Biochimica et Biophysica Acta, 641 (1981) 79—87

BBA 79117

EFFECT OF DIMYRISTOYL PHOSPHATIDYLCHOLINE ON INTACT ERYTHROCYTES

RELEASE OF SPECTRIN-FREE VESICLES WITHOUT ATP DEPLETION

PETER OTT [a,*], MICHAEL J. HOPE [b,**], ARIE J. VERKLEIJ [c], BEN ROELOFSEN [b], U. BRODBECK [a] and LAURENS L.M. VAN DEENEN [b]

[a] *Medizinisch-Chemisches Institut der Universität, P.O. Box, CH-3000 Bern (Switzerland),* [b] *Biochemistry Department and* [c] *Institute of Molecular Biology, State University of Utrecht, Transitorium III, Padualaan 8, De Uithof, 3508 TB Utrecht (The Netherlands)*

(Received August 25th, 1980)

Key words: Vesicle release; Dimyristoyl phosphatidylcholine; ATP depletion; Phospholipid; Membrane protein; Spectrin; (Erythrocyte membrane)

Summary

Incubation of human erythrocytes with suspensions of sonicated dimyristoyl phosphatidylcholine resulted in dramatic morphological changes of the cells and release of membrane vesicles. The shedding of membrane vesicles was not preceded by ATP depletion and only occurred at temperatures of incubation that were above the phase transition temperature of dimyristoyl phosphatidylcholine. Membrane vesicles were separated from intact erythrocytes and exogenous dimyristoyl phosphatidylcholine by a series of centrifugation steps. The lipid composition of the membrane vesicles was similar to that of the native erythrocyte, and the predominant membrane proteins were band 3, glycophorin and acetylcholinesterase. Spectrin was not detected. Freeze-fracture electron microscopy showed vesicles (150 nm in diameter) with protein particles embedded in the lipid bilayer.

Introduction

Erythrocytes are readily induced to undergo membrane vesiculation. Prolonged incubation of cells in a glucose-free medium results in the discocyte-

* To whom correspondence should be addressed.
** Present address: Department of Biochemistry, University of British Columbia, Vancouver, B.C. V6T 1W5, Canada.

to-spherocyte shape change which precedes the 'budding-off' of vesicles. This process has been correlated with the depletion of intracellular ATP [1] and the resulting dephosphorylation of spectrin, a major component of the erythrocyte cytoskeleton, has been considered as a possible mechanism by which these morphological changes are brought about [2,3]. Vesiculation of the plasma membrane has also been observed in erythrocytes stored for transfusion which experience a gradual loss of intracellular ATP [4]. However, in this case, an ATP-dependent accumulation of membrane-penetrating metabolites was postulated to be responsible for vesicle release [5,6]. In accord with this hypothesis are the morphological changes induced by agents which penentrate the erythrocyte membrane, such as anesthetics and lysophosphatidylcholine [7–9].

In most cases the membrane vesicles released from erythrocytes have a similar composition. Their lipid content resembles that of the native red cell and the membrane proteins band 3, glycophorin and acetylcholinesterase are generally present. A recent report, however, described an apparently selective extraction of erythrocyte membrane proteins and phospholipids from the outer monolayer of the plasma membrane following incubation with dispersions of dimyristoyl phosphatidylcholine [10]. The mechanism of this extraction has not yet been described.

The present paper offers an explanation on how the release of red blood cell membrane constituents proceeds during incubation with dimyristoyl phosphatidylcholine. Furthermore, it provides new aspects of the mechanism of membrane vesicle formation.

Materials and Methods

Materials

Blood samples were obtained from healthy adult donors. Erythrocytes were immediately separated from plasma by centrifugation for 15 min at 3000 rev./min, washed three times with 10 mM Tris-HCl, pH 7.4, containing 144 mM NaCl, and used within 2 h of collection. Dimyristoyl phosphatidylcholine, prepared according to standard procedures [11], was a kind gift of Dr. R.A. Demel, Utrecht. Firefly lantern extract was obtained from Sigma, St. Louis, MO, U.S.A. and ATP (crystallized) from Boehringer, Mannheim, F.R.G. Bovine serum albumin was from Poviet Producten, Amsterdam, The Netherlands. All other reagents were either from Fluka AG, Buchs, Switzerland, or from Merck, Darmstadt, F.R.G.

