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SHAPE AND VOLUME CHANGES IN RAT ERYTHROCYTES INDUCED BY SURFACE-ACTIVE ALKYLTRIMETHYLAMMONIUM SALTS AND SODIUM DODECYL SULPHATE

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Surface-active alkyltrimethylammonium salts (C_{12}, C_{14} and C_{16}) and sodium dodecyl sulphate (SDS) caused shape alterations and a volume increase in rat erythrocytes. The alkyltrimethylammonium salts caused echinocytic shapes at both prelytic and lytic concentrations during the first minutes of incubation at 37°C but as the incubation proceeded some of the echinocytes were transformed into stomatocytes. This transformation developed through the normal discocyte shape and it occurred only above certain concentrations. With C_{14} and C_{16} the concentration at which stomatocytic shapes appeared coincided with those at which the volume increase began. With the C_{12} homologue stomatocytic shapes did not appear until lytic concentrations were reached, whereas the volume increase began at prelytic concentrations. SDS caused only echinocytic shapes at 37°C and these appeared at prelytic concentrations, whereas the volume increase was associated with lytic concentrations. When erythrocytes crenated by SDS were cooled to room temperature they were transformed into stomatocytes and discocytes. Our results indicate that (a) even though ionic surfactants induce both swelling and shape alterations in erythrocytes these two changes are not necessarily connected, and that (b) the different shapes induced by cationic and anionic surfactants cannot be due to differences in the distribution of the surfactant molecules within the lipid bilayer of the erythrocyte membrane alone.

Introduction

A variety of chemical agents have been found to induce shape transformations in erythrocytes. Common to many of these agents is that they are amphiphilic compounds with significant surface activity. Anionic amphiphilic compounds are generally considered to produce crenated erythrocytes (echinocytes) and amphiphilic cations to produce cup-shaped erythrocytes (stomatocytes) [1–3]. At concentrations producing these shape alterations the amphiphilic compounds also decrease the osmotic fragility of the erythrocytes [4–6]. At higher concentrations both types of compound produce more or less spherical cells (sphero-echinocytes and sphero-stomatocytes). According to Deuticke [1] cells undergoing the early stages of echinocytic and stomatocytic shape alterations due to chemical agents do not change their volume. Yet Kwant and van Steveninck [4] found that chlorpromazine, a cup-forming compound, increased the mean volume by about 9%.

The mechanism whereby amphiphilic compounds produce shape transformations is still poorly understood. Echinocytic shape transformations in erythrocytes can also be obtained by elevating pH [7] and by virtue of ATP depletion [8,9]. Stomatocytic shape alterations, on the other hand, can be obtained by lowering pH [1]. In a recent study Sheetz and Singer [2] proposed that anionic amphiphilic compounds are preferentially intercalated into the outer half of the lipid bilayer of the cell membrane and cationic ones into the inner. The consequence of such an asymmetric distribution is, according to the authors, that anionic compounds expand the area of the outer

surface relative to the inner surface of the membrane and that this leads to crenation of the erythrocytes. The cationic compounds do the opposite and this in turn leads to cup-shaped erythrocytes.

In a previous study [10] it was found that surface-active alkyltrimethylammonium salts protect erythrocytes from osmotic haemolysis and that they cause a transformation of the erythrocyte shape into cup-shaped cells or invaginated spheres. The purpose of the present study was to examine further the shape and volume alterations induced by ionic surface-active agents.

Materials and Methods

Chemicals. Cetyltrimethylammonium bromide (CTAB, C_{16}) and sodium dodecyl sulphate (SDS), 99% pure, were obtained from E. Merck AG, Darmstadt, F.R.G. Dodecyltrimethylammonium bromide (C_{12}) and tetradecyltrimethylammonium bromide (C_{14}), 94–98% pure, were generously supplied by MoDoKemi AB, Stenungsund, Sweden. Trimethyl[1-^{14}C]cetyltrimethylammonium bromide ([^{14}C]CTAB) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. The specific activity of the compound was 6.4 mCi/mmol and the radiochemical purity was about 97%.

Erythrocytes. Blood was collected from female Sprague-Dawley rats (250–350 g). The rats were anaesthetized lightly with diethyl ether, the chest was rapidly opened and blood was collected by heart puncture in cold (4°C) phosphate-buffered saline (150 mM NaCl/10 mM phosphate, pH 7.4) without the use of an anticoagulant. The erythrocytes were washed four or five times in phosphate-buffered saline using at least 10 vol. washing solution per vol. erythrocytes. The erythrocytes were suspended in phosphate-buffered saline to a concentration of $(1.6–1.7) \cdot 10^9$ cells/ml. Cell counts were made using a Coulter Counter. All experiments were carried out on the day the blood was collected.

Incubation of the erythrocytes. Aliquots of 1 ml of the erythrocyte suspension were pipetted into glass tubes containing 9 ml phosphate-buffered saline (warmed to 37°C) with various concentrations of the surfactants. The contents of the tubes were briefly mixed with a vortex mixer and the tubes were incubated at 37 ± 0.5°C in a shaking thermostat bath.

