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Morphological characterization of exovesicles and endovesicles released from human erythrocytes following treatment with amphiphiles

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In order to morphologically characterize exo- and endovesicles released during treatment of erythrocytes with amphiphiles and to look for possible amphiphile-specific effects on the vesiculation pattern, human erythrocytes were treated at 37°C with amphiphiles at concentrations where they exhibit maximum protection against hypotonic haemolysis (cAH_{max}). Released exo- and endovesicles and treated cells were studied by means of transmission (TEM) and scanning (SEM) electron microscopy. All sphero-echinocytogenic amphiphiles induced a release of both spherical and tubular exovesicles. Dodecyl maltoside, a nonionic amphiphile with a bulky polar head, induced a release of predominantly tubular exovesicles, while all other sphero-echinocytogenic amphiphiles induced a release of predominantly spherical exovesicles. Some branched tubular exovesicles were released by a double-chained cationic amphiphile. Tail- and tongue-like structures were often seen on the exovesicles. Spherical exovesicles were frequently invaginated. Stomatocytogenic amphiphiles induced endovesiculation. In erythrocytes treated with most of the stomatocytogenic amphiphiles the endovesicles were clustered, but with some amphiphiles the endovesicles were randomly distributed. Large ringformed endovesicles (octaethyleneglycol alkyl ethers) and endovesicles in chains (octyl and decyl glucopyranoside) also occurred. The endovesicle membrane was often budding into the lumen of the vesicle and in some cases this could ultimately lead to a vesicle inside the endovesicle. We conclude that amphiphiles do not only trigger vesiculation, but may also specifically affect the vesiculation processes.

Introduction

In a study including about 40 amphiphilic compounds, we found that all amphiphiles inducing marked shape alterations at cAH_{max} in human erythrocytes also caused release of vesicles [1]. Sphero-echinocytogenic amphiphiles caused exovesiculation, whereas stomatocytogenic amphiphiles caused endovesiculation. If an amphiphile first induced sphero-echinocytic shapes which then, during incubation, were transformed to stomatocytic shapes, both exo- and endovesicles were released. The shape alteration induced by amphiphiles and thereby the membrane curvature, thus, determine to which side of the membrane the vesicles will be released. The mechanisms by which vesicles are released from the membrane are, however, unknown.

Although release of exo- and endovesicles has been reported following a variety of treatments of erythrocytes, there is little detailed information about the morphology of released vesicles and of treated cells. The fact that a wide variety of treatments may force erythrocytes to vesiculate, indicates that vesiculation is a generalized characteristic of the stressed erythrocyte membrane. It is, however, not known whether the type of treatment of erythrocytes or the type of compound used to cause vesiculation specifically affect the vesiculation pattern or the vesicle morphology. In a previous study [2], we obtained results indicating that the morphology of exovesicles released following treatment of erythrocytes with amphiphiles may vary with the type of amphiphile. The present work represents an extension of our previous study. The aim of the study was to find out whether the amphiphile used to cause vesiculation may have an influence on the vesiculation pattern or the vesicle morphology and if such an influence may be related to any molecular feature of the amphiphile. We here report that the morphology of released exovesicles, as well as the vesiculation pattern in case of endovesiculation, may be dependent on the type of amphiphile used. Furthermore, our study revealed several interesting findings which may shed some

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light on the events involved in the release of endo- and exo-vesicles from the erythrocyte membrane.

Materials and Methods

Chemicals. Alkyltrimethylammonium bromides, benzyl alcohol, sodium cholate, chlorpromazine hydrochloride, sodium deoxycholate, dibucaine hydrochloride, sodium glycocholate, myristoyl L-α-lysophosphatidylcholine (lysoPC), D,L-propranolol hydrochloride, sodium taurocholate, thymol and trifluoperazine dihydrochloride were purchased from Sigma. 3-(Alkyldimethylammonio)-1-propanesulphonates (Zwittergent^R), n-octyl β-D-glucopyranoside and decyl β-D-glucopyranoside were obtained from Calbiochem-Behring, sodium alkylsulphates and Triton X-100 from Merck, hexadecylpyridinium chloride and dodecyl D-maltoside from Fluka. Octaethyleneglycol mono n-alkyl ethers and pentaethyleneglycol mono n-dodecyl ether were obtained from Nikko. All amphiphiles and chemicals were standard commercial products, except the *N,N'*-bisdimethyl-1,2-ethanediamine dichloride derivative $[C_8H_{17}OOCCH_2(CH_3)_2NCH_2CH_2N(CH_3)_2CH_2COOC_8H_{17}]^{2+} \cdot 2Cl^-$ (dioctyldiQAS), which was synthesized at the Institute of Organic and Polymer Technology, Technical University of Wrocław, Poland [3]. ^{1}H-NMR spectra revealed no impurities in the dioctyldiQAS preparation. All compounds were dissolved in buffer, except dibucaine and thymol, which were dissolved in ethanol. The ethanol concentration used in the experiments was at most 0.5%. At this concentration, ethanol did not alter the shape of the erythrocytes. Haemolysis started at about 3% ethanol.

Erythrocytes. Blood was drawn from healthy donors by vein puncture into heparinized tubes. The erythrocytes were washed three times in a buffer 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 buffer at a cell density of $(1.6–1.7) \cdot 10^9$ cells/ml and kept at 4°C until used. All experiments were carried out within 30 h after the blood was drawn.