Methods

Acetylcholinesterase activity was determined according to the method of Ellman et al. [12]. Protein was measured following the procedure of Lowry et al. [13] using bovine serum albumin as standard. The ATP content in erythrocyte suspensions was determined following the procedure of Stanley and Williams [14]. Phospholipids were extracted according to the method of Renkonen et al. [15] and analyzed by the two-dimensional thin-layer chromatography technique of Broekhuize [16]. For a quantitative determination of phospholipid distributions, the spots, detected after staining with iodine

vapor, were scraped from the plate and transferred individually into test tubes for phosphorous determination according to the method of Rouser et al. [17]. Polyacrylamide gel electrophoresis was carried out following the procedure of Fairbanks et al. [18].

Treatment of erythrocytes and purification of released material were carried out as follows: 1 vol. of washed erythrocytes was incubated with 9 vol. of a dispersion of dimyristoyl phosphatidylcholine (0.5 mg lipid per ml in 10 mM Tris-HCl buffer, pH 7.4, containing 144 mM NaCl) which was obtained by sonication for 15 min using the MSE ultrasonic disintegrator MK2 operated at an amplitude of 16 μm. Unless stated otherwise, incubation of red cells was carried out at 30°C.

At appropriate times, the incubation mixture (or an aliquot) was centrifuged at 3000 rev./min for 20 min to remove the erythrocytes. The released material obtained in the supernatant of this low-speed centrifugation was concentrated and separated from dimyristoyl phosphatidylcholine by centrifugation at 30 000 $\times g$ for 30 min at 30°C in an MSE Hi-Spin 21 centrifuge equipped with an 8 $\times$ 50 ml angle rotor. The pellet was washed three times by resuspension in a 10 mM Tris-HCl buffer, pH 7.4, with 144 mM NaCl followed by centrifugation at 30 000 $\times g$ for 30 min. The supernatants which contained the bulk of dimyristoyl phosphatidylcholine were discarded. The pellet obtained after the final centrifugation step was resuspended in buffer and stored at either 4°C (overnight) or at −20°C. Erythrocytes were fixed for scanning electron microscopy with 2% glutaraldehyde in 0.05 M potassium phosphate, pH 7.4, dehydrated with solutions of increasing ethanol concentrations (0—100% ethanol) and then transferred into amyl acetate. Samples were dried with a Balzers critical-point apparatus, coated with gold using the Balzers sputtering outfit and then examined in a Philips SEM 500 scanning electron microscope. Freeze-fracture electron microscopy of released material was carried out according to the method of Ververgaert et al. [19]. Samples were quenched from 4°C and glycerol was added to prevent freeze damage.

Results

The effect of dimyristoyl phosphatidylcholine dispersions on the morphology of erythrocytes is clearly demonstrated in Fig. 1. In the absence of exogenous lipid, cells maintained a normal discocyte conformation throughout the period of incubation (Fig. 1A). However, in the presence of dimyristoyl phosphatidylcholine, erythrocytes underwent a transformation in shape from discocyte through echinocyte to sphero-echinocyte (Fig. 1B—D). Material in the process of being pinched off from the tips of membrane projections to form vesicles was easily detectable (Fig. 1D).

