validity of the bilayer couple hypothesis. The present study was designed to test the bilayer couple hypothesis by using chemically simple amphiphiles. For this purpose we selected some anionic, cationic, zwitterionic, and nonionic alkyl derivatives (C_{10}–C_{16}) to study the shape alterations induced by these agents.

Materials and Methods

Chemicals. Alkyltrimethylammonium bromides (C_{10}, C_{12}, C_{14}, C_{16}) and chlorpromazine hydrochloride were purchased from Sigma Chemical Co. Zwittergents (3-(alkyldimethylammonio)-1-pro-

panesulphonates) (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 and dodecyl D-maltoside from Fluka and dodecylamine hydrochloride from Eastman Kodak Co. Octaethyleneglycol mono *n*-alkyl ethers (C_{10}, C_{12}, C_{14}, C_{16}) and pentaethyleneglycol mono *n*-dodecyl ether were obtained from Nikko Chemicals Co. All the amphiphilic agents used, as well as all the other chemicals, were of reagent grade.

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 used. All experiments were carried out within 48 h after the blood was drawn.

Incubation of the erythrocytes. Aliquots of 0.1 ml of the erythrocyte suspension were pipetted into polystyrene tubes containing 0.9 ml medium and various concentrations of the amphiphiles (37 °C). The final erythrocyte concentration was $(1.6–1.7) \cdot 10^8$ cells/ml (about 1% haematocrit) and the incubations were carried out in a shaking thermostat bath at 37 °C. The amphiphiles were used in concentrations previously determined to result in maximum protection against hypotonic haemolysis (CAH_{max}), in half-maximum protection (CAH_{50}) and in a slight haemolysis (2–10%) following 60 min incubation [19]. The concentrations used are given in Table I.

Examination of erythrocyte morphology. At suitable time intervals between 0.5 and 120 min 10 μl of the samples were pipetted onto glass coverslips with a Finnpipette (polypropylene tips). The coverslip was inverted and immediately examined with a phase contrast microscope at a magnification of 500 or 800 ×. The shape of the erythrocytes was classified according to Bessis [20]. The proportions of different shapes were estimated and only cells floating freely in the suspension were regarded. The examination was completed within 30 s after pipetting the erythrocyte suspension onto the coverslips. Shape examinations were made with 5–10-min intervals and the evaluation of a sample was usually made on two successive observations. With each concentration of the amphiphiles two to four separate experiments were made with blood from two or more donors.

Examination of the stability of the shape alterations was carried out with erythrocytes treated with amphiphiles at concentrations corresponding to those resulting in maximum stabilization against hypotonic haemolysis. Following incubation for 60 min at 37 °C the erythrocytes were rapidly washed three times with medium (37 °C). The samples were reincubated and erythrocyte shape was examined at suitable time intervals.

Results

Shape of untreated erythrocytes

About half of the erythrocytes were slightly crenated following washing with medium and the crenation of the erythrocytes increased somewhat during incubation. Crenated erythrocytes were mainly echinocytes of stages I and II according to the terminology of Bessis [20]. It was not due to the 'glass effect' [21] since it was also seen in samples pipetted onto plastic films or in samples fixed with formaldehyde or glutaraldehyde. The crenation increased somewhat with the number of washes and it is therefore apparently caused by a washing off of plasma components coating the erythrocyte surface. It appears that some of the coating components are important in maintaining the discoid shape and that the removal of these components drives the stomatocyte-echinocyte equilibrium towards the echinocytic side. This slight crenation of control erythrocytes did not, however, seriously interfere with the evaluation of shape alterations caused by the amphiphiles.

Shape transformations induced by amphiphiles

The shape transformations induced by the amphiphiles following 1 h of incubation at concentrations corresponding to those resulting in maximum protection (CAH_{max}) and half-maximum protection (CAH_{50}) against hypotonic haemolysis and in a slight haemolysis (2–10%) are summarized in Fig. 1. The concentrations of the amphiphiles resulting in these effects are listed in Table I.