Incubation of erythrocytes. Aliquotes of a prewarmed (37°C) erythrocyte stock suspension were pipetted into polystyrene tubes or glass vials containing prewarmed (37°C) buffer and amphiphiles. The final cell density was $(1.6–1.7) \cdot 10^8$ cells/ml (about 1.5% haematocrit) and the incubations were carried out in a shaking thermostat bath at 37°C. The amphiphiles were used at sublytic concentrations found to result in maximum protection against hypotonic haemolysis (cAH_{max}) following 60 min incubation at 37°C (Tables I and II).

TABLE I

Morphological characteristics of exovesicles released from amphiphile-treated human erythrocytes

Erythrocytes (approx. $1.65 \cdot 10^8$ cells/ml) were incubated at 37°C for 60 min with amphiphiles at concentrations where they exhibit maximum protection against hypotonic haemolysis (cAH_{max}). Occurrence of spherical and tubular exovesicles; + + +, > 75%; +, < 25%.

Compound	Concn. (μM) (cAH_{max})	Morphological characteristics of exovesicles			
		spheres	occurrence of invaginated spherical exovesicles	presence of tail/tongue on spherical exovesicles	tubes
Alkylzwittergents^R					
C_{10}	3630	+ + + +	sparsely	'tongue'	+
C_{12}	263	+ + + +	sparsely	'tongue'	+
C_{14}	28	+ + + +	sparsely	not seen	+
C_{16}	14	+ + + +	sparsely	'tongue'	+
LysoPC, C_{14}	13	+ + + +	sparsely	tongue	+
Alkyltrimethylammonium bromides					
C_{10}	3300	+ + + +	frequently	tail	+
C_{12}	300	+ + + +	frequently	tail + tongue	+
C_{14}	40	+ + + +	sparsely	tail	+
C_{16}	14	+ + + +	sparsely	tail + tongue	+
Hexadecylpyridinium chloride	30	+ + + +	frequently	tail + tongue	+
DioctyldiQAS	100	+ + + +	not seen	tongue	+
Sodium alkylsulphates					
C_{10}	708	+ + + +	sparsely	tail + tongue	+
C_{12}	50	+ + + +	sparsely	'tongue'	+
C_{14}	23	+ + + +	sparsely	'tongue'	+
Sodium glycocholate	10000 [a]	+ + + +	sparsely	tail + tongue	+
Sodium taurocholate	13000 [a]	+ + + +	sparsely	tail + tongue	+
Dodecyl maltoside	40	+	sparsely	'tongue'	+ + + +

[a] Concentrations used as cAH_{max}-equivalents, see Materials and Methods.

These concentrations were, as previously described [4,5], estimated from dose-response curves from experiments carried out in the buffer diluted to an osmolarity giving about 80% haemolysis of untreated samples. In case of bile salts the dose-response curves were obscure, since the protective effect of the compounds in