Morphological observations of the erythrocytes. Following incubation, aliquots (10–30 μl) of the erythrocyte suspensions were pipetted on to glass coverslips. The coverslips were inverted and immediately (within 10 s) examined under a phase contrast microscope at a magnification of 500 or 800X. The proportions of the different shapes were estimated approximately and the shapes classified according to Bessis [11]. The estimations were completed within 90 s after pipetting the erythrocyte suspension on the coverslips and only cells floating freely in the suspension were regarded. By this technique it was possible to avoid the well-known echinocytogenic effect of glass surfaces. The fact that the cells moved freely during the examination made it possible to discriminate between different shapes with the exception, however, of sphero-echinocytes II and sphero-stomatocytes II, which are very difficult to distinguish from each other in an optical microscope. In Fig. 1 some echinocytic and stomatocytic shapes are illustrated.

Measurement of potassium and haemoglobin release. The haemolytic activity of SDS and the release of potassium caused by the surfactants were determined as previously described [10].

Cell volume measurements. Following incubation, aliquots of the erythrocyte suspensions were diluted to a cell concentration of about $4 \cdot 10^5$ cells per ml. Size distribution plots were obtained using a Coulter Counter (Model ZB) coupled to a Model P128 size distribution analyser (Coulter Electronics) and a *XY* plotter. An 70 μm orifice was used and the plots were made with the edit facility on. All counts were performed at 37°C in phosphate-buffered saline and 50 μl of the diluted erythrocyte suspensions were counted. Human erythrocytes with a known mean cell volume were used to calibrate the Coulter Counter.

Adsorption of [^{14}C]CTAB to erythrocytes. Erythrocytes were incubated as described above in phosphate-buffered saline containing 5 μM [^{14}C]-CTAB. At certain intervals samples of 0.5 ml were pipetted into ice-chilled polyethylene tubes and centrifuged in a Beckman Microfuge (about 10000 × *g*). The total centrifugation time was about 50 s. Aliquots of 0.4 ml of the supernatants were taken to determine radioactivity. The samples were counted in a liquid scintillation spectrometer using Aquasol (New England Nuclear, Boston, MA) as the scintillation fluid. The samples were checked for quenching

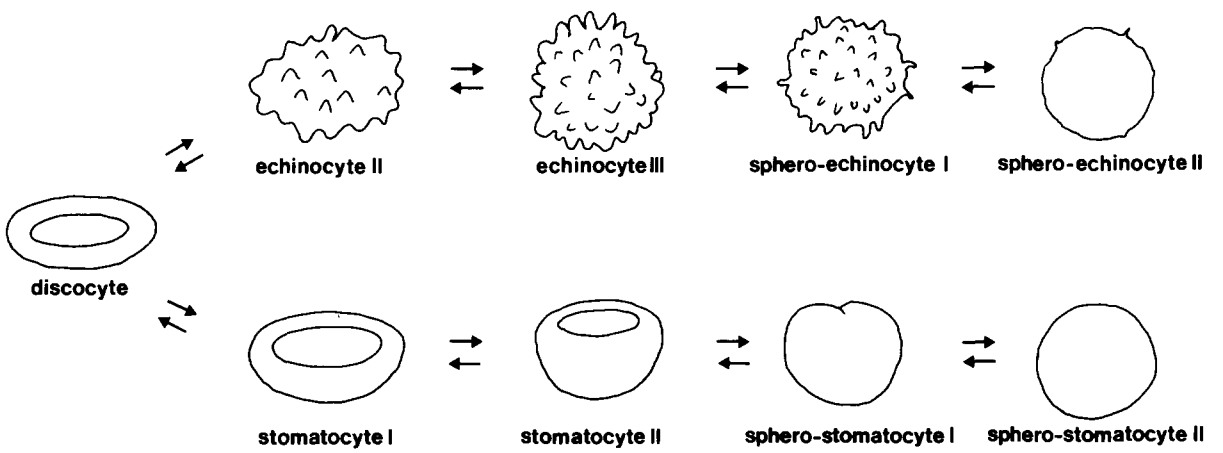

Fig. 1. Some shapes in erythrocytes classified according to Bessis [11].

using [^{14}C]toluene (New England Nuclear) as internal standard.

Treatment of glassware. Prior to each experiment the glassware was soaked with weak solutions of the surfactants used in the experiment and then rinsed with distilled water. This was done in an attempt to avoid a drop in the surfactant concentration due to adsorption of the surfactant to glass surfaces. The polyethylene tubes used in determining the adsorption of [^{14}C]CTAB to the erythrocytes were treated in a similar way.

Results

Shape transformations in untreated erythrocytes

About one-third of the erythrocytes were crenated following the washings with buffer solution and the amount of erythrocytes crenated increased somewhat during the incubation. The crenated cells were of echinocyte stages I and II according to the terminology used by Bessis [11]. In some experiments the erythrocytes were examined on siliconized coverslips, but this did not seem to reduce significantly the degree of crenation. The crenation of the erythrocytes was probably caused by the washing off of plasma components coating the erythrocytes, because the proportion of crenated erythrocytes increased with the number of washings.

