Biochimica et Biophysica Acta, 555 (1979) 102—110

BBA 78429

A MECHANISM OF ERYTHROCYTE LYSIS BY LYSOPHOSPHATIDYLCHOLINE

T.J. BIERBAUM, S.R. BOUMA and W.H. HUESTIS *

Department of Chemistry, Stanford University, Stanford, CA 94305 (U.S.A.)

(Received March 14th, 1979)

Key words: Lysophosphatidylcholine; Hemolysis; Tetrodotoxin; Na^+ channel; (Erythrocyte membrane)

Summary

Lysophosphatidylcholine micelles liberate several cell surface polypeptides from erythrocyte membranes, inducing a sodium-selective permeability defect which leads to colloid osmotic lysis. Evidence is presented to support the hypothesis that at the lowest lytic lysophospholipid concentrations, selective disruption of membrane protein function, rather than gross structural reorganization of the membrane, is the primary lytic mechanism.

Introduction

The hemolytic action of lysophospholipids has often been attributed to general structural changes induced in the phospholipid matrix of cell membranes [1]. Incorporation of lysolipids produces numerous kinds of abnormal behavior in such membranes, including gross morphological changes [2] and membrane fusion [3]. In studies of phospholipid interactions with cells [4,5], we observed that sonicated fluid phase phosphatidylcholine vesicles induce colloid osmotic lysis in erythrocytes by creating a sodium-specific membrane permeability defect. One consistently observed event preceding hemolysis was the transfer of a few cell surface proteins, including the acetylcholinesterase, from cell to vesicle membrane. An early study [6] of lysophospholipid-induced hemolysis revealed that lysolipids also cause release of erythrocyte acetylcholinesterase from the cells, concurrent with lysis. The present investigation was undertaken to determine whether mechanistic similarities exist between lysolipid-induced hemolysis and the process described for phospholipid vesicles.

* To whom correspondence may be addressed.

Materials and Methods

Materials. Human erythrocytes were obtained from adult donors and used within 12 h of collection. Dimyristoyl phosphatidylcholine, lauroyl lysophosphatidylcholine, and palmitoyl lysophosphatidylcholine were products of Sigma Chemicals. Tetrodotoxin was a product of Sankyo Ltd., Tokyo. Rhodamine B was purchased from MCB.

Unless otherwise noted, all experiments were conducted in 310 mosM of phosphate-buffered saline of the following composition: 140 mM NaCl, 5 mM KH_2PO_4, 2.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM glucose. pH was adjusted to 7.38 with 1 M NaOH.

Methods. Lauroyl or palmitoyl lysophosphatidylcholine suspensions in phosphate-buffered saline were prepared by brief sonication (5 min) in a bath sonicator (Heat Systems Ultrasonics model 9H) at 25°C. The sonicated concentrates were diluted with saline buffer to achieve desired concentrations (see Figs. 1 and 5), resonicated briefly, and allowed to stand at 4°C for at least 12 h. The last step was necessary to insure complete micelle-monomer equilibrium [7]. Sucrose and tetrodotoxin were added as concentrates to lysophosphatidylcholine suspensions.

Erythrocytes were separated from plasma by centrifugation, then washed three times by suspension in 4 vols. of 0.15 M NaCl, followed by centrifugation. A final wash in saline buffer followed.

Suspensions of lysophospholipids were added to packed erythrocytes (in equal volumes unless otherwise indicated) at 2°C, with vortex mixing. Zero-time aliquots were centrifuged immediately ($t < 3$ min). Cell/lysolipid mixtures were incubated in ice, and aliquots were removed and separated by centrifugation (30 s at 3000 × *g*). Supernatants were removed immediately and stored at 2°C. Separation of soluble protein from protein-lipid complexes was achieved by gel filtration through Sepharose 4B. Acetylcholinesterase activity, total protein, and lipid content of supernatants were analysed as described [4]. Cation permeability and hemolysis of the lysolipid-treated erythrocytes were determined as described [5].