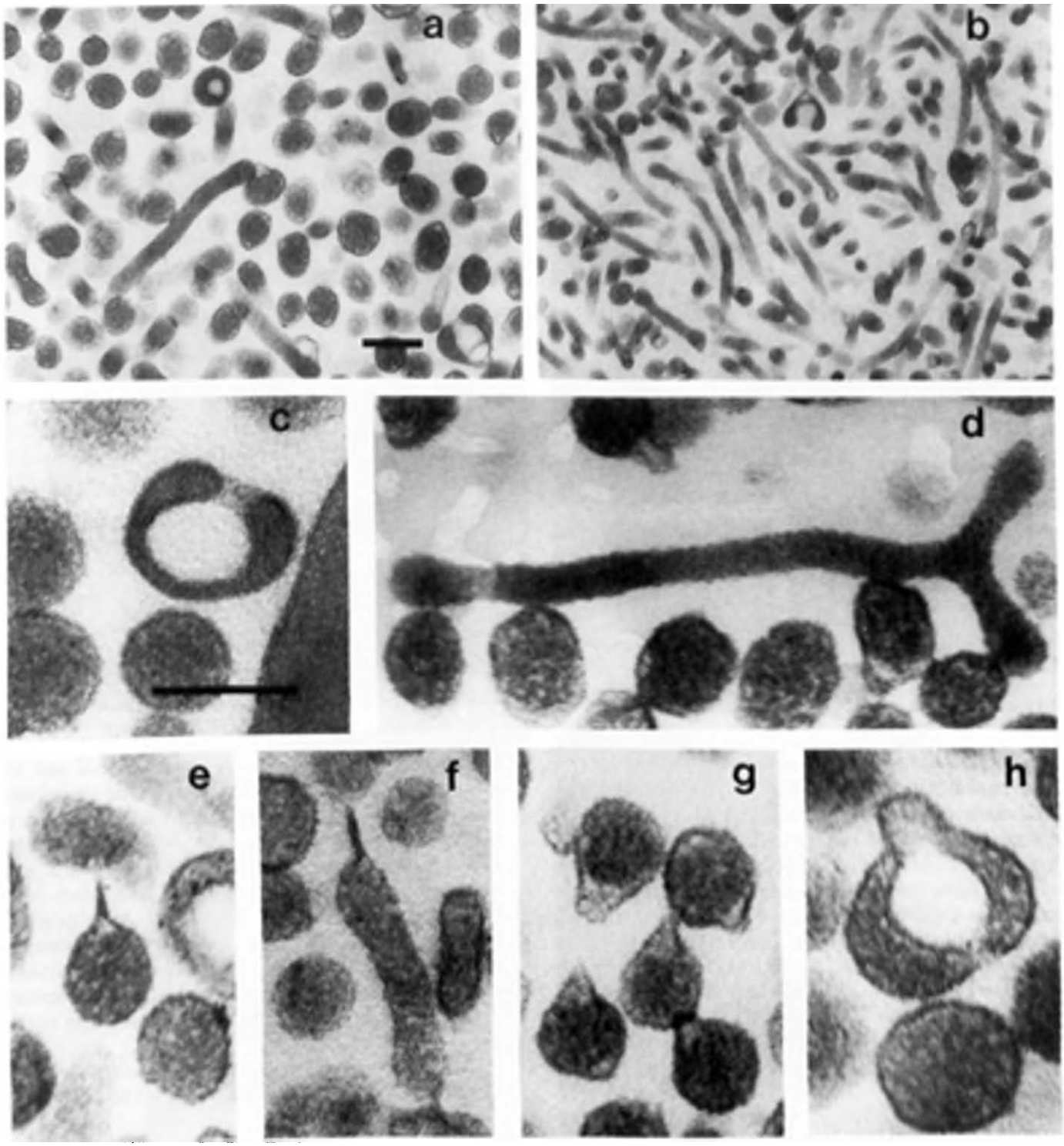


Fig. 1. TEM micrographs showing exovesicles released from human erythrocytes during incubation with amphiphiles at concentrations corresponding to cAH_{max} for 60 min at 37°C. Amphiphile concentrations and cell density as in Table I. (a), predominantly spherical exovesicles released following treatment with lysophosphatidylcholine; (b), predominantly tubular exovesicles released following treatment with dodecyl maltoside; (c), an extremely invaginated spherical exovesicle (dodecyltrimethylammonium bromide); (d), a branched tubular exovesicle (dioctyldiQAS); (e), a tail on a spherical exovesicle (hexdecylpyridinium chloride); (f), a tail on a tubular exovesicle (hexadecylpyridinium chloride); (g), tongues on exovesicles (dioctyldiQAS); (h), a complex protrusions on an invaginated spherical exovesicles (dodecyltrimethylammonium bromide). In (a) and (b) the bar represents 200 nm, in (c–h) 150 nm.

TABLE II

Pattern of endovesicles and occurrence of invaginations on endovesicles in amphiphile-treated human erythrocytes

Cell density and incubation conditions as in Table I.

Compound	Concn. (μM) (cAH_{max})	Dominating endovesiculations pattern	Invagination in endovesicles
Alkyltrimethylammonium bromides			
C_{10}	3300	cluster/random	not seen
C_{12}	300	cluster/random	not seen
C_{14}	40	cluster/random	not seen
C_{16}	14	cluster/random	not seen
Hexadecylpyridinium chloride	30	random	sparsely
Propranolol hydrochloride	3000	cluster	sparsely
Chlorpromazine hydrochloride	20 [a]	cluster	frequently
Trifluoperazine dihydrochloride	25 [a]	cluster	sparsely
Dibucaine hydrochloride	600	cluster	frequently
Sodium glycocholate	10000 [b]	cluster/random	not seen
Sodium taurocholate	13000 [b]	cluster/random	sparsely
Sodium cholate	3000 [b]	random	not seen
Sodium deoxycholate	700 [b]	random	not seen
Octyl glucopyranoside	9000	string	sparsely
Decyl glucopyranoside	740	string	sparsely
Triton X-100	0.008%	cluster/random	sparsely
Pentaethyleneglycol dodecyl ether	40	cluster/random	sparsely
Octaethyleneglycol alkyl ethers			
C_{10}	300	ring	
C_{12}	44	ring	
C_{14}	24	ring	
C_{16}	23	ring	
Benzyl alcohol	60	random	sparsely
Thymol	800	random	sparsely

[a] With trifluoperazine and chlorpromazine sublytic concentrations higher than cAH_{max} (we used 50 and 200 μM, respectively) were requires to obtain endovesiculation.