Shape transformations caused by the surface-active alkyltrimethylammonium salts

The shape transformations caused by these agents were complex. Within 1 min of incubation the erythrocytes were transformed into sphero-echinocytes (stages I and II), and these were the dominating shapes during the first 5–20 min of incubation (Table I). The shape transformations which followed were dependent on the length of the alkyl chain of the surfactant and on the concentration of the surfactant. At prelytic concentrations of C_{14} and C_{16} some of the erythrocytes were gradually transformed into stomatocytes and sphero-stomatocytes. The transformation from echinocytes into stomatocytes apparently developed through the normal discocyte shape, since at a given time the erythrocyte population was a mixture of discocytes and of different stages of echinocytes and stomatocytes. The lowest concentration which enabled a transformation of the majority of the erythrocytes into stomatocytes within 60 min was for C_{14} 10–15 μM and for C_{16} 7.5–10 μM (Table II). When the concentration of the surfactants was increased the transformation rate from echinocytes into stomatocytes was enhanced and the stages involving mixed shapes became less and less distinctive. The transformation into stomatocytes and sphero-stomatocytes was virtually completed within 20–60 min, depending on the concentration of the surfactants, and during the following 60 min there was only a slight increase in the stomatocytic shapes. The ghosts appearing at lytic concentrations were stomatocytes and sphero-stomatocytes.

The pattern of shape alterations induced by the C_{12} homologue differed from that of the two other homologues. At prelytic concentrations C_{12} did not

TABLE I

TIME COURSE OF SHAPE TRANSFORMATIONS OF RAT ERYTHROCYTES INCUBATED WITH SURFACTANTS

The proportion of the different shapes was approximately estimated as described in Materials and Methods. +, a particular shape present in about 25% of the cells in the sample; ++, in about 50%; +++, in about 75%; ++++, in about 100%; (+), a particular shape present in a significant amount but less than 25%. C_{12}, dodecyltrimethylammonium bromide; C_{16}, cetyltrimethylammonium bromide [a]. Erythrocyte concentration was $(1.6–1.7) \cdot 10^{11}$ cells/l and incubation temperature 37°C. The lower concentration is for C_{12} non-haemolytic, for C_{16} and SDS low-haemolytic (under 5%) and the higher concentration for each surfactant corresponds to a concentration resulting in about 50% haemolysis following 1 h incubation.

Surfactant	μM	Time after addition of surfactant (min)	Shape of erythrocytes [b]								
			sphero-echinoc. II	sphero-echinoc. I	echinoc. III	echinoc. II	discoc.	stomatoc. I	stomatoc. II	sphero-stomatoc. I	sphero-stomatoc. II
C_{12}	200	1	+++	+							
		20	+++	+							
		40	++	++							
		60		+	+	+	+				
	550	1	+++	+							
		20	++	++							
		40	+	+	+	+	(+)	(+)			
		60		+	+	(+)	(+)	+	+		
C_{16}	15	1	++	++							
		20		+	+	+	(+)	+	(+)		
		40						+	++	+	
		60						(+)	++	++	
	33	1	+	+	+	+		(+)			
		20		+	+	(+)		+	+		
		40							++	+	+
		60							+	+++	+
SDS	50	1	+++	+							
		20	++	++	(+)	(+)					
		40	+	++	+						
		60	+	+	+	+					
	150	1	+++	+							
		20	+	++	+						
		40	+	++	+	+					
		60	+	+	(+)	(+)			+ [c]	+ [c]	

[a] The shape transformations induced by C_{14} followed the same pattern as that caused by C_{16}.

[b] See Fig. 1 for classification key.

[c] Erythrocytes having these shapes were ghosts.

TABLE II

SHAPE OF RAT ERYTHROCYTES FOLLOWING TREATMENT WITH SURFACTANTS AT 37°C FOR 1 h

The proportion of the different shapes was approximately estimated as described in Materials and Methods. +, a particular shape present in about 25% of the cells in the sample; ++, in about 50%; +++, in about 75%; ++++, in about 100%; (+), a particular shape present in a significant amount but less than 25%. C_{12}, dodecyltrimethylammonium bromide; C_{14}, tetradecyltrimethylammonium bromide; C_{16}, cetyltrimethylammonium bromide. Erythrocyte concentration was (1.6–1.7) · 10^{11} cells/l. The two highest concentrations for each surfactant correspond to concentrations resulting in about 50% and about 100% haemolysis, respectively.