The zwittergents (3-(alkyldimethylammonio)-

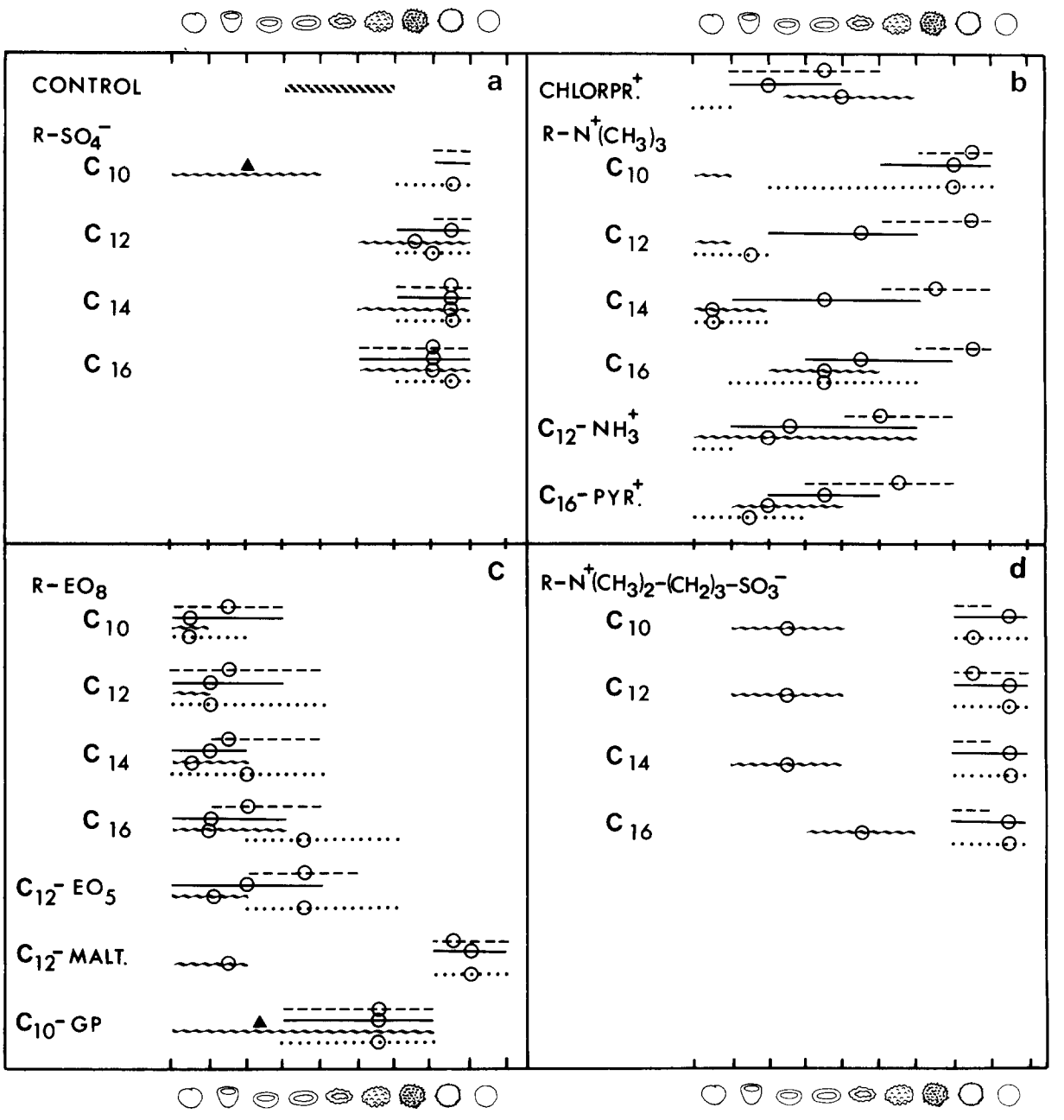

Fig. 1. Shape of erythrocytes following 1 h of incubation with amphiphiles at antihaemolytic concentrations (CAH_{50} and CAH_{max}) and a slightly lytic concentration (2–10% haemolysis). Erythrocyte concentration and incubation temperature as in Table I. The presentation is based on two to four separate experiments with each concentration of the amphiphiles. (a) Anionic, (b) cationic, (c) nonionic, (d) zwitterionic. - - - - - -, CAH_{50}; ———, CAH_{max}; · · · · · ·, lytic; ~~~, washed three times following incubation at CAH_{max} and then incubated for 30 min in a medium without amphiphile; O, dominating shape; ▲, haemoglobin released during washings. CHLORPR$^+$, chlorpromazine hydrochloride; C_{16}-PYR$^+$, hexadecylpyridinium chloride; R-EO_8, octaethyleneglycol alkyl ethers, C_{12}-EO_5, pentaethyleneglycol dodecyl ether; C_{12}-MALT, dodecyl D-maltoside; C_{10}-GP, decyl β-D-glucopyranoside.

TABLE I

THE CONCENTRATIONS OF THE AMPHIPHILES USED IN THE SHAPE TRANSFORMATION STUDIES

The erythrocyte concentration was $(1.6–1.7)\cdot10^8$ cells/ml and the erythrocytes were incubated with the amphiphiles for 1 h at 37 °C.

Amphiphile	Concentration (μM)		
	AH_{50}	AH_{max}	lytic (2–10%)
Sodium alkyl sulphates			
C_{10}	153	708	950
C_{12}	20.8	50	100
C_{14}	8	23.3	35
C_{16}	18	40	60
Alkyltrimethylammonium bromides			
C_{10}	720	3300	6000
C_{12}	76.7	300	500
C_{14}	15.3	40	60
C_{16}	7.5	14.2	20
Dodecylamine hydrochloride	7.5	26.8	45
Cetylpyridinium chloride	9	32.7	50
Chlorpromazine hydrochloride	10	20	300
Octaethyleneglycol mono *n*-alkyl ethers			
C_{10}	113	300	400