The course of vesiculation was followed by measuring the amount of acetylcholinesterase released from the red blood cells and by determining the phospholipid content of the erythrocytes during the incubation period (Fig. 2). An initial lag phase of 2 h was observed prior to the appearance of acetylcholinesterase in the supernatant of the incubation mixture. During this initial period the phospholipid content of the red cells increased by approx. 5%. As there was no decrease in cell number during the incubation (results not

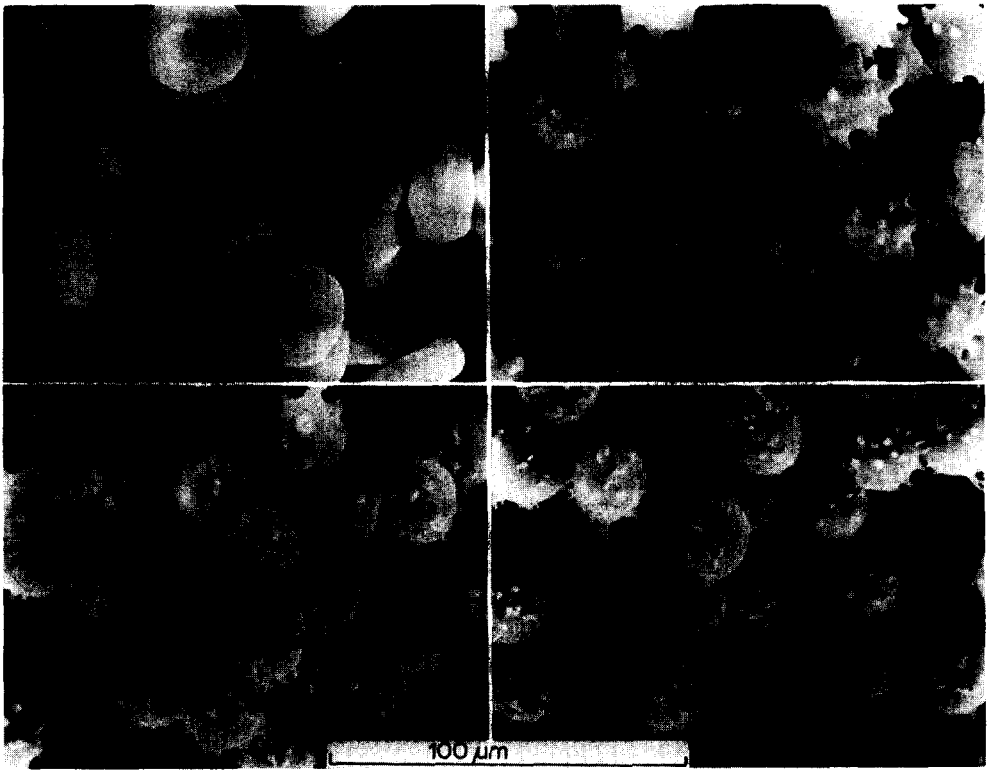

Fig. 1. Scanning electron micrographs of erythrocytes. Cells were incubated and prepared for microscopy as described in Materials and Methods. (A) Control after 90 min incubation in the absence of dimyristoyl phosphatidylcholine. (B) Cells incubated with dimyristoyl phosphatidylcholine for 60 min. (C) Same preparation as B but incubated for 90 min. (D) Same preparation as in B but incubated for 120 min.