Surfactant (μM)		Shape of erythrocytes [a]								
		sphero-echinoc. II	sphero-echinoc. I	echinoc. III	echinoc. II	discoc.	stomatoc. I	stomatoc. II	sphero-stomatoc. I	sphero-stomatoc. II
C_{12}	20		+	+	+	+				
	40		+	+	+	+				
	80		+	+	+	+				
	120		+	+	+	+				
	200		+	+	+	+				
	550		+	+	(+)	(+)	+	+		
	800		(+)	(+)				+	++	+
C_{14}	5			(+)	+	++	+			
	7.5			(+)	+	++	+			
	10			(+)	+	+	++			
	15					+	++	+		
	25					(+)	+	++	+	
	80					(+)	(+)	++	++	
	120						(+)	+	++	+
C_{16}	5			(+)	+	++	+			
	7.5			(+)	+	+	++			
	10				(+)	(+)	+	++	+	
	15						(+)	++	++	
	20						(+)	+	++	+
	33							+	+++	+
	50							++	++	
SDS	10	+	++	+	(+)	(+)				
	20	+	++	+	(+)	(+)				
	30	+	++	+	(+)	(+)				
	40	+	++	+	(+)	(+)				
	100	+	++	+	(+)				(+) [b]	
	150	+	+	(+)	(+)			+ [b]	+ [b]	
	175							+ [b]	+++ [b]	

[a] See Fig. 1 for classification key.

[b] Erythrocytes having these shapes were ghosts.

induce stomatocytic shapes. Following the initial stages involving sphero-echinocytes there was a gradual transformation of part of the sphero-echinocytes into other types of crenated erythrocyte and very few of the erythrocytes were stomatocytes. A significant amount of stomatocytic shapes did not appear below lytic concentrations (400–500 μM). Some of the stomatocytes induced by C_{12} had an irregular shape and were possibly stomato-acanthocytes. Similar irregularly shaped stomatocytes were also induced by C_{14} but they occurred more frequently with the C_{12} homologue.

Shape transformations caused by SDS

The anionic surfactant SDS caused, like the cationic surfactants, a transformation of the erythrocytes into sphero-echinocytes (stages I and II) within 1 min of incubation (Table I). Past this there was a gradual change of part of the sphero-echinocytes into other types of echinocyte, and within 10–30 min sphero-echinocytes I and echinocytes III dominated the erythrocyte population. During the following 60 min there was no essential change in the shape of the erythrocytes. The transformation from sphero-echinocytes into other types of crenated cells was enhanced when the concentration of the surfactant was increased, but there was no distinct difference in the proportion of different types of crenated cells at various concentrations of SDS (Table II). Ghosts produced at lytic concentrations of SDS were stomatocytes and sphero-stomatocytes.

When erythrocytes treated with SDS were cooled to room temperature (about 20°C) a very interesting effect was observed. The decrease in temperature caused a transformation of part of the crenated erythrocytes into stomatocytes. This effect was most pronounced at concentrations of 50–100 μM. At these concentrations most of the erythrocytes were transformed into stomatocytes and discocytes by this procedure. When the erythrocytes were warmed to 37°C they were re-transformed into echinocytic shapes. This alteration in shape caused by a change in temperature could be repeated several times. Erythrocytes treated with the alkyltrimethylammonium salts did not show shape alterations following cooling to room temperature.

This effect of temperature upon the SDS-crenated erythrocytes was rather unexpected. It appears that experiments performed in order to demonstrate the reversal of shape transformations by washing off the transforming agent from erythrocytes with cold buffer solution and then fixing the cells in cold buffer solution [2] must be interpreted with some caution.

TABLE III

MODAL VOLUME OF RAT ERYTHROCYTES TREATED WITH SURFACTANTS FOR 1 h

C_{12}, dodecyltrimethylammonium bromide; C_{14}, tetradecyltrimethylammonium bromide; C_{16}, cetyltrimethylammonium bromide. The two last concentrations for each surfactant correspond to concentrations resulting in 50 and 100% haemolysis, respectively.

C_{12}		C_{14}		C_{16}		SDS	
Concn. (μM)	Volume (μm^3)	Concn. (μM)	Volume (μm^3)	Concn. (μM)	Volume (μm^3)	Concn. (μM)	Volume (μm^3)
0	53.1 ± 0.4 [a]	0	53.1 ± 0.4	0	53.3 ± 1.0	0	52.2 [b]
20	53.1 ± 0.4	5	53.6 ± 0.2	5	54.9 ± 0.7	10	52.2
40	55.6 ± 0.8	7.5	54.5 ± 0.5	7.5	57.2 ± 0.6	20	52.2
80	59.1 ± 0.5	10	56.0 ± 0.3	10	58.4 ± 0.9	30	53.1
120	67.9 ± 1.1	12.5	59.4 ± 0.4	15	70.5 ± 0.9	40	54.8
160	71.5 ± 1.1	15	63.0 ± 0.4	17.5	72.4 ± 0.6	50	60.7
200	71.5 ± 1.1	25	70.6 ± 0.5	20	74.1 ± 0.8	100	70.1
550	80.1 ± 1.6	80	72.6 ± 1.0	33	76.9 ± 1.1	150	83.2
800	81.6 ± 1.6	120	84.0 ± 1.1	50	81.6 ± 0.8	175	88.5

[a] Mean and S.E. of 3–5 separate experiments.
[b] Mean of two separate experiments.