C_{12}	9.9	43.8	52
C_{14}	7.9	24.4	31
C_{16}	6.4	20.5	34
Pentaethyleneglycol mono *n*-dodecyl ether	8.9	40	66
Decyl β-D-glucopyranoside	170	740	950
Dodecyl D-maltoside	16.6	40	58
3-(Alkyldimethylammonio)-1-propanesulphonates			
C_{10}	1430	3630	10000
C_{12}	103	263	850
C_{14}	8	27.5	90
C_{16}	5	13.8	20

1-propanesulphonates) (Fig. 1d) and the nonionic derivative dodecyl D-maltoside (Fig. 1c) were the most echinocytogenic agents. They induced sphero-echinocytes at CAH_{50} and spherocytes or a mixture of sphero-echinocytes and spherocytes at the higher concentrations. There was no significant difference in the shape transforming potency between the different homologues of the zwittergents at equiprotecting concentrations.

The sodium alkyl sulphates (Fig. 1a) were slightly less echinocytogenic than the zwittergents and dodecyl D-maltoside. The C_{16} homologue was slightly less echinocytogenic than the other homologues. Decyl β-D-glucopyranoside (Fig. 1c) had a weak echinocytogenic effect at all the concentrations studied.

With the C_{12} and C_{14} homologues of the sodium alkyl sulphates a striking effect was observed. If the samples pipetted onto coverslips were spread to a thin layer with the side of the pipette tip a considerable part of the echinocytes were transformed to discocytes and stomatocytes. This was especially evident with the C_{14} homologue and it also occurred in samples spread on plastic surfaces. The examination of samples treated with the C_{14} and C_{12} had thus to be carried out with caution. This phenomenon is obscure. It did not occur with the C_{10} and C_{16} homologues or with any other of the amphiphiles used in our study.