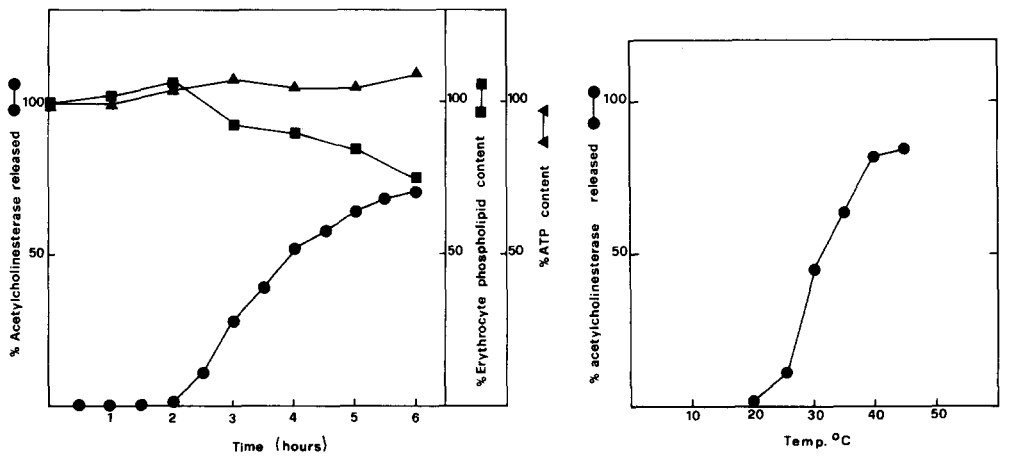

Fig. 2. Time course of vesicle fromation. Erythrocytes were incubated with dimyristoyl phosphatidylcholine and the following parameters determined as described in Materials and Methods. (●——●) Acetylcholinesterase activity determined in the supernatant after the removal of erythrocytes by centrifugation. Acetylcholinesterase activity of the incubation mixture including erythrocytes at the beginning of the experiment was taken as 100%. (■——■) Phospholipid content of erythrocytes removed from the incubation mixture by centrifugation. (▲——▲) ATP content of incubation mixture.

Fig. 3. Temperature dependences of vesicle formation. Incubation was carried out as described in Materials and Methods for 4 h at the temperatures indicated. Erythrocytes were removed by centrifugation and acetylcholinesterase activity in the supernatant, representative for vesicle release, was determined. Acetylcholinesterase activity of the total incubation mixture at the beginning of each experiment was taken as 100%.