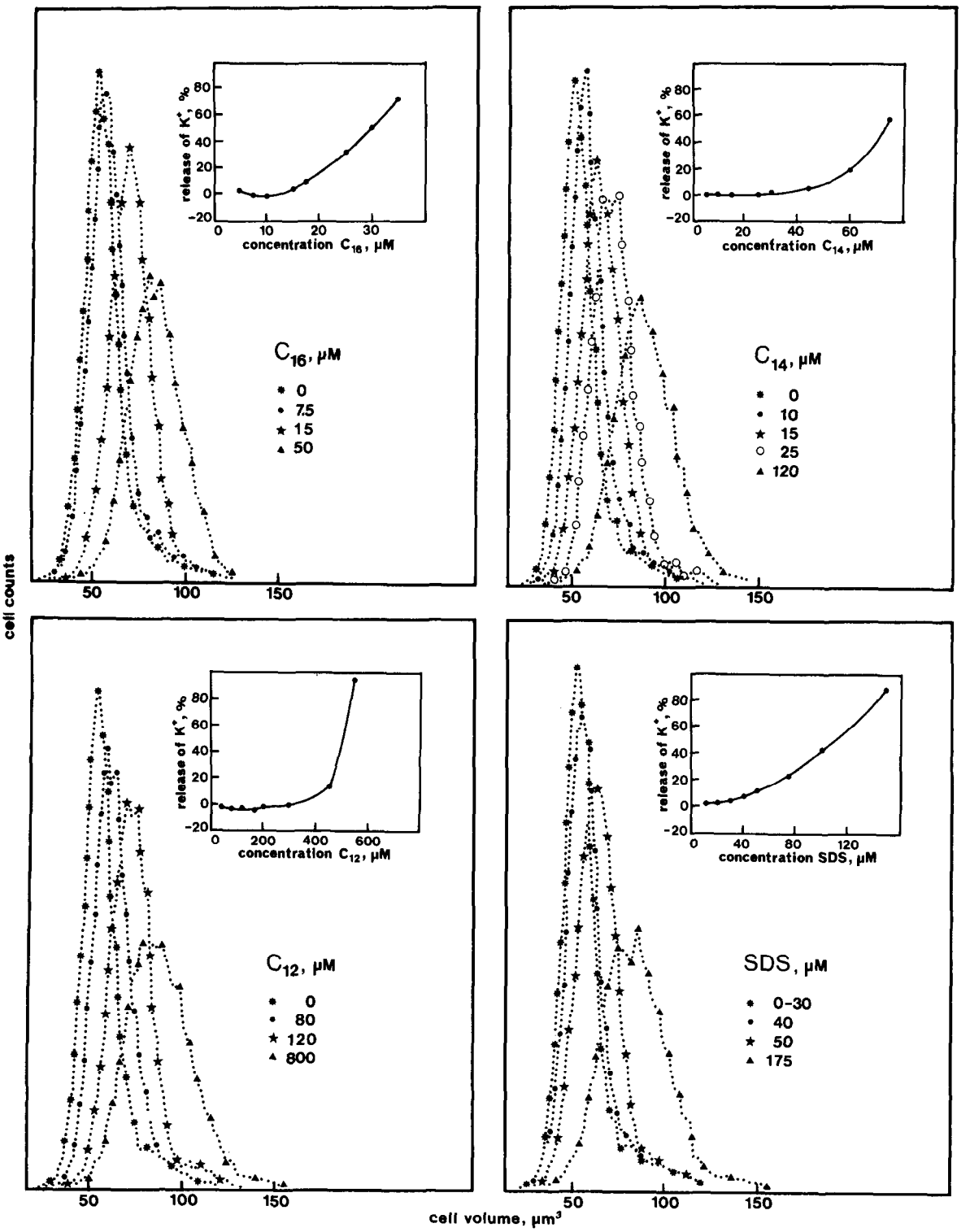

Fig. 2. Volume-distribution plots as monitored by a Coulter Counter and a size distribution analyser of rat erythrocytes treated with surface-active agents for 1 h. C_{16}, cetyltrimethylammonium bromide; C_{14}, tetradecyltrimethylammonium bromide; C_{12}, dodecyltrimethylammonium bromide. Each curve represents a single experiment in which about 20 000 cells were counted. The highest concentration of each surfactant corresponds to a concentration resulting in 100% haemolysis. The insets show the release of potassium, as compared with a control, in rat erythrocytes during 1 h incubation. Each point represents the mean of 2–4 separate experiments. Erythrocyte concentration was $(1.6–1.7) \cdot 10^{11}$ cells/l and incubation temperature 37 ± 0.5°C.

Effect of the surfactants on the erythrocyte volume

As shown in Table III, the alkyltrimethylammonium salts caused a concentration-dependent increase in the modal volume of the erythrocytes. This increase in the cellular volume began prior to the release of potassium (Fig. 2) and continued up to concentrations corresponding to 100% haemolysis. At equilytic concentrations of the surfactants there was about the same degree of volume increase. SDS also caused a concentration-dependent increase in the cell volume, but as for this surfactant the increase in the cell volume did not begin until lytic concentrations were reached (Table III and Fig. 2). As can be seen from the volume-distribution plots in Fig. 2, there is a marked change in the shape of the curves with an increase in the concentration of the surfactants, indicating an non-uniform response among the erythrocytes to the surfactants. There was no change in the number of particles registered by the Coulter Counter with an increase in the concentration of the surfactants. The shift of the volume-distribution curves toward higher volumes is thus due to an increase in the volume of the erythrocytes and not to an aggregation of erythrocytes or/and an aggregation of fragmented erythrocytes.