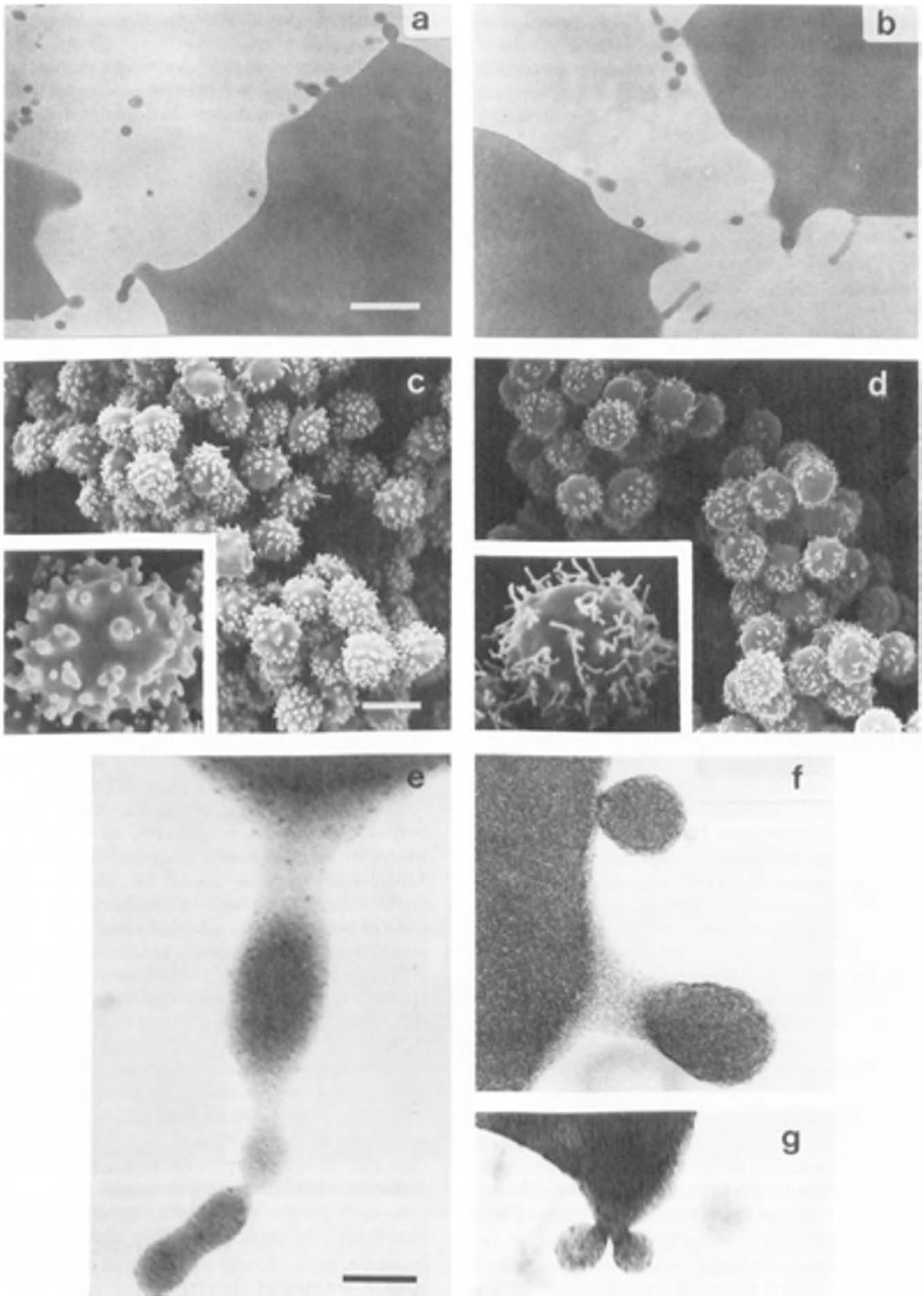
[b] Concentration used as cAH_{max}-equivalents, see Materials and Methods.

hypotonic buffer increased over concentrations which gave haemolysis in isotonic buffer. It is likely that this is due to differences in their solubility or in their association colloidal behaviour in hypotonic and isotonic buffer. As an equivalent to cAH_{max} for the bile salts we have, therefore, used the highest prelytic concentration ($\leq 2\%$ haemolysis). For those amphiphiles of which the critical micellar concentration (CMC) in buffer were found in the literature, the concentrations used were, except in case of glyco- and taurocholate [6], well below their CMC-values [7,8].

Processing of erythrocytes and exovesicles for TEM. Following incubation with amphiphiles at cAH_{max} for 60 min (in some cases 5 min) erythrocytes were pelleted by centrifugation at $100 \times g$ for 7 min and exovesicles were, after an additional similar centrifugation of the supernatant, pelleted by centrifugation of the supernatant at $40000 \times g$ for 30 min. Exovesicles and erythrocytes were separately fixed in 2% glutaraldehyde in buffer for 1 h at 4°C and postfixed in 1% OsO_4 in buffer for 30 min at 4°C. After dehydration in a graded series of acetone/water, the samples were embedded in Epon. The sections were stained with lead citrate and post-stained with uranyl acetate before examination in a JEOL 100SX electron microscope.

Processing of erythrocytes and exovesicles for SEM. Erythrocytes were treated with amphiphiles at cAH_{max}

Fig. 2. TEM and SEM micrographs showing protrusions and prestage exovesicles on human erythrocytes treated with amphiphiles at concentrations corresponding to cAH_{max} at 37°C. Amphiphile concentrations and cell density as in Table 1. (a), erythrocytes treated with dodecylzwittergent[R] for 5 min; (b), erythrocytes treated with dodecyl maltoside for 5 min; (c), erythrocytes treated with dodecyltrimethylammonium bromide for 30 s; (d), erythrocytes treated with dodecyl maltoside for 30 s; (e), a tubular protrusion apparently in the process of breaking up into spherical vesicles, note the electron lucent necks (dioctyldiQAS, 60 min); (f), a spherical vesicle connected to the erythrocyte membrane by a electron lucent neck (dioctyldiQAS, 60 min); (g), twin vesicles which apparently are going to be a branched tubular vesicle (dioctyldiQAS, 60 min). In (a) and (b) the bar represents 500 nm, in (c) and (d) 5 μm and in (e–g) 150 nm.


a
b
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for 0.5, 2 or 5 min and then pelleted as above. Exovesicles were pelleted as above, following treatment of erythrocytes with amphiphiles at cAH_{max} for 60 min. Erythrocytes and vesicles were then fixed, postfixed and dehydrated as above. After critical-point drying and gold-sputtering, erythrocytes and vesicles were examined in a Cambridge Instruments S360 microscope.