Critical micelle concentrations of lysophospholipids were determined by dye inclusion, using rhodamine B. Fluorescence of rhodamine B solutions containing various concentrations of lysolipids were measured at 577 nm, using a Perkin-Elmer MPF-2A spectrofluorimeter.

Results

Effects of sucrose and tetrodotoxin on lysis

At concentrations greater than their critical micelle concentrations, lauroyl and palmitoyl lysophosphatidylcholine induced hemolysis in erythrocytes (Fig. 1). At 2°C this lysis was slow enough to permit some mechanistic studies. Fig. 2a shows that hemolysis induced by 700 μM lauroyl lysophosphatidylcholine could be inhibited by sucrose (50 mM) or by tetrodotoxin (10 nM). Suppression of the lysis by sucrose was indicative of colloid osmotic imbalance, suggesting that a membrane permeability defect was responsible. The effect of tetrodotoxin suggested that the permeability defect was specific for Na^+, as

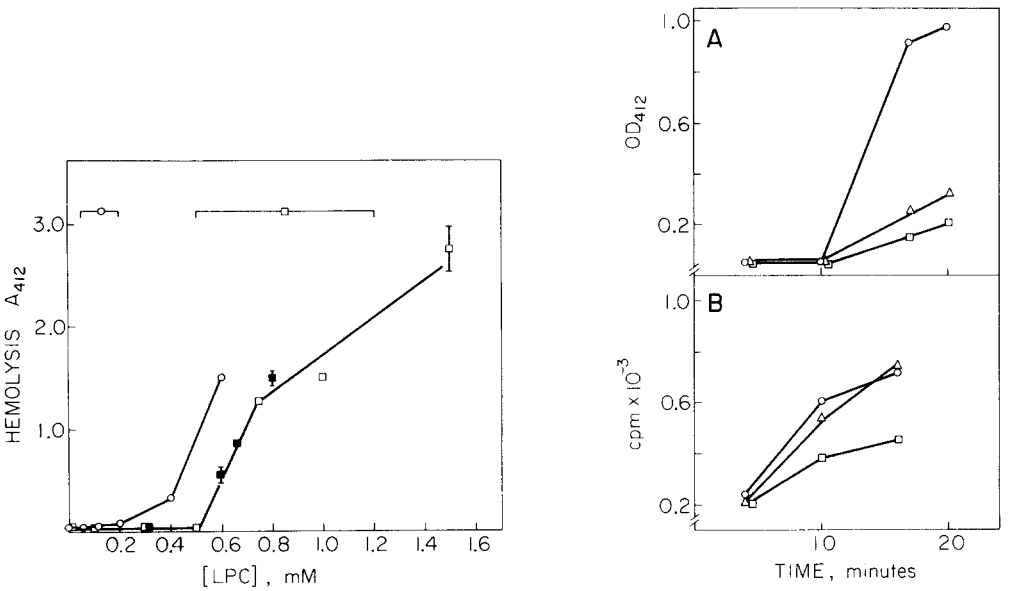


Fig. 1. Hemolysis as a function of lysophospholipid concentration after 30 min of incubation at 2°C. ○, palmitoyl lysophosphatidylcholine; □, lauroyl lysophosphatidylcholine; ■, lauroyl lysophosphatidylcholine after 4 h. Hemolysis was measured as the absorption at 412 nm of cell-free supernatant diluted 1 : 30 with buffer. Micellization ranges (⌐— —¬, palmitoyl lysophosphatidylcholine; ⌐—□—¬ lauroyl lysophosphatidylcholine) are shown for reference.

