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# PRESERVATION OF BILAYER STRUCTURE IN HUMAN ERYTHROCYTES AND ERYTHROCYTE GHOSTS AFTER PHOSPHOLIPASE TREATMENT

## A $^{31}$P-NMR STUDY

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## Summary

1. Fresh human erythrocytes were treated with lytic and non-lytic combinations of phospholipases $A_2$, C and sphingomyelinase. The $^{31}$P-NMR spectra of ghosts derived from such erythrocytes show that, in all cases, the residual phospholipids and lysophospholipids remain organized in a bilayer configuration.

2. A bilayer configuration of the (lyso)phospholipids was also observed after treatment of erythrocyte ghosts with various phospholipases even in the case that 98% of the phospholipid was converted into lysophospholipids (72%) and ceramides (26%).

3. A slightly decreased order of the phosphate group of phospholipid molecules, seen as reduced effective chemical shift anisotropy in the $^{31}$P-NMR spectra, was found following the formation of diacylglycerols and ceramides in the membrane of intact erythrocytes. Treatment of ghosts always resulted in an extensive decrease in the order of the phosphate groups.

4. The results allow the following conclusions to be made:

a. Hydrolysis of phospholipids in intact red cells and ghosts does not result in the formation of non-bilayer configuration of residual phospholipids and lysophospholipids.

b. Haemolysis, which is obtained by subsequent treatment of intact cells with sphingomyelinase and phospholipase $A_2$, or with phospholipase C, cannot be ascribed to the formation of non-bilayer configuration of phosphate-containing lipids.

c. Preservation of bilayer structure, even after hydrolysis of all phospholipid, shows that other membrane constituents, e.g. cholesterol and/or membrane

proteins play an important role in stabilizing the structure of the erythrocyte membrane.

d. A major prerequisite for the application of phospholipases in lipid localization studies, the preservation of a bilayer configuration during phospholipid hydrolysis, is met for the erythrocyte membrane.

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## Introduction

Phospholipases have been used frequently to study the distribution of phospholipids over the inner and outer layer of membranes (for a recent review see Ref. 1). The most convincing results have been obtained with erythrocyte membranes in which a relative asymmetric distribution of phospholipids was established: choline-containing phospholipids appeared to be located mainly in the outer layer whereas aminophospholipids were found to reside mostly in the inner layer of the erythrocyte membrane [2]. These data obtained with various phospholipases are in good agreement with chemical labelling studies [3—6], and were confirmed by subsequent studies on right side-out and inside-out resealed ghosts [7,8].

A prerequisite for phospholipid localization studies is that the phospholipids in the membrane are arranged in a bilayer configuration and that this organization is not significantly modified under the experimental conditions. That this prerequisite is not always fullfilled is obvious from recent studies on microsomal membranes. Application of $^{31}$P-NMR revealed that some of the phospholipid molecules experience a high isotropic motion indicating that part of the phospholipids in these membranes are not arranged in a classical bilayer [9,10]. In addition it has been observed that the formation of lysophospholipids, free fatty acids, diacylglycerol and ceramides, all of which are known to destabilize bilayers, can result in lysis or desintegration of the membrane [2,11—16].

The present study was initiated to investigate the effects of phospholipid degradation in erythrocytes on the fate of the lipid bilayer. $^{31}$P-NMR which was shown to be a powerful technique to study the phase behaviour of membrane phospholipids [17], has been applied to monitor the arrangement of phospholipids in the red cell membrane and to detect possible alterations following phospholipase treatment of intact erythrocytes and erythrocyte ghosts.

## Materials and Methods

*Phospholipases.* Phospholipase $A_2$ (EC 3.1.1.4) from *Naja naja* venom was purified as described by Cremona and Kearney [18] with the exception that gel filtration on Sephadex G-75 was replaced by gel filtration on Sephadex G-100. Phospholipase $A_2$ from bee venom was obtained from Sigma Chem. Co. (St. Louis, MO) and used without further purification. Phospholipase C (EC 3.1.4.3) from *Bacillus cereus* was purified according to Zwaal et al. [19] and phospholipase C from *Clostridium welchii* according to Zwaal et al. [20]. The procedure of Colley et al. [21] was used to purify sphingomyelinase C (EC 3.1.4.12) from *Staphylococcus aureus.*