Results

For the TEM studies, erythrocytes were treated with amphiphiles at cAH_{max} for 60 min (in some cases 5 min), whereafter released exovesicles and treated erythrocytes were further processed. Morphological features of released exovesicles are presented in Table I. The dominating endovesiculation pattern, as well as the occurrence of invaginations on endovesicles are presented in Table II. The tables also show the concentrations of amphiphiles used. For the SEM studies erythrocytes were pelleted after treatment with amphiphiles at cAH_{max} for 0.5, 2 or 5 min and exovesicles were pelleted after treatment of erythrocytes for 60 min. All experiments in the TEM and SEM studies were repeated at least two times.

Exovesicles

In these studies, erythrocytes were treated with amphiphiles which previously had been shown to cause a release of acetylcholinesterase (AChE) from erythrocytes at cAH_{max} [1,2]. AChE is a membrane-bound enzyme frequently used to monitor the release of membrane vesicles from the erythrocyte membrane [9–12]. Amphiphiles causing a release of exovesicles are sphero-echinocytogenic [1,2]. As previously shown, the amount of AChE released is, depending on the type of amphiphile used, about 5–20% of the total AChE activity of the erythrocytes [2]. It should, however, be noticed that AChE may be enriched in exovesicles [9,11,12]. The amount of AChE released can, thus, not be regarded as a reliable measurement of the membrane area released.

The exovesicles released were of two types, spherical and tubular. All the sphero-echinocytogenic amphiphiles caused a release of both types of vesicles. Predominantly spherical vesicles were released by all the amphiphiles (Fig. 1a, Table I), except with dodecylmaltoside where the vesicles were predominantly tubular (Fig. 1b).

The diameter of the spherical exovesicles was about 150 nm and no differences in size between spherical exovesicles released by the different amphiphiles could be observed. The length of the tubular exovesicles was usually 400 to 850 nm, but a tubular exovesicle as long as 1120 nm was seen (dodecyl maltoside). The diameter of the tubular exovesicles varied with the length of the vesicle so, that it was smaller when the vesicle was longer. Tubular exovesicles induced by dodecyl maltoside had a smaller diameter (approx. 37 nm) than tubular exovesicles induced by other amphiphiles (approx. 55 nm).

Several interesting features were observed on the exovesicles. Invaginated spherical exovesicles were seen in all preparations, except in samples treated with dioctyldiQAS, and they were especially abundant in samples treated with decyltrimethylammonium bromide, dodecyltrimethylammonium bromide and hexadecylpyridinium chloride. Some of the invaginated exovesicles had the appearance of extremely cup-shaped 'mini-stomatocytes' (Fig. 1c). In samples treated with dioctyldiQAS many of the tubular exovesicles were branched. The branched exovesicles were of different shapes but they were mostly Y-shaped (Fig. 1d). A tail (Fig. 1e) or a tongue-like protrusion (Fig. 1g) was frequently seen on spherical vesicles released by most of the amphiphiles. Tails (Fig. 1f) and protrusions also occurred on the tips of some of the tubular vesicles. The tails had a rod-like appearance and seemed to consist of electron-dense material. They seemed to be slightly twisted structures with a length of 45–80 nm. The tongue-like protrusions often contained less electron dense material than the rest of the vesicle. On exovesicles induced by some amphiphiles small and diffuse tongue-like protrusions (marked in Table I as 'tongues') was seen. Occasionally, intermediate forms of tails and tongues were seen. In samples treated with alkyltrimethylammonium bromides complex, protrusions were seen on some of the invaginated vesicles (Fig. 1h). One large membrane domain seems to be involved in creating the invagination as well as the protrusion. Exovesicles released by glycocholate and taurocholate were often haemoglobin-depleted.

In order to visualize the release of the exovesicles from the membrane, erythrocytes were fixed after a treatment for 5 min with amphiphiles. Two types of vesiculation patterns could be distinguished. In erythrocytes treated with amphiphiles inducing a release of predominantly spherical vesicles, spherical exovesicles or a chain of a few spherical vesicles appeared to be pinched off from the membrane (Fig. 2a). In erythrocytes treated with dodecyl maltoside, which gave rise to predominantly tubular exovesicles, both tubular and spherical vesicles appeared to be pinched off from the membrane (Fig. 2b).

The SEM studies confirmed the results from the TEM studies and gave further information about the extent of cell surface involved in the vesiculation process. Fig. 2c shows erythrocytes treated for 30 s with dodecyltrimethylammonium bromide, which causes a release of predominantly spherical vesicles. In these cells small echinocytic protrusions, often collected into bouquets, were seen and there were only a few long tubular protrusions. Interestingly a large bald area,

free of echinocytic protrusions, were seen on many erythrocytes. Following treatment with dodecyltrimethylammonium bromide for 5 min, most of the erythrocytes had left the sphero-enchinocytic stage and were transformed to echinocytes and discocytes. Few cells with protrusions were seen in these preparations (results not shown). Fig. 2d shows erythrocytes treated with dodecyl maltoside for 30 s. These cells had predominantly tubular protrusions which were collected into bouquets and they had frequently a large area free