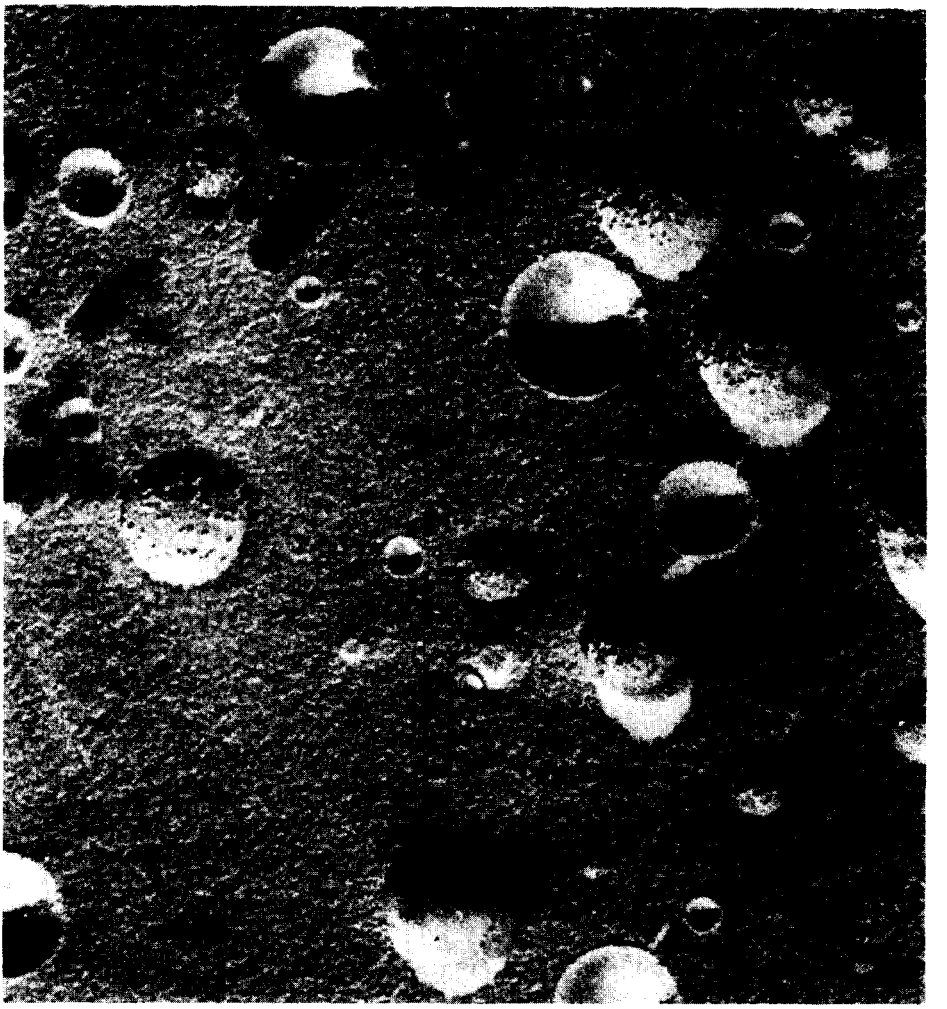

Fig. 4. Freeze-fracture electron microscopy of released vesicles. Vesicles were prepared and purified as described in Materials and Methods. The bar represents 100 nm.

shown), the additional phospholipid must have arisen from the exogenous dimyristoyl phosphatidylcholine rather than from a change in the assayed cell population. Vesiculation initiated after 2 h incubation at 30°C. The data presented in Fig. 2 show the appearance of acetylcholinesterase in the supernatant and a concomitant decrease in red cell membrane phospholipid. The release of vesicles continued without detectable hemolysis for up to 6 h, when 60—70% of the total acetylcholinesterase activity could be recovered in the supernatant of the first centrifugation step, and the level of erythrocyte phospholipid was approx. 75% of the initial concentration. This indicates that about 25% of the total membane lipid is released with the vesicles. No loss of ATP could be measured (Fig. 2).

In control experiments performed under similar conditions, but without dimyristoyl phosphatidylcholine in the incubation medium, no acetylcholin-

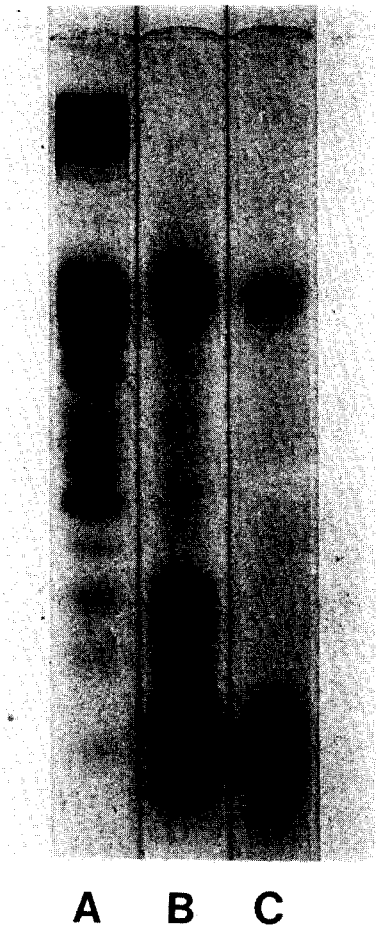

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified vesicles. Electrophoresis was carried out as described in Materials and Methods. (A) Ghosts (control) stained with Coomassie brilliant blue. (B) Vesicles stained with Coomassie brilliant blue. (C) Vesicles, stained for carbohydrates (periodic acid Schiff stain).

TABLE I

COMPARISON OF PHOSPHOLIPID COMPOSITION IN RELEASED VESICLES, ERYTHROCYTES AFTER INCUBATION WITH DIMYRISTOYL PHOSPHATIDYLCHOLINE AND INTACT ERYTHROCYTES

Lipids were separated and determined as described in Materials and Methods. Data are expressed as mol% of total phospholipid fraction (mean of three experiments ±S.D.)

Component	Vesicles	Incubated erythrocytes	Erythrocytes *
Sphingomyelin	27.9 ± 4.1	23.3 ± 4.4	25.8 ± 1.7
Phosphatidylcholine	42.4 ± 2.6	43.6 ± 5.8	28.3 ± 2.1
Phosphatidylethanolamine	21.3 ± 1.3	24.2 ± 3.9	26.7 ± 1.0
Phosphatidylserine	8.2 ± 1.9	8.9 ± 1.5	12.7 ± 1.3

* Taken from Ref. 20.

esterase release could be observed. Total acetylcholinesterase activity remained constant in all experiments.