*Treatment of erythrocytes.* Human red blood cells were isolated, immediately after collection, from standard acid/citrate/dextrose-treated blood by centrifugation for 10 min at 2500 × *g*. Cells were washed three times with a buffer containing 150 mM NaCl, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4. Incubations were carried out as follows: 50-ml packed cells were suspended in 750 ml of the above buffer, phospholipase was added and the mixtures were incubated at 37°C for 2 h with slow rotation. The amounts of phospholipases added were 460 IU phospholipase $A_2$, 150 IU sphingomyelinase or 100 IU phospholipase C from *C. welchii.* After incubation the mixtures were centrifuged for 10 min at 2500 × *g* and the supernatants were collected to assay haemolysis as described before [22]. EDTA at a final concentration of 30 mM was added to stop phospholipase activity. After incubations with phospholipases in which lysis was observed the supernatants were centrifuged for 20 min at 30 000 × *g* to prevent loss of membrane material.

*Treatment of erythrocyte ghosts.* Erythrocyte ghosts were prepared by a modified Dodge procedure [23] in a buffer containing 10 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4. The ghosts isolated from 50-ml of packed cells were suspended in 200 ml of a buffer containing 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 1 mM Tris-HCl, pH 7.4, and incubated as described above for 1 h with 300 IU phospholipase $A_2$, 125 IU of sphingomyelinase or 500 IU of phospholipase C from *B. cereus.* To stop phospholipase activity EDTA was added up to a final concentration of 20 mM and the ghosts were collected by centrifugation for 20 min at 30 000 × *g*.

*Preparation of samples for NMR measurements.* After phospholipase incubations the cells were lysed and ghosts were isolated as described above. All membrane suspensions were washed four times with 300 ml of the EDTA-containing buffer and frozen. Immediately before the NMR measurements the membrane preparations were thawed, washed and suspended in a 20% $^2H_2O$-containing (150 mM NaCl, 25 mM Tris-HCl) buffer, pH 7.0. During incubation, and the subsequent washing procedures the loss of membrane material, measured as lipid phosphorus, was always less than 10%.

*NMR measurements.* $^{31}P$ NMR measurements were performed at 37°C under conditions of proton decoupling on a Bruker WH-90 spectrometer at a frequency of 36.4 MHz (as described in detail in Ref. 17). Free induction decays were obtained from 50 000 to 100 000 transients employing 45° rF pulses with a 0.17 s interpuls time.

*Phospholipid analysis.* Samples from the incubation mixtures were taken before and after phospholipase treatment. Lipids were extracted by the method of Rose and Oklander [24], concentrated, dissolved in a small volume of $CHCl_3/CH_3OH$ (2 : 1, v/v) and separated by two-dimensional thin-layer chromatography [25]. Precoated silica gel plates (Merck 5721 Kieselgel 60 DC-Fertigplatten) were used with $CHCl_3/CH_3OH$/25% ammonia/$H_2O$ (90 : 54 : 5.7 : 5.3, by vol.) as the first solvent system, followed by $CHCl_3/CH_3OH$/acetic acid/$H_2O$ (90 : 30 : 8 : 2.85, by vol.) in the second direction. Lipids were visualized by $I_2$ vapour and scraped off the plates in order to measure the amount of phosphate present [26]. The extent of phospholipid degradation was calculated using various lipids as internal standards. After phospholipase $A_2$ and phospholipase C (*B. cereus*) incubations sphingomyelin served as

standard, whereas phosphatidylcholine was used following sphingomyelinase and phosphatidylserine following phospholipase C from *C. welchii* incubations. After the combined action of phospholipase $A_2$ and sphingomyelinase the lipids from the incubation mixtures and corresponding controls were quantitatively applied to the thin-layer plates.

## Results

*Treatment of intact erythrocytes with phospholipases*

In a first control experiment cells were incubated without the addition of enzyme. The phospholipid composition remained unaltered during this incubation. The $^{31}P$ NMR spectrum of the membranes derived from such cells is shown in Fig. 1A. The spectrum shows a low-field shoulder and a high-field peak which is characteristic for a membrane in which all the phospholipids are arranged in a bilayer configuration [17]. The effective chemical shift anisotropy ($\Delta\nu$) being related to the order in the phosphate region [27], determined as the distance between the low-field shoulder and the high-field peak has a value of 37.7 ppm (Table I). The latter value closely corresponds to the value of 38 ppm reported before [28].