Fig. 2. Effect of sucrose and tetrodotoxin on hemolysis (A) and $^{22}Na^+$ uptake (B) of lysolipid-treated cells. ○, control; △, 50 mM sucrose added; □, 10 mM tetrodotoxin. Hemolysis was measured as in Fig. 1. Na^+ uptake was measured as cpm in 25 μl of packed, washed erythrocytes.

was the defect induced in erythrocytes by phospholipid vesicle treatment [5]. Studies with $^{22}Na^+$ showed that lysolipid-treated cells exhibited increased sodium permeability, which was also inhibited by 10 nM tetrodotoxin (Fig. 2b). Uptake of $^{86}Rb^+$, a potassium analog, was not inhibited similarly (Table I).

TABLE I

UPTAKE OF $^{22}Na^+$ AND $^{86}Rb^+$ BY NORMAL AND LYSOPHOSPHATIDYLCHOLINE-TREATED ERYTHROCYTES

Lysophosphatidylcholine-treated cells were incubated with 700 μM lauroyl lysophosphatidylcholine in the presence of the radioisotope.

	$^{22}Na^+$ uptake (cpm/25 μl)	$^{86}Rb^+$ uptake
Normal cells		
In buffer	70 ± 10	1700 ± 200
+ 10^{-8} M tetrodoxin	75	1500
Lysophosphatidylcholine-treated cells		
In buffer	570 ± 50	2500 ± 200
+ 10^{-8} M tetrodotoxin	110	2150

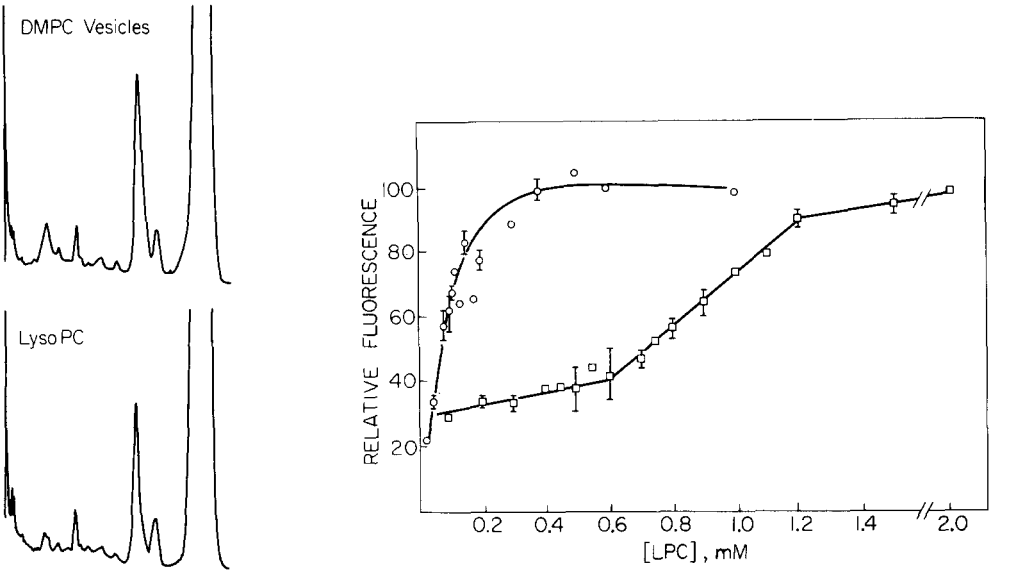


Fig. 3. Polyacrylamide gel electrophoresis of protein liberated from erythrocytes by phospholipid vesicles (dimyristoyl phosphatidylcholine, 20 mM, incubated 30 min at 37°C) and by lauroyl lysophosphatidylcholine (700 μM, 5 min at 2°C).

Fig. 4. Determination of critical micellization ranges of lauroyl lysophosphatidylcholine (□) and palmitoyl lysophosphatidylcholine (○). Relative fluorescence of rhodamine B is plotted as a function of lipid concentration.