The influence of incubation temperature on the rate of release is shown in Fig. 3. Up to 80% of total acetylcholinesterase activity could be released within 4 h at 45°C. No release of membrane vesicles was promoted by dimyristoyl phosphatidylcholine below 20°C, which corresponds approximately to the phase transition temperature for this phospholipid.

Membrane vesicles which were released from the erythrocytes could be separated from the cells and the bulk of dimyristoyl phosphatidylcholine by centrifugation (see Materials and Methods). Freeze-fracture electron microscopy performed on the released structures revealed the presence of vesicles with an average diameter of 150 nm. Intramembranous particles were detectable in an arrangement similar to that of the particles found in the native erythrocyte membrane (Fig. 4). This suggested that the major membrane-spanning proteins of the red blood cell membrane, band 3 and glycophorin were also present, which was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 5). From this figure it is also clear that the membrane vesicles contained hemoglobin, which was located in the lumen of the vesicles as it was not detectable in the supernatants obtained from the purification procedure. The phospholipid composition of the membrane vesicles and of the parent erythrocytes are presented in Table I. Clearly, the vesicles are representative of the total phospholipid content of the red cell membrane and are not composed of lipids from the outer leaflet only.

Discussion

The results presented here demonstrate that human erythrocytes undergo membrane vesiculation as a response to interacting with dispersions of dimyristoyl phosphatidylcholine at temperatures above the phase transition for this phospholipid. Previous studies on membrane vesiculation from erythrocytes have implied that the series of events leading to the release of membrane fragments is the result of low concentrations of intracellular ATP [1,4—6]. It is thought that high levels of ATP are required to keep the cytoskeletal network of proteins in the correct conformation, and thus to maintain cell shape and deformability [1,4—6].

However, the present study showed that a 6 h incubation with dispersions of dimyristoyl phosphatidylcholine resulted in a discocyte-to-sphero-echinocyte shape change (Fig. 1) and the loss of plasma membrane in the form of vesicles (Fig. 4), although intracellular ATP concentrations did not decrease. Furthermore, incubation of erythrocytes with dimyristoyl phosphatidylcholine in a buffer containing glucose, inosine and adenine, which previously had been used to prevent ATP depletion [1], did not change the time course and the extent of the vesiculation process.

Previous studies have shown that incubation of red cells with substances such as local anesthetics and sublytic concentrations of lysophosphatidylcholine [7,9] induce formation of echinocytes. Sheetz and Singer [2] have proposed that the action of such agents can be explained by a bilayer couple

hypothesis, whereby compounds which penetrate the outer monolayer of the cell membrane will expand this half of the bilayer relative to the inner monolayer, producing a crenated cell shape. Similarly, dimyristoyl phosphatidylcholine, which is known to exchange readily between two membranes at 37°C [21], may penetrate and expand the outer monolayer of the erythrocyte, thus inducing echinocyte formation.

The mechanism by which a crenated cell progresses to a point where it sheds membrane in the form of vesicles at present can only be speculated on. It is possible that interactions of the cytoplasmic part of the membrane with dimyristoyl phosphatidylcholine undergoing 'flip-flop' to the inner monolayer are of importance. Such transbilayer movements of phospholipids have been shown to be enhanced by an asymmetrical perturbation of the bilayer in model membrane systems [22,23]. Therefore, once a certain concentration of dimyristoyl phosphatidylcholine has penetrated the outer monolayer, movement of phospholipid to the cytoplasmic half of the bilayer could occur. This exogenous phospholipid then may induce the lateral phase separation of phosphatidylserine and phosphatidylethanolamine which has been considered as a prerequisite of the membrane fusion preceding the vesicle release [24]. Such a scheme would explain the lag time observed prior to vesiculation (Fig. 2) and the dependence of the release velocity on the concentration of added phospholipid (Ott, P., unpublished observations).