Cells were incubated with bee venom phospholipase $A_2$ under non-lytic conditions with various degrees of phospholipid hydrolysis (Table I). In all cases a bilayer spectrum could be observed by $^{31}P$ NMR (see for an example Fig. 1B).

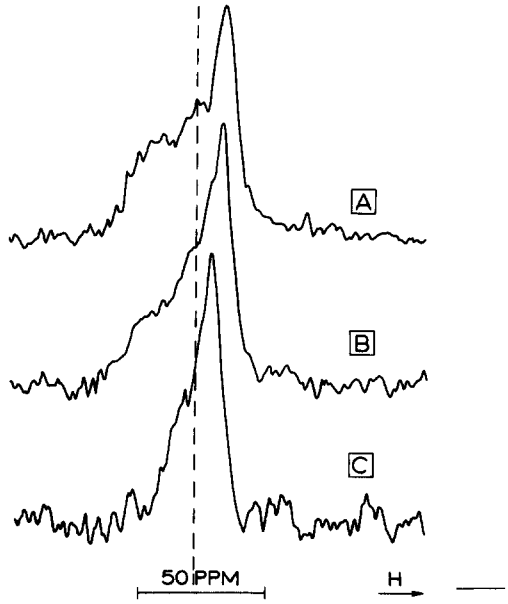


**Fig. 1. $^{31}P$ NMR spectra of erythrocyte membranes. Spectrum A is obtained from membranes derived from cells incubated without phospholipases and shows a $\Delta\nu$ value of 39.1 ppm. Spectrum B is obtained with membranes derived from intact erythrocytes treated with phospholipase $A_2$ (*N. naja*) ($\Delta\nu$ = 37.1 ppm). Spectrum C is from isolated membranes treated with phospholipase A (bee venom) and sphingomyelinase ($\Delta\nu$ = 15). The dotted line indicates the position of the signal which can arise from phospholipids undergoing isotropic motion.**

TABLE I

$^{31}$P NMR MEASUREMENTS ON MEMBRANES DERIVED FROM PHOSPHOLIPASE-TREATED ERYTHROCYTES

Incubations of intact erythrocytes with various enzymes was carried out as described in Materials and Methods. The extent of phospholipid degradation is expressed as percent of the total amount of each phospholipid present. Phosphatidylserine was not hydrolyzed. The effective chemical shift anisotropy ($\Delta\nu$) is expressed in ppm. The extent of haemolysis is given between brackets. B.V., bee venom; N.N., *N. naja*; C.W., *C. welchii*; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin.

| Phospholipase treatment | Phosphalipid hydrolysed | | | | $\Delta\nu$ |
|---|---|---|---|---|---|
| | PC | PE | Sph | Total | |
| Non-lytic | | | | | |
| — | — | — | — | — | 39 |
| Phospholipase A2 (B.V.) | 26 | — | — | 10 | 35 |
| Phospholipase $A_2$ (B.V.) | 42 | — | — | 13 | 33 |
| Phospholipase $A_2$ (B.V.) | 50 | 7 | — | 17 | 36 |
| Phospholipase $A_2$ (N.N.) | 50 | — | — | 16 | 37 |
| Sphingomyelinase | — | — | 85 | 22 | 33 |
| Phospholipase $A_2$ (N.N.) + sphingomyelinase | 65 | 15 | 73 | 41 | 26 |
| Lytic | | | | | |
| Sphingomyelinase + phospholipase $A_2$ (N.N.) (13) | 57 | 29 | 83 | 48 | 25 |
| Sphingomyelinase + phospholipase $A_2$ (N.N.) (80) | 69 | 50 | 86 | 59 | 16 |
| Phospholipase C (C.W.) (76) | 90 | 56 | 78 | 63 | 20 |

At the position of isotropic $^{31}$P no (narrow) signal was observed indicating that all the residual phospholipids as well as the formed lysophospholipids are still arranged in a bilayer. The measured $\Delta\nu$ values show that despite the conversion of 17% of the total phospholipid (corresponding to 35% of the phospholipid in the outer membrane layer) the ordering of phosphate headgroups remains preserved. Similar results were obtained after treatment of intact cells with phospholipase $A_2$ from *N. naja*, and sphingomyelinase from *S. aureus* (Table I).