The polyethyleneglycol alkyl ethers were all stomatocytogenic (Fig. 1c). The homologues of the octaethyleneglycol alkyl ethers had about equal shape transforming potency with the exception of the C_{16} homologue which at the lytic concentration only had a weak stomatocytogenic effect.

The cationic amphiphiles induced rather complex and clearly concentration dependent shape alterations. At CAH_{50} all the derivatives were echinocytogenic (Fig. 1b). With increasing concentration the shape of the erythrocytes shifted more and more towards stomatocytic forms. At lytic concentrations all the derivatives, except the C_{10} homologue of the alkyltrimethylammonium derivatives, were stomatocytogenic. Samples treated with lytic concentrations of the C_{16} homologue, however, contained both stomatocytes and echinocytes but since no stomatocytes appeared in control samples this homologue was classified as a weak stomatocytic agent at lytic concentrations. Chlorpromazine hydrochloride has often been used as a stomatocytogenic agent and for this reason we included it in our study as a reference agent. As can be seen (Fig. 1b) chlorpromazine hydrochloride is a potent stomatocytogenic agent and our observations concerning its shape transforming ability are in agreement with those previously reported [6,9].

Summarizing our findings above it is evident that one cannot solely on the basis of the net charge of the polar head of amphiphilic agents predict the shape transformation induced by the

agents. Among the nonionic derivatives, for example, there are both potent stomatocytogenic (octaethyleneglycol alkyl ethers) and potent echinocytogenic (dodecyl D-maltoside) agents as well as weak echinocytogenic agents (decyl β-D-glucopyranoside).