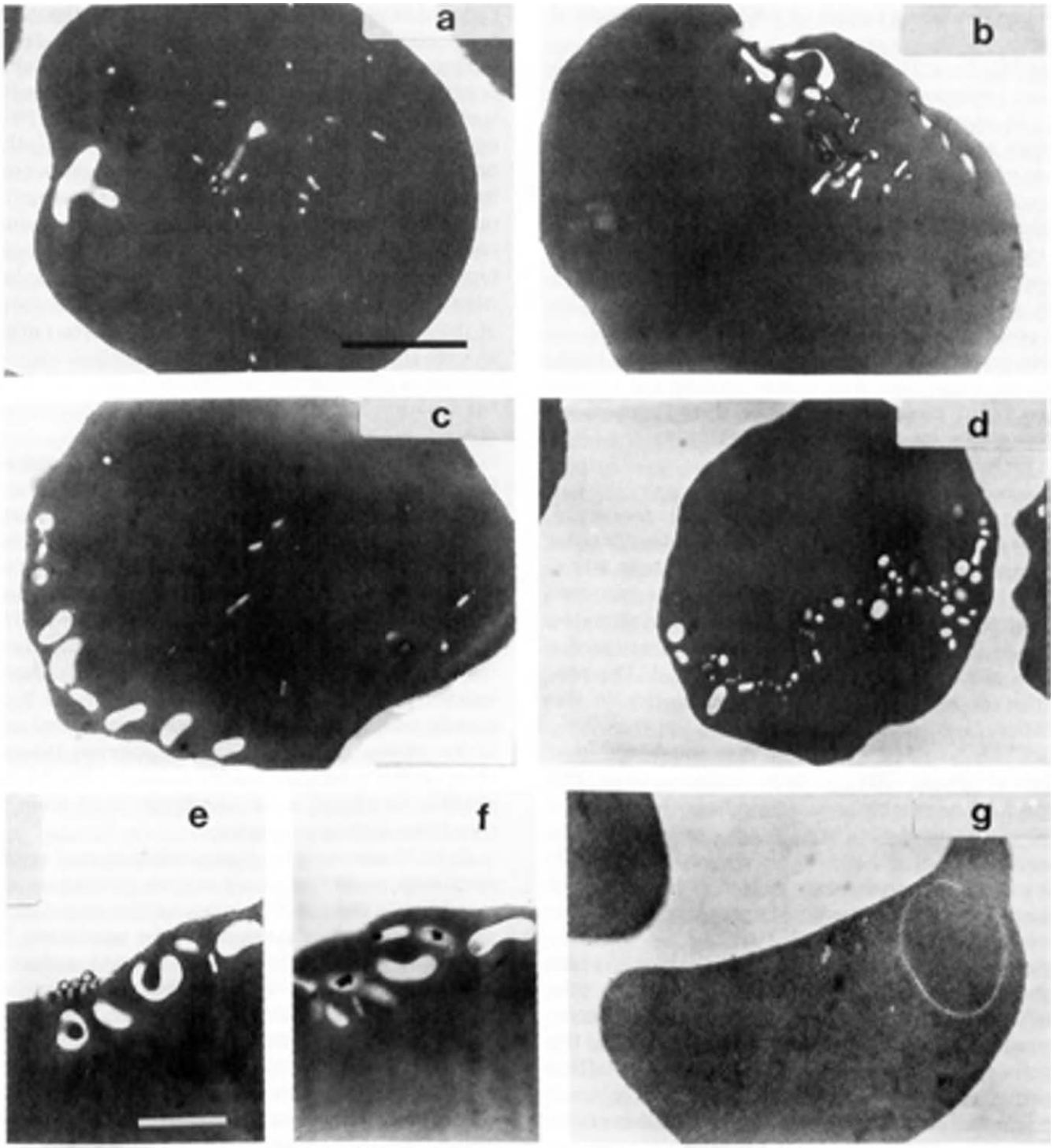

Fig. 3. TEM micrographs showing endovesicles in human erythrocytes treated with amphiphiles at concentrations corresponding to cAH_{max} for 60 min at 37°C. Cell density and amphiphile concentrations as in Table I and II. (a), randomly distributed endovesicles (cholate); (b), a cluster of vesicles in one pole of the cell (propranolol); (c), a cluster of vesicles near the rime of the cell plus randomly scattered vesicles (Triton X-100); (d), a string of clustered vesicles (octyl glucopyranoside); (e), invaginations on endovesicles (dibucaine); (f), vesicles formed within the lumen of endovesicles (chlorpromazine); (g), a large ringformed endovesicle (octaethyleneglycol dodecyl ether). In (a–d) and (g) the bar represents 1 μm, in (e) and (f) 500 nm.

from protrusions. Erythrocytes treated for 5 min with dodecyl maltoside had a similar appearance (results not shown). SEM micrographs of exovesicles collected following treatment of erythrocytes with dodecyl maltoside and dodecyltrimethylammonium bromide confirmed that dodecyl maltoside causes a release of predominantly tubular vesicles and dodecyltrimethylammonium bromide a release of predominantly spherical vesicles (results not shown).

In Fig. 2e–g TEM-micrographs of prestage exovesicles on erythrocytes treated with dioctylQAS are shown. Fig. 2e shows a tubular protrusion probably in the process of breaking up into spherical exovesicles. The areas which apparently are going to be separate spherical vesicles are separated by necks. Fig. 2f shows the shedding of spherical vesicles from the membrane. One of the vesicles is connected to the erythrocyte membrane with a long neck. Fig. 2g shows the shedding of twin vesicles from the membrane. These twin vesicles are possibly going to be a branched tubular vesicle.