In the following set of experiments the combined action of phospholipase $A_2$ and sphingomyelinase on intact erythrocytes was investigated. Human erythrocytes stay intact when the enzymes are added in the above order. It was found that, despite hydrolysis of 50% of the membrane phospholipids, which under non-lytic conditions corresponds to the complete hydrolysis of the phospholipids in the outer membrane layer [29], no phosphorus-containing lipid is found which experiences isotropic motion. A stable bilayer structure is preserved which consists of a monolayer of lysophospholipids at the outside and a monolayer of intact phospholipids at the inside.

When the erythrocytes are incubated first with sphingomyelinase during 1 h followed by phospholipase $A_2$ treatment, the cells lyse [2]. The phenomenon was again observed (Table I) but despite the extensive hydrolysis of phospholipids from both outer and inner layer, the bilayer structure is still present. Similar results were obtained after the treatment of red cells with phospholipase C from *C. welchii*. The NMR spectra resulting from treatment with non-lytic and lytic combinations of phospholipase $A_2$ and sphingomyelinase are similar. These spectra are, in contrast to the spectra obtained after incubations with phospholipase $A_2$ and spingomyelinase alone, clearly narrowed which is shown in the measured values for $\Delta\nu$. Especially in those experiments in which

TABLE II

$^{31}P$ NMR MEASUREMENTS OF PHOSPHOLIPASE-TREATED ERYTHROCYTE GHOSTS

A description of the incubation conditions is given in Materials and Methods. For abbreviations see Table I. In addition: B.C., *B. Cereus*; PS, phosphatidylserine.

| Phospholipase treatment | Phospholipid hydrolysed | | | | | $\Delta\nu$ |
|---|---|---|---|---|---|---|
| | PC | PE | PS | Sph | Total | |
| Phospholipase $A_2$ (B.V.) | 85 | 90 | 80 | — | 62 | 17 |
| Phospholipase $A_2$ (B.V.) + sphingomyelinase | 95 | 98 | 100 | 90 | 97 | 17 |
| Phospholipase $A_2$ (B.V.) + sphingomyelinase | 99 | 99 | 98 | 50 | 83 | 15 |
| Sphingomyelinase + phospholipase $A_2$ (B.V.) | 98 | 98 | 96 | 75 | 88 | 16 |
| Phospholipase C (B.C.) | 96 | 100 | 76 | — | 70 | 25 |

haemolysis did occur, low values for the effective chemical shift anisotropy are found corresponding with the values obtained after treatment of erythrocyte ghosts (Table II). The data obtained with the two combinations of phospholipase $A_2$ and sphingomyelinase indicate that haemolysis is not due to a transition of residual phospholipid from a bilayer configuration into another type of phospholipid organization within the erythrocyte membrane.

*Treatment of erythrocyte ghosts with phospholipases*

During those treatments of erythrocytes which resulted in lysis, a large amount of phosphatidylethanolamine was hydrolyzed, indicating that the enzymes had access to the interior phospholipids. The NMR data showed already that even under those conditions the bilayer structure is preserved. Evidence to substantiate this was provided by treatment of erythrocyte ghosts in which both sides of the membrane are accessible for enzymic attack. The results are presented in Table II and Fig. 1. Under all conditions the residual phospholipids as well as the phosphate-containing hydrolysis products give rise to $^{31}P$ NMR spectra with the characteristic bilayer shape irrespective of the extent of hydrolysis. Even after the combined action of phospholipase $A_2$ and sphingomyelinase which resulted in complete hydrolysis of the phospholipids a bilayer spectrum was recorded. The effective chemical shift anisotropy appears to be reduced considerably. A minimum value is obtained in those membranes in which substantial amounts of lysophospholipids are formed as was found already after treatment of intact cells with the lytic combination of sphingomyelinase + phospholipase $A_2$ (Table I).