Protein liberation accompanying hemolysis

After incubation with erythrocytes, lysolipid suspensions contained cell proteins. When these suspensions were subjected to gel filtration through Sepharose 4B, two fractions were obtained. The first contained the bulk of the lipid and the five polypeptide species shown in Fig. 3. Their apparent molecular weights on sodium dodecyl sulfate-polyacrylamide gels [8] were 90 000, 80 000, 65 000, 30 000, and 15 000 ± 5000. The second Sepharose fraction contained polypeptides of 50 000, 30 000, and 15 000 daltons. Thus, some cell polypeptides apparently associated specifically with lipid in a macromolecular aggregate. Among these proteins was acetylcholinesterase, an enzyme normally bound to the erythrocyte surface. Acetylcholinesterase was removed from the cells efficiently by lysolipid suspensions, in agreement with an early report [6].

Critical micelle concentrations

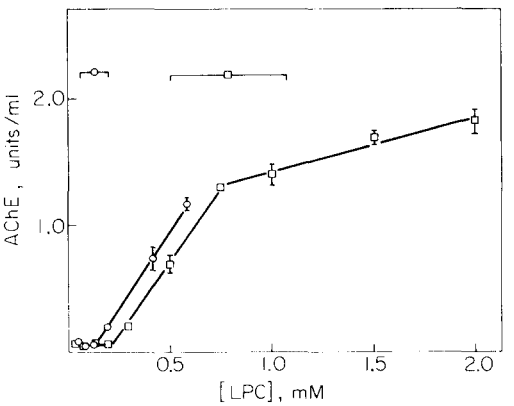
The critical micelle concentrations of the lysolipids were determined by dye inclusion, using rhodamine B. Rhodamine B absorbs in the ultraviolet with a wavelength maximum at 356 nm and fluoresces in the visible with a maximum at 577 nm. The fluorescence is enhanced but not shifted in non-polar media. Fig. 4 shows data from determination of critical micelle concentrations for palmitoyl and lauroyl lysophosphatidylcholine under the temperature and ionic strength conditions of the above experiments. Rhodamine fluorescence exhibited sharp increases at 50 μM and 500 μM for palmitoyl and lauroyl lysophosphatidylcholine. The palmitoyl lysophosphatidylcholine critical micelle

concentration range obtained agreed with reported values [9]. Varying rhodamine concentrations from 50 to 300 nM did not change the observed values, nor did the presence of 50 mM sucrose.

Effects of lysolipid concentration on protein liberation and hemolysis

The effects of lysolipid concentration on acetylcholinesterase liberation from erythrocytes is shown in Fig. 5. For both lysolipids, no acetylcholinesterase release was observed at concentrations below a discrete threshold. Above that threshold (100 μM for palmitoyl lysophosphatidylcholine, approx. 300 μM for lauroyl lysophosphatidylcholine), supernatant acetylcholinesterase activity increased with lysolipid concentration up to 2 mM. For any lipid concentration, little further increase in supernatant acetylcholinesterase was observed after about 10 min; thus when the process occurred at all it was rapid. At lysolipid concentrations below these thresholds, no acetylcholinesterase release was observed after 2 h of incubation.

A similar concentration effect was observed for hemolysis, although the threshold concentrations were higher. Hemolysis was observed in less than 10 min at 2°C for palmitoyl lysophosphatidylcholine concentrations greater than or equal to 400 μM and for lauroyl lysophosphatidylcholine concentra-



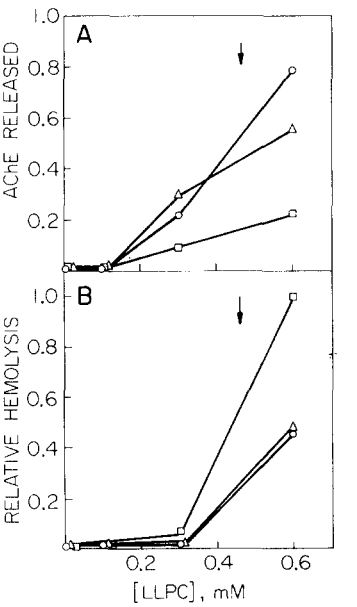


Fig. 5. Acetylcholinesterase liberation as a function of lysophospholipid concentration after 15 min of incubation at 2°C. ○, palmitoyl lysophosphatidylcholine; □, lauroyl lysophosphatidylcholine. Enzyme activity in cell-free supernatants was determined as described by Ellman [15]. Critical micelle concentration ranges are indicated for reference.