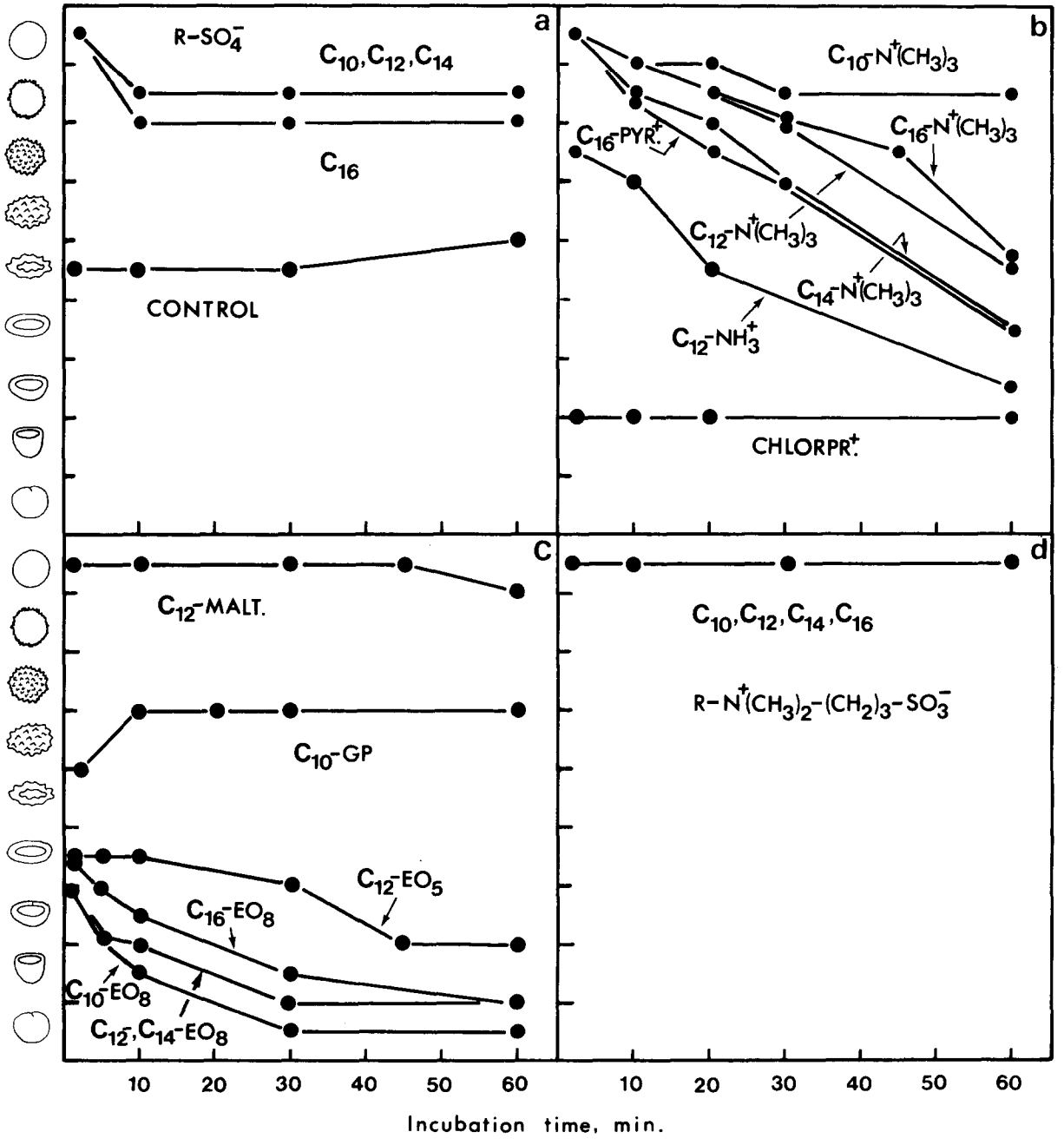

Fig. 2. Time-course of shape alterations in erythrocytes incubated with the amphiphiles at CAH_{max}. Only the dominating shape is shown in the figure and for a correct information of the shapes induced following 1 h of incubation see Fig. 1. For abbreviations concerning the amphiphiles see Fig. 1. Erythrocyte concentration and incubation time as in Table I. The presentation is based on two to four separate experiments with each amphiphile.

Time-course of shape alterations

In Fig. 2 the dominating shapes induced by the amphiphiles at CAH_{max} are plotted against incubation time. With the zwittergents (Fig. 2d) and chlorpromazine hydrochloride (Fig. 2b) no time-dependent shape alterations were seen. Erythrocytes treated with these derivatives were transformed within 30 s of incubation and the induced shapes were maintained during 60 min of incubation.

Minor time-dependent shape alterations were seen in erythrocytes treated with the sodium alkyl sulphates (Fig. 2a), dodecyl D-maltoside (Fig. 2c) and decyl β-D-glucopyranoside (Fig. 2c).

Pronounced time dependent shape alterations were seen with the polyoxyethyleneglycol alkyl ethers and the cationic amphiphiles (except chlorpromazine hydrochloride). During the first minutes of incubation erythrocytes treated with the polyoxyethyleneglycol alkyl ethers (Fig. 2c) were discocytes or slightly cupped cells which during incubation were transformed to more cupped forms or to invaginated spheres. The rate of the shape alterations increased with increasing concentration of the homologues (not shown). The cationic amphiphiles (Fig. 2b) showed complex time-dependent shape alterations. The most striking with these derivatives was that they induced spherocytes or sphero-echinocytes during the first minutes of incubation, but as the incubation proceeded the degree of crenation decreased and discocytes and stomatocytes (not with decyltrimethylammonium bromide) appeared. The rate of the transformation was accelerated with an increased concentration of the amphiphiles and the shape transformations were not fully completed within 60 min.

Summarizing the time-dependent shape alterations it is interesting to note that among the stomatocytogenic amphiphiles some induced stomatocytes immediately (chlorpromazine hydrochloride, C_{10}-EO_8, C_{12}-EO_8 and C_{14}-EO_8), some following a short lag time (C_{16}-EO_8 and C_{12}-EO_5) while others first passed the erythrocytes through echinocytic stages before stomatocytic shapes were attained (cationic amphiphiles). With the echinocytogenic amphiphiles on the other hand, the echinocytic shapes were immediately induced and they remained unchanged or shifted towards slightly less evaginated forms during incubation.