Time course of the volume changes

A shift of the volume-distribution curves occurred within 5 min of incubation. The volume increase of the erythrocytes was virtually completed within 30–60 min of incubation. At lytic concentrations, however, there was still some shift of the volume-distribution curves past 60 min of incubation. No significant differences in the time-dependent shift of the volume-distribution curves were observed between the different surfactants. Fig. 3 shows the time-related shift of the volume distribution curves for C_{12} and SDS at a low-haemolytic concentration and at a concentration corresponding to 50% haemolysis.

The adsorption of [^{14}C]CTAB (C_{16}) to the rat erythrocytes

The adsorption study was carried out at a prelytic concentration of CTAB (5 μM) in order to avoid any

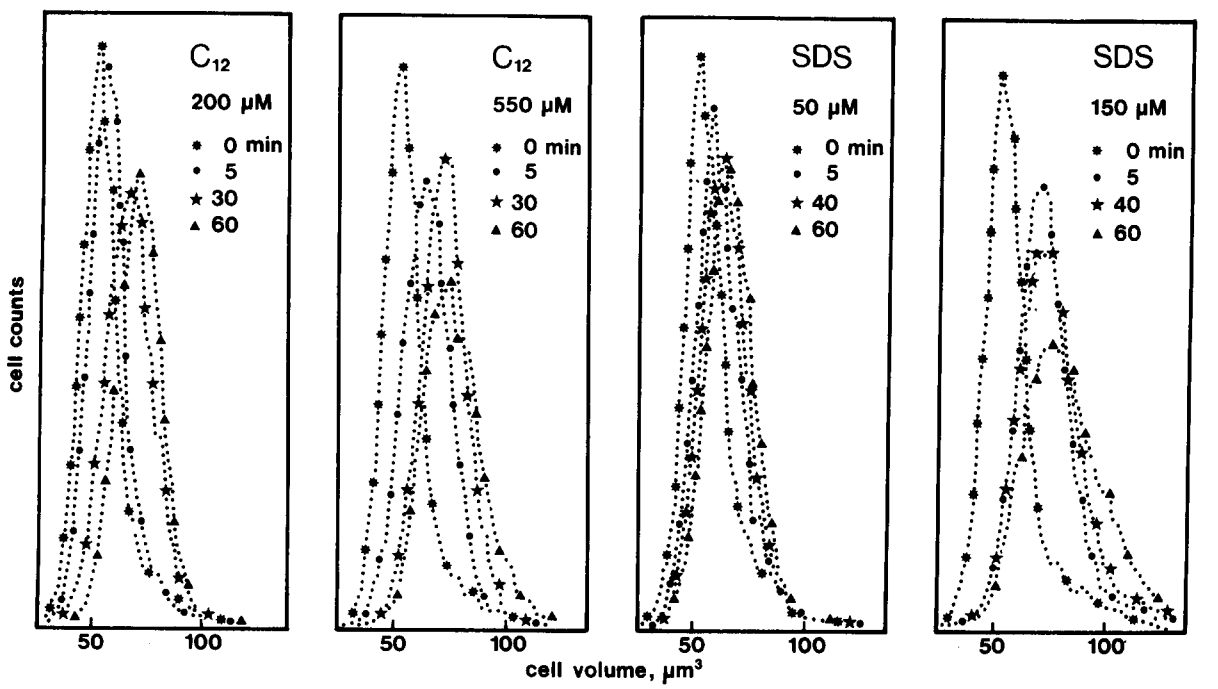

Fig. 3. Time related shift in volume-distribution plots. The lower concentration is for dodecyltrimethylammonium bromide (C_{12}) non-haemolytic and for sodium dodecyl sulphate (SDS) low-haemolytic (under 5% haemolysis). The higher concentration gives 50% haemolysis following 1 h incubation. Each curve represents a single experiment. Erythrocyte concentration at incubation was $(1.6–1.7) \cdot 10^{11}$ cells/l and temperature 37 ± 0.5°C.