## Discussion

The polymorphic phase behaviour of phospholipids in biological membranes can be studied successfully with $^{31}P$ NMR. Whenever phospholipids are arranged in a bilayer structure a typical spectrum, as depicted in Fig. 1, is obtained whereas non-bilayer configurations give rise to completely different spectra which can be clearly discriminated from the bilayer spectrum [17]. It is possible therefore also to recognize the co-existence of different phases of phospholipids within one membrane. Such a phenomenon was illustrated recently in studies on rat liver microsomal membranes in which a sizeable frac-

tion of the phospholipids appeared to be in a non-bilayer configuration [9,10, 12]. The data presented here did not show non-bilayer configurations of phospholipids within the erythrocyte membrane and furthermore indicate that the phosphate-containing lipids remain arranged in a bilayer upon hydrolysis by various phospholipases.

However, it has to be emphasized that this conclusion is based, in part, on indirect evidence. $^{31}P$ NMR measurements on intact erythrocytes are impossible at present, because of the limited sensitivity of the technique. The data were obtained therefore with isolated erythrocyte ghosts. The fact, however, that even a phospholipase treatment of ghosts, resulting in more than 90% of phospholipid hydrolysis, does not introduce non-bilayer configurations of residual phospholipids and lysophospholipids justifies an extrapolation from the data obtained with ghosts towards the native membrane.

Since lysophospholipids, free fatty acids and ceramides do not form stable bilayers itself, other membrane constituents have to exert an important influence on bilayer stability when phospholipid is hydrolysed in one or in both monolayers of the erythrocyte membrane. Cholesterol, which is present in high amounts in erythrocyte membranes, seems to be a most suitable candidate for such a function. Studies on model membrane systems containing high amounts of lysophosphatidylcholine already showed that the presence of cholesterol may affect the architecture [30], stability and permeability [31] of these systems. In the presence of more than 50% cholesterol, lysophosphatidylcholine was found to form stable bilayers impermeable for glucose [31]. $^{31}P$ NMR measurements furthermore showed that non-bilayer arrangements of lysophosphatidylcholine/phosphatidylcholine mixtures can be transformed to bilayer structures in the presence of cholesterol (van Echteld, C., de Kruyff, B. and de Gier, J., unpublished results). Furthermore, intrinsic membrane proteins as well as those proteins which are supposed to interact with the interior membrane layer (spectrin, actin) could play a role in the stabilization of the bilayer structure as well. It should be noted here that Cullis and Gratewohl already showed that pronase treatment of erythrocyte ghosts, followed by ether extraction, hardly altered the $^{31}P$ NMR bilayer spectra [32].

The present data corroborate with the earlier observations of Verkleij et al. [2] who showed that freeze-fracturing of phospholipase-treated erythrocytes and erythrocyte ghosts is still possible and thus indicated that a bilayer structure is preserved. Coleman et al. [33] as well as the above authors furthermore showed that sphingomyelinase and phospholipase C treatment resulted in the formation of ceramide and diacylglycerol droplets, respectively. It can be concluded now that these lipid droplets do not contain phosphate-containing lipids nor lyso derivatives undergoing isotropic motion. The formation of such lipid droplets indicates that ceramides and diacylglycerols segregate out of the plane of the monolayer in which they are formed. It is tempting to speculate that this results in an increase in the space available for the residual (lyso)phospholipids which could explain the observed decrease in phosphate headgroup order. On the other hand the measured $\Delta\nu$ values reach minimal values whenever high amounts of lysophospholipids are formed in the membrane. At present it is not possible to determine the precise effects of both hydrolysis products on the

degree of order of phospholipids within this membrane. It cannot be excluded, however, that the smaller $\Delta\nu$ value is brought about by a different orientation of the phosphate segment with respect to the bilayer axis. In addition, the possibility that in these latter systems micellar configurations exist, in which the lipids rapidly exchange with the bilayer part cannot be excluded.

The data presented above confirm that phospholipases can be successfully used to localize phospholipids in the erythrocyte membrane. A major prerequisite for the application of phospholipases in such localization studies is met: the bilayer configuration of the membrane is maintained even after extensive phospholipid hydrolysis. It is obvious that under these conditions exogenous phospholipases do not have access to phospholipids from the interior membrane layer and hydrolyse only the outer layer phospholipids. This statement, however, is correct only when the rate of transbilayer movements of phospholipids is slow relative to the time required for the phospholipase incubation. That this is indeed the case for intact erythrocytes was shown already by Rousselet et al. [34] and by Renooy and van Golde [35] and could be confirmed recently (van Meer, G. et al., unpublished results).