Shape alterations following washing and reincubation

Following the 60 min incubation at CAH_{max} the erythrocytes were rapidly washed three times with medium (37 °C) and then reincubated in amphiphile-free medium at 37 °C for about 1 h. The shapes of the erythrocytes following 30 min of reincubation are shown in Fig. 1.

No or only a weak alteration of the transformed shape occurred with the sodium alkyl sulphates ($> C_{10}$) and the polyoxyethyleneglycol alkyl ethers (Fig. 1a,c). Erythrocytes treated with sodium alkyl sulphates were shifted slightly towards less crenated forms and those treated with polyoxyethyleneglycol alkyl ethers towards more invaginated forms. With the octaethyleneglycol alkyl ethers the degree of the shift decreased somewhat with increasing length of the alkyl chain. In erythrocytes treated with the C_{10} homologue of the sodium alkyl sulphates the sphero-echinocytes were transformed to invaginated spheres or stomatocytes and the washings were accompanied with a release of haemoglobin. A similar release of haemoglobin was also observed in erythrocytes treated with decyl β-D-glucopyranoside (Fig. 1c).

The spherocytes and sphero-echinocytes induced by the C_{10}, C_{12} and C_{14} homologues of the zwittergents were transformed by the washes to stomatocytes and discocytes (Fig. 1d). Following washing of the samples treated with the C_{16} homologue no stomatocytes appeared, only discocytes and echinocytes. Also in the case of dodecyl D-maltoside (Fig. 1c) the spherocytes and sphero-echinocytes were shifted towards stomatocytic shapes.

In erythrocytes treated with chlorpromazine hydrochloride the first wash transformed the erythrocytes to crenated forms but following the second wash the samples had the same appearance as control samples (Fig. 1b). Erythrocytes treated with the other cationic amphiphiles were all shifted towards stomatocytic shapes by the washes (Fig. 1b). For the alkyltrimethylammonium derivatives there was a clear decrease in the degree of the shift with an increasing length of the alkyl chain.

Some general trends emerged from the washing experiments. First, if an alteration of the transformed shape occurred following the washes, the shape was always shifted towards the stomatocytic

side (more invaginated or less evaginated shapes) independent of the shapes primarily induced. The only exception from this was chlorpromazine hydrochloride. Second, the shift following washing was more pronounced with the C_{10}, C_{12} and C_{14} homologues than with the C_{16} homologue. Third, the erythrocytes treated with the amphiphiles (except chlorpromazine hydrochloride) did not attain normal shape following washings and a reincubation of up to 1 h.

Discussion

The bilayer couple hypothesis [9,10], stating that amphiphiles induce different shape alterations in erythrocytes through a preferential intercalation in either monolayer of the lipid bilayer, has received much attention and the current opinion seems to be that the shape alterations are best explained by this hypothesis [14,15,22–24]. However, several findings in the present study are very difficult to explain within the framework of the bilayer couple hypothesis. The statement that the charge of the polar head of the amphiphile is of crucial importance for the type of shape alteration induced is apparently not correct. According to the bilayer couple hypothesis the cationic amphiphiles should at equilibrium be intercalated mainly into the inner monolayer and give stomatocytes [10], but as was found in our study none of the cationic amphiphiles (except chlorpromazine) induced stomatocytes at CAH_{50}. Stomatocytes appeared at CAH_{max} or at slightly lytic concentrations following about 30 min of incubation. Decyltrimethylammonium bromide induced echinocytes only, even at lytic concentrations. The nonionic octaethyleneglycol alkyl ethers on the other hand were stomatocytogenic at all the concentrations studied, and contrary to the alkyltrimethylammonium bromides these derivatives induced stomatocytes almost immediately. If stomatocytes arise through a preferential intercalation of cationic amphiphiles into the inner monolayer due to an excess of phospholipids with a negative net charge in this monolayer, as proposed in the bilayer couple hypothesis, then the alkyltrimethylammonium bromides, and not the nonionic octaethyleneglycol alkyl ethers, should be the most potent stomatocytic agents. Furthermore, the finding that there are both potent stomatocytogenic and potent echinocytogenic as well as weak echinocytogenic agents among the nonionic amphiphiles is puzzling in the context of the bilayer couple hypothesis. Thus the description of the shape transformations in erythrocytes as being merely due to a selective intercalation of differently charged amphiphiles into either monolayer is apparently not correct.