Fig. 6. Relative release of acetylcholinesterase (A) and hemolysis (B) for cells treated with lauroyl lysophosphatidylcholine at various cell/lysolipid ratios. ○, packed cells and lysolipid suspensions mixed 1 : 1 (by vol.); △, packed cells and lysolipid suspensions, 1 : 2 (by vol.); □, packed cells and lysolipid suspensions, 1 : 10 (by vol.). Onset of the critical micelle concentration range is indicated by the arrow.

tions greater than or equal to 500 μM (Fig. 1). At lower lysolipid levels no lysis was observed even after 7 h of incubation.

The threshold lipid concentrations for these phenomena were not consistently similar to the critical micelle concentration for either lysolipid. The onset of hemolysis was about 500 μM for lauroyl lysophosphatidylcholine, which is reasonably close to the critical micelle concentration. However, this may have been coincidental, since palmitoyl lysophosphatidylcholine-induced lysis was not observed until well above its critical micelle concentration range.

The threshold lysophosphatidylcholine concentrations for acetylcholinesterase liberation and hemolysis were found to be independent of the number of cells in suspension over an order of magnitude range. The effects of lauroyl lysophosphatidylcholine concentration were studied for three cell concentrations (Fig. 6), ranging from equal volumes of packed cells and lysolipid suspension to a ten-fold excess of lysolipid suspension. In this range of cell/lysolipid ratios, the threshold concentrations for acetylcholinesterase liberation and hemolysis were unchanged.

Sodium dodecyl sulfate-polyacrylamide gels of lysolipid supernatants revealed significant differences in the number and quantity of proteins released from cells exposed to lytic and sublytic lipid concentrations. In the sublytic range, proteins of 15 000 and 20 000 daltons were observed in the supernatants, but similar species were observed to dissociate when cells were incubated in lipid-free buffer. The higher molecular weight polypeptides characteristic of lytic lysolipid supernatants were not observed in sublytic supernatants. The same patterns of protein release were observed where lysis was suppressed by sucrose or tetrodotoxin; thus lysis did not produce the protein release.

Discussion

These studies were initiated to discover whether lysolipid-induced erythrocyte lysis is mechanistically similar to the hemolysis induced by sonicated phosphatidylcholine vesicles. In a previous report [5], evidence was presented to support the hypothesis that sonicated phosphatidylcholine vesicles 'extract' erythrocyte membrane proteins which regulate Na^+ permeability. Loss of these factors results in massive sodium-specific ion uptake and eventual colloid osmotic lysis of the cells. The proteins lost from the cell membrane associated specifically with the vesicle lipid aggregates, apparently in native orientation. (The transferred acetylcholinesterase hydrolysed impermeant substrates, thus its active site was exposed to the outside medium. Its activity was undiminished by transfer to the vesicle membrane.) Reverse transfer of vesicle-bound proteins also was demonstrated, with consequent inhibition of abnormal sodium permeability and lysis. The present investigation of lysophosphatidylcholines was prompted by several considerations. First, an early report [6] indicated that erythrocyte lysis by crotalid venom phospholipase A was preceded by substantial 'solubilization' of membrane acetylcholinesterase. It is well known that the operant agent in such hemolysis is the lysolipid product of enzyme action on serum phospholipids, thus lysolipid action on erythrocytes resembled phosphatidylcholine vesicle-mediated hemolysis in one intriguing particular. Second, recent work in this laboratory (Cook, S.L. and Huestis, W.H., unpublished

results) has shown that phospholipid vesicle lipid fluidity is a critical factor governing the rates of transfer of various membrane proteins to vesicles. If cell membrane proteins were transferred to lysolipid aggregates similarly, lysophosphatidylcholine might provide a useful instance of highly fluid system. Finally, it was of interest to investigate the role of lipid monomers in these events. Since the critical aggregation concentrations of lysolipids are five orders of magnitude higher than those of phospholipids [10], the lysolipid monomer concentration range is more amenable to study.