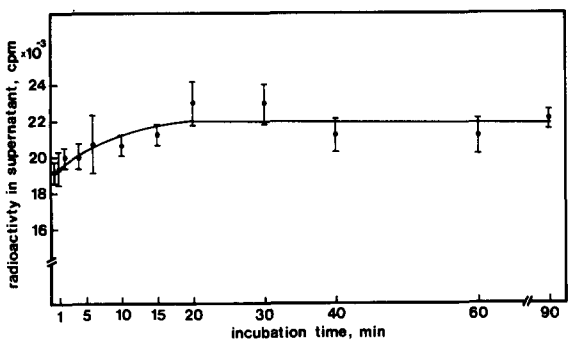

Fig. 4. Adsorption of [^{14}C]CTAB to rat erythrocytes at a prelytic concentration of CTAB (5 μM). Erythrocyte concentration was (1.6–1.7) · 10^{11} cells/l, incubation temperature 37 ± 0.5°C and the total amount of radioactivity in a sample 183 000 cpm.

interference by released haemoglobin with the adsorption of CTAB to the erythrocytes. As can be seen from Fig. 4, equilibrium was achieved within the first minutes of incubation. During the following 90 min the amount of radioactivity in the supernatant was constant. At the concentration used about 88% of the added amount of [^{14}C]CTAB was adsorbed to the erythrocytes. This corresponds to about 1.6 · 10^7 CTAB molecules per erythrocyte.

Discussion

The crenation of the erythrocytes (in about 30% of the cells) occurring prior to the addition of the surfactants may to some extent have interfered with the evaluation of the shape transformations induced by the surfactants. This crenation of the erythrocytes was probably caused by the washing off of the plasma components coating the erythrocytes, and by a less extensive washing it could have been avoided. However, in order to minimize possible effects on the erythrocyte shape caused through interaction of the surfactants with the coating components we considered it important to remove most of these components. To ensure that uncontrolled cooling during the examination of the samples under the microscope did not interfere with our evaluation of the shape transformations some experiments were performed with warmed (37°C) coverslips. The samples were examined immediately (within 5–10 s) under the micrscope. The results obtained with this procedure did not differ from those obtained with the procedure routinely used.

It is generally assumed that anionic amphiphilic compounds induce echinocytic and cationic amphiphilic compounds stomatocytic shape changes in erythrocytes [1,7]. Several observations made in the present study show that this generalization is an oversimplification. Firstly, the first shape changes to occur with both the alkyltrimethylammonium salts (cationic) and SDS (anionic) were echinocytic. Secondly, the shape alterations which followed in the case of the alkyltrimethylammonium salts were dependent on the concentration of the surfactants. Stomatocytic shapes appeared only above certain concentrations and after a considerable time of incubation (10–30 min). The C_{12} homologue caused stomatocytic shapes only at haemolytic concentrations. A similar complex effect of quaternary ammonium compounds on the erythrocyte shape has previously been reported by Sheetz and Singer [2] and by Fujh and associates [3]. Thirdly, SDS cannot unconditionally be considered an echinocytogenic agent since erythrocytes crenated by SDS at 37°C were transformed into stomatocytes when they were cooled to room temperature. Fourthly, ghosts produced at lytic concentrations of SDS were stomatocytes and not echinocytes.

The shape alterations in erythrocytes induced by amphiphilic compounds seem to be complex and the mechanisms underlying them are still poorly understood. According to a theory presented by Sheetz and Singer [2,12] anionic amphiphilic compounds are preferentially bound to the outer half of the bilayer and cationic ones to the inner. This discrimination between anionic and cationic compounds is attributed to electrostatic interaction with the negatively charged phosphatidylserine which is mainly localized to the inner half of the bilayer. The consequence of such an asymmetric distribution is that anionic compounds expand the area of the outer half of the bilayer relative to the inner and cause echinocytic shapes. The cationic compounds do the opposite and cause stomatocytic shapes. Cationic compounds with a low rate of diffusion across the lipid bilayer, such as quaternary ammonium compounds, should, according to Sheetz and Singer [2], first cause echinocytic shape alterations but when equilibrium is achieved they are intercalated mainly into the inner half of the

bilayer and thus cause stomatocytic shape alterations.

The theory developed by Sheetz and associates [2, 12,13] is indeed attractive. It appears to explain in a convincing way the complex shape alterations induced by the surface-active alkyltrimethylammonium salts, because these surfactants first produced echinocytic shapes but as incubation proceeded the echinocytes were transformed into stomatocytes. The present study, however, does not entirely support this theory. Firstly, the shape changes of SDS-crenated erythrocytes into stomatocytes upon cooling and the fact that ghosts produced at lytic concentrations of SDS were stomatocytes and not echinocytes do not coincide with the reasoning. Secondly, in the adsorption study involving [^{14}C]CTAB it was found that equilibrium was achieved very rapidly. A slow flip of CTAB molecules to the inner half of the bilayer should have been reflected in the supernatant radioactivity but this was not the case. On the whole it is difficult to imagine a flip of the charged alkyltrimethylammonium surfactants to the inner half of the lipid bilayer in a relatively short time. Recent data indicate that cationic amphiphilic agents can penetrate into the inner part of the lipid bilayer [14,15]. However, a translocation of such agents between the outer and inner part of the bilayer is apparently rather slow, and the kinetics and details of such a process are still not known.