In an attempt to conceptualize shape alterations induced by amphiphiles it is necessary to take into account current views on the stability and the dynamics of lipid bilayers. A great variety of different lipid molecules are present in the plasma membrane of mammalian cells [25]. Many of these lipids form lamellar crystalline phases with water, but there are also lipids in the membrane which form non-bilayer structures. Amphiphiles with the shape of 'cones' (large surface area of the polar head and a small hydrophobic volume) tend to form micellar aggregates, whereas amphiphiles with the shape of 'inverted cones' (small surface area and large hydrophobic volume) tend to form inverted micelles or hexagonal phases (H_{II} phases) [26–28]. Lipids with the shape of 'cones' and 'inverted cones', present in the membrane, are thought to combine in a complementary fashion to arrive at a net bilayer structure [27–29]. The shape of the lipid molecules is also considered to be important in the regulation of the shape of erythrocytes because it has been shown that lipid manipulations resulting in an increase of the number of either 'cones' or 'inverted cones' alter the curvature of the membrane and thereby the shape of the erythrocyte [13,30]. Furthermore, theoretical calculations and lipid manipulations have shown that an expansion or shrinking of one monolayer relative to the other with about 1% results in a marked alteration of the erythrocyte shape [31,32].

The picture emerging from these studies is that the geometry of lipid molecules is of vital importance for the stability of the bilayer and that a stable bilayer implies a subtle balance between the areas of the monolayers. In the light of these views, data available on the binding of amphiphiles to the erythrocyte membrane are obscure. A number of binding studies carried out with different types of amphiphiles show that $(1–3) \cdot 10^7$ mole-

cules are bound per erythrocyte at CAH_{50} or at concentrations resulting in pronounced shape alterations [15,22,33–36]. In a previous study with rat erythrocytes about $2.3 \cdot 10^7$ molecules of the C_{16} homologue of the alkyltrimethylammonium bromides (CTAB) were found to be bound per erythrocyte at CAH_{50} [36]. However, according to current concepts amphiphiles having a bulky charged polar head, such as the alkyltrimethylammonium bromides, are unable to rapidly flip from one monolayer to the other. Such amphiphiles are thought to be kinetically trapped in the outer monolayer. Through a still unknown mechanism they are thought to flip slowly to the inner monolayer [10]. Assuming that $2.3 \cdot 10^7$ CTAB molecules are bound to the outer monolayer at CAH_{50} [36] the ratio between the number of amphiphile and phospholipid molecules in the monolayer will, as previously calculated [19], be about 1 : 4. At CAH_{max} the corresponding number will be about 1 : 2. The alkyltrimethylammonium bromides, like all the charged amphiphiles used in the present study, have the shape of 'cones'. It seems very unlikely that the bilayer could be stable and maintain its integrity with such a great amount of 'cones' in the outer monolayer. Furthermore, assuming the area of the trimethylammonium group in a monolayer to be 40 $Å^2$ [37] the total area occupied by the intercalated amphiphiles will amount to 6.6% of the membrane area at CAH_{50} and to about 13% at CAH_{max} [19]. It is very unlikely that the bilayer could maintain its stability with such an imbalance in the areas between the two monolayers, considering that a difference in the relative area of the two monolayers of about 1% is sufficient to cause marked shape alterations [31,32]. However, if one considers the protecting effects amphiphiles have against osmotic stress it is evident that no pronounced destabilization of the bilayer occurs following treatment with amphiphiles. When erythrocytes are suspended in a hypotonic buffer solution containing sublytic concentrations of the amphiphiles (as was done in the haemolysis protection experiments [19]) they are immediately protected against hypotonic haemolysis. The bilayer apparently attains a stable configuration immediately when treated with sublytic concentrations of the amphiphiles, otherwise the erythrocyte could not show an increased osmotic resistance. Consequently there must be some events which immediately correct the imbalance within the outer monolayer due to the presence of a large amount of 'cones', and the imbalance between the two monolayers due to the difference in the area of the two monolayers.