Under the conditions described, lysophosphatidylcholine lysis of erythrocytes resembled that induced by phosphatidylcholine vesicles in several critical respects. The same set of cell surface proteins was transferred from the cell membrane to exogenous lipid aggregates. In both cases the cells developed increased sodium permeability which could be inhibited by tetrodotoxin. The ensuing hemolysis could be suppressed by tetrodotoxin or by 50 mM sucrose, suggesting that osmotic imbalance created by abnormal Na^+ uptake was the primary effector of lysis.

Several mechanisms might be advanced to explain the membrane disruption induced by lysolipids. General structural reorganization due to incorporation of foreign lipid ('detergent' action) might render the membrane permeable to ions and small molecules, but such generalized disruption would not be expected to produce a sodium-specific, tetrodotoxin-inhibitable lesion. (Indeed, in the concentration range where tetrodotoxin inhibits erythrocyte sodium uptake, it also acts as a specific blocking agent for sodium channels in excitable tissue [11].) This specificity suggests that the membrane alteration is limited to a component or components pharmacologically similar to sodium channels in other cells. In the similar lytic process induced by phosphatidylcholine vesicles, considerable evidence is available to suggest that the lesion is related to loss of membrane proteins to the lipid aggregates. In either case, this loss of proteins could arise from disruptions caused by incorporated foreign lipid, or from a reversible association between exogenous lipid and the cell membrane during which membrane components are exchanged. The experiments of Verkleij and coworkers [16] suggest that the latter mechanism is more likely. These workers showed that erythrocytes can withstand lysophospholipid in their membranes (generated in situ by phospholipase action) in concentrations which would be lytic if introduced exogenously. This suggests that the effects of incorporated lysolipids are qualitatively different from those of external lysolipids.

To investigate this hypothesis and to discover whether the aggregation state of exogenous lipid affects it lytic action, we studied the concentration dependence of lysophosphatidylcholine lysis. Monomeric lysophosphatidylcholine should exist in equilibrium with the cell membranes and, above its critical micelle concentration, with lysophosphatidylcholine aggregates. Generalized effects arising from foreign lipid lysolipid incorporation into the cell membrane should appear in proportion to lysolipid concentration in the monomer range. As is shown in Figs. 1 and 5, this was not found. For both lysolipids tested, hemolysis was not observed in submicellar concentration ranges regardless of the duration of exposure. At discrete threshold concentrations, hemolysis occurred and thereafter increased with increasing lysolipid concentration. The lauroyl lysophosphatidylcholine lysis threshold appeared near its critical

micelle concentration (500 μM), but palmitoyl lysophosphatidylcholine lysis ensued at substantially higher concentrations (400 μM; the critical micelle concentration is approximately 50 mM). This finding is consistent with the report of Reman et al. [12] that minimum lytic concentrations of various lysolipids did not correspond systematically to their respective critical micelle concentrations. However, under our conditions, the lytic thresholds lay in micellar concentration ranges in all cases.

In contrast, acetylcholinesterase liberation was detectable at a lauroyl lysophosphatidylcholine concentration (300 μM) slightly below the critical micelle concentration of the pure lysolipid (500 μM). However, even at this lysolipid concentration the liberated acetylcholinesterase activity was found associated with a lipid complex which eluted from Sepharose 4B in the exclusion volume. It is possible that the presence of protein and other cell components altered the critical aggregation concentration of the lysolipid, producing the observed high molecular weight complexes. The fact that the thresholds for cholinesterase liberation and hemolysis were different supports the earlier observation [5] that cholinesterase loss per se does not produce lysis. Rather, development of the permeability lesion appears to be associated with loss of more slowly extracted species (65 000 and 30 000 daltons) which are released by 600 μM but not 300 μM lauroyl lysophosphatidylcholine.