The interpretation of the drug-induced shape alterations made by Sheetz and associates seems to suffer from some uncertainty. In developing their view they based their reasoning mainly on the two following conclusions drawn from experimental observations: (a) if erythrocytes crenated with a drug are rapidly washed at 0°C the drug in the outer part of the bilayer is largely removed and the erythrocytes will be transformed into stomatocytes because the drug left in the inner part of the bilayer now expands the inner part of the bilayer relative to the outer part [2]; (b) when erythrocytes are simultaneously exposed to a stomatocytogenic and an echinocytogenic agent these agents will independently and simultaneously bind to the membrane and in an appropriate combination there will be a compensatory effect on the cell shape [13]. The first of these assumptions is in our opinion uncertain. In order to demonstrate the reversal of shape transformations, drug-crenated erythrocytes were washed once in cold drug-free buffer and then fixed [2]. The shape transformation observed following this procedure may be due to a temperature effect, since, as was found in the present investigation, erythrocytes crenated with SDS were transformed into stomatocytes upon cooling. Furthermore, it appears unlikely that a single rapid wash in a cold buffer solution could remove most of an amphiphilic compound intercalated in the outer part of the lipid bilayer. In the case of C_{16} only small amounts of the surfactant were removed from the erythrocyte membrane by repeated washes [14]. Concerning the second assumption it appears uncertain whether two amphiphilic compounds with opposite charge are independently bound to the erythrocyte membrane when simultaneously present in the buffer solution. It is known that amphiphilic compounds of opposite charges at concentrations below critical micelle concentration may form ion-pairs or aggregates. It remains to be shown that this did not occur as regards the oleate and chlorpromazine used by Sheetz and Singer in their study [13]. If the compounds are not independently bound one cannot speak of the shape transformation observed as a result of a compensating effect.

Interpretation of the shape alterations induced by amphiphilic compounds entirely on the basis of an asymmetric distribution in the lipid bilayer is in our opinion not sufficient as such. The molecular basis of the shape transformations induced by amphiphilic compounds is probably very complex. A considerable body of evidence suggests that the erythrocytes contain a 'cytoskeleton' which influences the cell shape [15,16], and it appears that this 'cytoskeleton' is involved also in shape transformations induced by amphiphilic agents.

The increase in the cell volume prior to an increased loss of K^+ in the presence of the alkyltrimethylammonium salts is very puzzling. At a concentration giving maximal protection against hypotonic haemolysis [10] there was no increased loss of K^+, but all the alkyltrimethylammonium homologues increased the volume by about 30%. The C_{14} and the C_{16} homologue induced at this concentration (15 and 25 μM, respectively) stomatocytes and sphero-stomatocytes, whereas the C_{12} homologue (120 μM) caused echinocytes and sphero-echinocytes. SDS, on the other hand, behaved quite differently in that no volume increase occurred prior to the loss of K^+,

although a transformation into echinocytes and sphero-echinocytes occurred before the loss of K^+ increased.

The volume increase induced at lytic concentrations of the surfactants is easily described in terms of a colloid osmotic process. The surface-active agents studied should orient in the lipid bilayer of the cell membrane in such a way that the alkyl chains are situated amongst the hydrocarbon chains of the phospholipids and the polar groups at the lipid/water interface. As the adsorption proceeds, electrostatic repulsion between the polar group of the surfactant molecules in the lipid bilayer should tend to break up the lipid bilayer into 'micellar' aggregates [10,17]. The increase in cation permeability leads to faster leaks than the pumps can cope with and consequently to a colloid osmotic swelling of the cell. The volume increase induced by prelytic concentrations of the alkyltrimethylammonium salts is, however, obscure as it seems difficult to outline a mechanism leading to a volume increase without an increase in the loss of K^+. It appears that the surface-active alkyltrimethylammonium salts in concentrations below those disrupting the lipid bilayer may induce swelling by altering the transport capacity of the membrane without increasing the cation efflux. A decreased cation efflux is in fact indicated by our data since the loss of K^+ was somewhat below the control at prelytic concentrations of the surfactants. A decreased ion permeability following the treatment with prelytic concentrations of the surface-active alkyltrimethylammonium salts is also indicated by the finding made in our laboratory that phosphate efflux is decreased in rat erythrocytes treated with a prelytic concentration of the C_{16} homologue (unpublished finding). However, with the evidence available at present, it is not possible to speculate on the mechanism whereby the alkyltrimethylammonium salts at prelytic concentrations induce swelling of the erythrocytes.

Several studies indicate that the response of the Coulter Counter is complex and there seems to be some controversy regarding the response of the Coulter Counter to particles of different shapes. Some authors [18,19] have stated that the shape of the particle affects to a great extent the pulse produced in the Coulter Counter. It is thus tempting to attribute the volume increase observed at prelytic concentrations of the alkyltrimethylammonium salts to an artefact caused by the shape transformations that occurred in the presence of the surfactants. However, the studies of Batch [20] and Eckhoff [21] and several studies made on human erythrocytes [22–26] indicate that the error introduced in our measurements due to differences in shape or in rigidity of the erythrocytes is not significant. The fact that the echinocytes induced at prelytic concentrations of C_{12} had an increased volume whereas those produced at prelytic concentrations of SDS had not, seems to exclude the possibility that an artefact is involved in the volume measurements.

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