However, a rapid transverse diffusion of amphiphiles having a charged polar head to the inner monolayer is according to present knowledge impossible. Thus the events stabilizing the bilayer must apparently involve some temporal rearrangement of the bilayer structure including a redistribution of some of the intercalated amphiphiles as well as some of the lipid species of the bilayer. Several authors have shown that non-bilayer structures may occur in lipid bilayers [38–42]. These non-bilayer structures have been suggested to be hexagonal phases (H_{II} phases) or inverted micelles located between the two monolayers of the lipid bilayer and they are thought to be involved in membrane fusion, exo- and endocytosis, and in transbilayer transport of phospholipids [38]. It seems likely that non-bilayer phases, possibly inverted mixed micelles, are involved as intermediates when amphiphiles are intercalated into the erythrocyte membrane. If mixed inverted micelles, consisting of some of the intercalated amphiphiles and lipid species from the bilayer, were formed between the two monolayers the imbalance between the two monolayers and the imbalance within the outer monolayer could be temporarily coped with and the bilayer could maintain its integrity. The intrabilayer inverted micelles could then rapidly redistribute intercalated amphiphiles and bilayer lipids between the two monolayers.

We thus suggest a refinement of the current view on the intercalation of amphiphiles into the lipid bilayer of membranes and on the shape alterations induced by amphiphiles in erythrocytes by introducing the concept of transient non-bilayer phases as essential elements in the intercalation process. We feel that the erythrocyte membrane due to its diverse lipid composition has an inbuilt capacity to respond to the intercalation of exogenous amphiphiles by a rapid formation of non-bilayer phases between the two monolayers followed by a consequent redistribution of some of the intercalated amphiphiles as well as a redistri-

bution of lipid species of the bilayer. The 'aim' of the process is to secure the bilayer structure and thereby the barrier properties of the membrane which is of vital importance to the cell. If formation of intrabilayer H_{II} phases is the mechanism whereby a breakdown of the bilayer can be prevented upon intercalation of amphiphiles then this leads to a possible explanation of the lipid diversity in membranes, i.e. the requirement for lipids with a variety of shapes.

As to the shape alterations resulting from the rearrangements, the ratio between the areas of the monolayers may be the main factor determining the shape attained by the erythrocytes, as suggested in the bilayer couple hypothesis. The imbalance between the monolayers, however, is not due to the intercalated amphiphiles alone but is the net result of the rearrangements. The rearrangements may also involve a release of certain lipids to the external medium, because we detected in a preliminary study phosphatidylcholine, phosphatidylserine and sphingomyelin in the external medium following treatment of erythrocytes with amphiphiles at CAH_{max}. Similar findings have recently been reported by Maher and Singer [43]. They found that erythrocytes treated with amphiphiles, in concentrations corresponding to those resulting in maximum protection against hypotonic haemolysis, released lipids and proteins to the external medium. However, a prerequisite for reaching a molecular description of shape transformations induced by amphiphiles is a detailed understanding of the events governing the formation of the non-bilayer phases and the transbilayer distribution of bilayer lipids and amphiphiles.

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