Thus, release of membrane proteins and cell lysis were observed only at lysophospholipid concentrations above certain thresholds near or above the critical micelle concentrations. This finding was consistent with the transfer of cell membrane components to exogenous micelles, but did not exclude the possibility that lysolipid incorporated into the cell membrane was primarily responsible for these events. For example, the cell membrane might be able to accommodate lysolipid up to a discrete level without damage, above which unstable micellar regions would form which would disrupt membrane structure and eject lipid-protein aggregates into the supernatant. The experiment described in Fig. 6 was addressed to this question.

The effects of lauroyl lysophosphatidylcholine concentration on cells were studied at three different cell concentrations. If the lysis threshold represents a saturation point for the cell membrane, then the threshold would be expected to appear at lower lysolipid concentrations when fewer cells are present. This was not observed; the thresholds for protein liberation and hemolysis remained constant over an order of magnitude change in cell concentration. Thus, the thresholds appear to reflect characteristics of the exogenous lipid which govern protein liberation and lysis. Even at the highest lysolipid/cell ratios studied, no protein transfer or hemolysis were observed through most of the monomeric concentration range. This is consistent with a requirement for some sort of exogenous lipid aggregate.

Using cell concentrations 10^2—10^3 lower than those employed in our experiments, Weltzien and coworkers [13,14] found that erythrocyte lysis was proportional to incorporated lysolipid monomer, and concluded that lysolipid micelles played no role in hemolysis. In our studies of cell concentrations below 20% (v/v) we observed little or no lysis at monomeric lysolipid concentrations. Above the usual lytic threshold, however, hemolysis was less sensitive to sucrose suppression than at higher cell concentrations. Since the

micelle-monomer equilibrium is reached slowly on the time scale of our experiments [7], the monomer incorporated/cell presumably is much greater at the low cell concentration employed by Weltzien. Thus, a different lytic mechanism may predominate under those conditions.

Acknowledgements

This study was supported by the National Institutes of Health (USPHS HL 18660 and HL 00258), the American Heart Association (77-828) and the Sloan Foundation.

References

1 Jeannet, M. and Hässig, A. (1964) Vox Sang. 9, 113—127
2 Sato, T. and Fuju, T. (1974) Chem. Pharmacol. Bull. 22, 156—156
3 Kroprowski, H. and Croce, R.M. (1973) Methods Cell. Biol. 7, 251—260
4 Bouma, S.R., Drislane, F.W. and Huestis, W.H. (1977) J. Biol. Chem. 252, 6759—6763
5 Huestis, W.H. (1977) J. Biol. Chem. 252, 6764—6768
6 Grieg, M.E. and Gibbons, A.J. (1956) Arch. Biochem. Biophys. 61, 335—340
7 Schneider, A.B. and Edelhoch, H. (1972) J. Biol. Chem. 247, 4986—4991
8 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
9 Haberland, M.E. and Reynolds, J.A. (1975) J. Biol. Chem. 250, 6636—6639
10 Smith, R. and Tanford, C. (1972) J. Mol. Biol. 67, 75
11 Hille, B. (1970) Prog. Biophys. Mol. Biol. 21, 1
12 Reman, F.C., Demel, R.A., de Gier, J., van Deenen, L.L.M., Eible, H. and Westphal, O. (1969) Chem. Phys. Lipids 3, 221—233
13 Weltzien, H.U., Arnold, B. and Reuther, R. (1977) Biochim. Biophys. Acta 466, 411—421
14 Weltzien, H.U., Arnold, B. and Kalkoff, H.G. (1976) Biochim. Biophys. Acta 455, 56—65
15 Ellman, J.L., Courtney, K.D., Andres, Jr.V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
16 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