No preparations of exovesicles released after treatment of erythrocytes with sodium cholate and sodium deoxycholate were done, because these amphiphiles induce a very small release of AChE [1].

Endovesicles

Stomatocytogenic amphiphiles induce endovesiculation [1,2]. There are, however, amphiphiles (hexadecylpyridinium chloride, alkyltrimethylammonium bromides, bile salts) which first induce sphero-echinocytic shapes and a release of exovesicles, whereafter the shape of the erythrocytes reverts towards stomatocytic shapes and endovesiculation occurs [1,2,13]. The proportion of erythrocytes showing endovisicles in the TEM-preparations was in several cases less than 20%, which may be taken to indicate that endovesiculation starts at about cAH_{max}. With trifluoperazine and chlorpromazine sublytic concentrations higher than cAH_{max} (we used 50 μM and 200 μM, respectively) were required to obtain endovesiculation. No endovesicles were seen in erythrocytes treated with amphiphiles inducing sphero-echinocytic shapes only or in untreated cells.

Several different endovesiculation patterns, which probably reflects differences in the vesiculation process, could be distinguished (Fig. 3). In erythrocyte treated with cholate (Fig. 3a) and deoxycholate, the vesicles were randomly scattered within the cell. This vesiculation pattern indicates that the sites from which the vesicles were formed, were scattered all over the cell surface. With most of the amphiphiles the endovesicles were clustered to one (Fig. 3b) or sometimes two poles of the cell. The number of endovesicles in these clusters was usually 10–40. These clusters of vesicles were probably formed from large membrane invaginations from which individual vesicles were shed. With some amphiphiles there were, besides a cluster of vesicles near the rim of the cell, randomly scattered vesicles (Fig. 3c). In erythrocytes treated with octyl glucopyranoside (Fig. 3d) and decyl glucopyranoside, the endovesicles often formed a pattern like a string of pearls. In a part of the endovesicles in erythrocytes treated with several amphiphiles the membrane of the endovesicle was budding off into the lumen of the vesicle. This phenomenon was specially abundant in erythrocytes treated with dibucaine (Fig. 3e) and chlorpromazine and this budding process could ultimately lead to a vesicle inside the endovesicles (Fig. 3f). In erythrocytes treated with octaethyleneglycol alkyl ethers one or a few large and narrow ringformed vesicles were formed (Fig. 3g). In many of these cells the ringstructure was closed. These vesicles look like long tubular vesicles, but they are more likely large vesicles, possibly formed when an invagination has filled up a large stomatocytic invagination. In this case, a large domain of the membrane seems to be involved in the creation of large single vesicles.

Discussion

The present study shows that exovesicles released from amphiphile-treated erythrocytes can be released from the membrane both as spheres and tubes. Formation of tubular projections on erythrocytes has previously been observed following a variety of treatments [14,15] and it is thought that single spherical vesicles may be shed from the tubular structures following their release from the erythrocytes [15,16]. This may also be the case for some of the amphiphile-induced spherical vesicles, since chains of spherical vesicles were occassionally seen and some tubular vesicles appeared to be in the process of breaking up into spheres. However, most of the spherical vesicles formed following amphiphile treatment were apparently shed from the membrane surface as spheres.

In SEM micrographs of amphiphile-treated erythrocytes large areas free of echinocytic protrusions were seen (Fig. 2c and 2d). This indicates that vesiculation is restricted to certain domains on the membrane. The release of a vesicles from the membrane surface requires a membrane fusion at the base of the vesicle to released and the initiation of the fusion is apparently dependent on some kind of bilayer destabilizing molecular events. Since most of the amphiphiles used in the present study have a molecular shape that differs from that of the phospholipids of the lipid bilayer, one may expect that they cause a segregation of lipids in the plane of the membrane or that the amphiphiles themselves are segregated from the membrane lipids. Long-chained alcohols have been found to induce the formation of rod-shaped membrane projections in human erythrocytes [17]. The process of rod formation

was associated with an accumulation of sphingomyelin and the alcohol in the rod-shaped projections. It seems reasonable to assume that a segregation of membrane lipids and/or intercalated amphiphiles to certain domains of the membrane may cause a bilayer destabilization, leading to membrane fusion and vesicle release. If vesiculation is initiated by a segregation of membrane lipids, one would expect the lipid composition of vesicles to differ from that of the native erythrocyte membrane. According to lipid analyses made in our laboratory (Hägerstrand, H., unpublished data), and by others [10], the phospholipid composition of exovesicles released by amphiphile-treatment is similar to that of the red cell membrane. However, our analyses (Hägerstrand H., unpublished data) showed the exovesicles are depleted in cholesterol. Thus, some segregation of membrane lipids seems to occur following treatment of erythrocytes with amphiphiles Whether such a segregation, possibly accompanied by an alteration in the membrane lipid asymmetry [18], is enough to initiate vesiculation or whether it is the intercalated amphiphiles which by themselves destabilize the bilayer remains to shown.