Bouma et al. [10] recently reported that acetylcholinesterase could be selectively extracted from erythrocytes, along with lipids from the outer monolayer only, by incubating cells with dispersions of dimyristoyl phosphatidylcholine. It is possible that in their studies the presence of erythrocyte phospholipids from both monolayers was masked by the experimental procedure they used, as the molar ratio of exogenous phospholipid to erythrocyte phospholipid was approx. 3 : 1 and the whole supernatant, thus containing a considerable excess of (dimyristoyl) phosphatidylcholine, was analysed by thin-layer chromatography. In the procedure reported here, the bulk of exogenous lipid was removed prior to any analysis. The present study demonstrates that the extraction process proposed by Bouma et al. [10] is in fact a release of membrane vesicles (Figs. 1 and 4) which contain acetylcholinesterase as well as band 3 protein and glycophorin. A slight increase of phospholipids from the outer monolayer, in particular sphingomyelin, may be observed (Table I) in the vesicles, but they definitely also contain considerable amounts of phospholipids originating from the inner leaflet of red blood cells (phosphatidylserine and phosphatidylethanolamine).

Acknowledgements

Part of this study was carried out during a stay of P.O. at the Rijksuniversiteit, Utrecht, which was made possible by a scholarship of the Swiss National Science Foundation. M.J.H. acknowledges financial support from EMBO. The support of the Swiss National Science Foundation grant No. 3.032-0.76 to U.B. is gratefully acknowledged. The authors are indebted to Miss Y. Amrein for skilfull technical assistance during part of this study.

References

1 Lutz, H.U., Liu, S.-C. and Palek, J. (1977) J. Cell Biol. 73, 548—560
2 Sheetz, M.P. and Singer, S.J. (1977) J. Cell. Biol. 73, 638—646
3 Birchmeier, W. and Singer, S.J. (1977) J. Cell. Biol. 73, 647—659
4 Rumsby, G.M., Trotter, J., Allan, D. and Michell, R.H. (1977) Biochem. Soc. Trans. 5, 126—128
5 Allan, D. and Michell, R.H. (1975) Nature 258, 348—349
6 Allan, D., Billah, M.M., Finean, J.B. and Michell, R.H. (1976) Nature 261, 58—60
7 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4457—4461
8 Fortes, G.P.A. and Ellory, J.C. (1975) Biochim. Biophys. Acta 413, 65—78
9 Weltzien, H.U. (1979) Biochim. Biophys. Acta 559, 259—287
10 Bouma, S.R., Drislane, F.W. and Huestis, W.H. (1977) J. Biol. Chem. 252, 6759—6763
11 Van Deenen, L.L.M. and de Haas, G.H. (1974) Adv. Lipid Res. 2, 167—234
12 Ellman, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmakol. 7, 88—95
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Stanley, P.E. and Williams, S.G. (1969) Anal. Biochem. 29, 381—392
15 Renkonen, O., Kosunen, T.U. and Renkonen, O.V. (1963) Ann. Med. Exp. Biol. Fenniae 41, 375—381
16 Broekhuize, R.M. (1969) Clin. Chim. Acta 23, 457—461
17 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494—496
18 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
19 Ververgaert, P.H.J.T., Elbers, P.F., Luithingh, A.J. and van de Berg, H.J. (1972) Cytobiology 6, 86—96
20 Van Deenen, L.L.M. and de Gier, J. (1974) in The Red Blood Cell (Surgenor, D.M., ed.), 2nd edn., Vol. 1, pp. 147—211, Academic Press, New York
21 De Kruijff, B. and van Zoelen, E.J.J. (1978) Biochim. Biophys. Acta 511, 105—115
22 De Kruijff, B. and Baken, P. (1978) Biochim. Biophys. Acta 507, 38—47
23 De Kruijff, B. and Wirtz, K.W.A. (1977) Biochim. Biophys. Acta 468, 318—326
24 Hope, M.J. and Cullis, P.R. (1979) FEBS Lett. 107, 323—326