In contrast to the erythrocyte membrane a large number of other membrane systems seem to be less suitable for the phospholipase approach which could reveal phospholipid localization (see Ref. 1 for a review). The data obtained with phospholipases are, in many cases, not conclusive [36] and even contradictory [16,37,38]. In view of the present data the maintenance of phospholipid bilayer structure of the various membrane structures under study must be verified, both in the native state and after phospholipase treatment.

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## References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47—71
2 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178—193
3 Bretscher, M.S. (1972) Nat. New Biol. 236, 11—12
4 Bretscher, M.S. (1972) J. Mol. Biol. 71, 523—528
5 Gordesky, S.E. and Marinetti, G.V. (1973) Biochem. Biophys. Res. Commun. 50, 1027—1031
6 Whiteley, N.M. and Berg, M.C. (1974) J. Mol. Biol. 87, 541—561
7 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83—96
8 Kahlenberg, A., Walker, C. and Rohrlick, R. (1974) Can. J. Biochem. 52, 803—806
9 De Kruijff, B., van den Besselaar, A.M.H.P., Cullis, P.R., van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 514, 1—8
10 Stier, A., Finch, S.A.E. and Bösterling, B. (1978) FEBS Lett. 91, 109—112
11 Simpson, R. and Hauser, R. (1966) Virology 30, 684—697
12 Wahlström, A. (1971) Toxicon 9, 45—56
13 Duckworth, D.H., Bevers, E.M., Verkley, A.J., Op den Kamp, J.A.F. and van Deenen, L.L.M. (1974) Arch. Biochem. Biophys. 165, 379—387
14 Op den Kamp, J.A.F., Kauerz, M.Th. and van Deenen, L.L.M. (1972) J. Bacteriol. 112, 1090—1098

15 Nanninga, N., Tijssen, F.C. and Op den Kamp, J.A.F. (1973) Biochim. Biophys. Acta 298, 184—194
16 Nilsson, O.S. and Dallner, G. (1977) Biochim. Biophys. Acta 464, 453—458
17 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207—218
18 Cremona, T. and Kearney, E.B. (1964) J. Biol. Chem. 239, 2328—2334
19 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 233, 474—479
20 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83—96
21 Colley, C.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 307, 74—82
22 Roelofsen, B., Zwaal, R.F.A., Comfurius, P., Woodward, C.B. and van Deenen, L.L.M. (1971) Biochim. Biophys. Acta 241, 925—929
23 Dodge, C.T., Mitchell, C.D. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
24 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428—431
25 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457—461
26 Rouser, G., Fleischer, S. and Yamamoto, (1970) Lipids 5, 493—496
27 Seelig, J. (1978) Biochim. Biophys. Acta 515, 105—140
28 Cullis, P.R. (1976) FEBS Lett. 68, 173—176
29 Zwaal, R.F.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159—182
30 Inoue, K., Suzuki, K. and Nojima, S. (1977) J. Biochem. (Tokyo) 81, 1097—1106
31 Kitagana, T., Inoue, K. and Nojima, S. (1976) J. Biochem. (Tokyo) 79, 1123—1133
32 Cullis, P.R. and Gratewohl, C. (1977) Biochim. Biophys. Acta 471, 213—226
33 Coleman, R., Finean, J.B., Knutton, S. and Limbrick, A.R. (1970) Biochim. Biophys. Acta 219, 81—92
34 Rousselet, A., Colbeau, A., Vignais, P.M. and Deveaux, P.F. (1976) Biochim. Biophys. Acta 426, 372—384
35 Renooy, W. and van Golde, L.M.G. (1977) Biochim. Biophys. Acta 470, 465—474
36 Bishop, D.G., Op den Kamp, J.A.F. and van Deenen, L.L.M. (1977) Eur. J. Biochem. 80, 381—391
37 Higgins, J.A. and Dawson, R.M.C. (1977) Biochim. Biophys. Acta 470, 342—356
38 Sundler, R., Sarcione, S.L., Alberts, A.W. and Vagelos, P.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3350—3354