Both spherical and tubular exovesicles were seen in all vesicle preparations. This indicates that whether the vesicle attains a spherical or a tubular shape is not simple determined by the type of amphiphile used to induce vesiculation or by an effect exerted by the amphiphile on the vesicle membrane. This view is supported by results from other laboratories, showing that both tubular and spherical exovesicles may be released also in non-amphiphile-induced (A23187/Ca^{2+}-treatment) exovesiculation [16] and that the age of the erythrocyte treated may influence the shapes of the exovesicles released [14]. However, two amphiphiles in our study seem to be able to specifically affect the shape of the vesicles released. Dodecyl maltoside, a nonionic amphiphile with a bulky polar head group, induced predominantly tubular vesicles, while all other sphero-echinocytogenic amphiphiles induced predominantly spherical exovesicles. The tubular exovesicles induced by dodecyl maltoside also had a smaller diameter than the tubular exovesicles induced by other amphiphiles. Thus, it seems as if the charge or the size of the poral head of the amphiphile may influence the vesicle shape. Since dodecyl maltoside was the only nonionic amphiphile in our study which induced exovesiculation, it is not possible to decide whether or not the nonionic character of the amphiphile has any significance in determining the shape of the vesicles. The bulky polar head of dodecyl maltoside gives the amphiphile a marked wedge-shaped character. It is tempting to assume that the wedged shape of the amphiphile is the factor that directly or indirectly influence the vesicle shape and leads to smaller diameter (high curvature) of the tubular vesicles. In this context, it is interesting to note that dodecyl maltoside differs from all the other nonionic amphiphiles used in the present study by being the only amphiphile causing exovesiculation. All the other nonionic amphiphiles induced stomatocytosis and endovesiculation. Dodecyl maltoside, on the other hand, is a potent echinocytogenic amphiphile. In the context of the bilayer couple hypothesis [19] this should be taken to indicate that dodecyl maltoside is trapped in the outer monolayer of the bilayer, while the other nonionic amphiphiles are translocated to the inner monolayer. Dodecyl maltoside has a more bulky polar head than the other nonionic amphiphiles. The bulky polar head of dodecyl maltoside is, assuming the bilayer couple hypothesis to be correct in explaining shape alterations caused by amphiphiles, apparently the reason for the inability of dodecyl maltoside to translocate to the inner monolayer. Interestingly, decyl glucopyranoside, which differs from dodecyl maltoside mainly by having only one hexose ring in the polar head instead of two, appears to be translocated to the inner monolayer. The other amphiphile that seems to specifically influence the vesicle shape is dioctyldiQAS, because branched tubular vesicles were only seen among vesicles induced by this amphiphile. DioctyldiQAS differs from the other alkyl derivatives used in this study by having two alkyl chains. Since the length of the alkyl chains is short, about half that of the acyl chains of the bilayer phospholipids, it is difficult to predict the effect of dioctyldiQAS in the membrane according to the shape-structure concepts [20]. By being charged this amphiphile should, however, be trapped in the outer monolayer of the bilayer and, due to the two short alkyl chains, it should probably exert a considerable stress on the outer monolayer.

A tail or a tongue was often seen on exovesicles. Tails have previously been seen on vesicles released from erythrocytes treated with A23187/Ca^{2+} [16,21] and following in vitro ageing of erythrocytes [21,22]. These tails have been found to be enriched in polypeptide 4.1 and it has been proposed that the tails are relics of the membrane fusion events that led to the release of vesicles [21]. It is thought that segregation of polypeptide 4.1 into the tail region may be significant in the membrane fusion process necessary for the release of the vesicle [21]. It is possible that both tails and tongues are relics of the membrane fusion event that lead to the release of the vesicles from the membrane, and that they reflect differences in the molecular events in the release process. It is tempting to assume that the tongues are relicts of the necks observed between vesicles and the erythrocyte membrane (Fig. 2f) and of the necks observed on some tubular vesicles which apparently were going to be broken up into spherical vesicles (Fig. 2e).

The invaginations seen one some of the exovesicles

are obscure. The shape of invaginated exovesicles resemble 'mini-stomatocytes'. Similar invaginations on vesicles have previously been reported to be formed on egg phosphatidylcholine vesicles upon removal of the detergent (octyl glucoside) from the phospholipid/detergent mixture [23]. It has also been reported that small biconcave erythrocytelike vesicles may be formed when extracting the alcohol from rod-shaped membrane projections induced on human erythrocytes by incubation with phosphatidylcholine/cholesterol/C_{16-18}-alcohol vesicles [17]. In erythrocytes, stomatocytosis is thought to arise from an increase in the inner area of the lipid bilayer relative to the outer [19]. If the invaginations on the exovesicles are interpreted according to the bilayer couple hypothesis, they can be taken to indicate that some exovesicles with an imbalance between the area of the inner and the outer monolayer are formed during the shedding process. It has, however, been shown that exovesicles are depleted in cytoskeletal proteins [10,24,25]. The erythrocyte cytoskeleton is thought to be important in stabilizing the erythrocyte shape [26]. It is, therefore, possible that an absence of a cytoskeleton makes the vesicle shape unstable and that other factors (see Ref. 27), besides an imbalance in area between the two monolayers, may affect the shape of the vesicles. Interestingly, invaginations also occurred on endovesicles. The endovesicles formed should be inside-out and in the lumen of these vesicles, right-side-out vesicles could be formed. This indicates that some of the endovesicles formed are unstable and that they after being shed from the membrane can undergo complex alterations.

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Recent observations in our laboratory show that exovesicles released from human erythrocytes following treatment with decyl maltoside and sucrose monolaurate at cAH_{max} have predominantly a tubular structure similar to that of the vesicles released by dodecyl maltoside, which are described in the present study. Since these three amphiphiles have a large hydrophilic group, the results support the idea the large hydrophilic group is the main factor determining the tubular shape of the exovesicles.

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