J. Membrane Biol. 66, 1–7 (1982)

The Journal of
Membrane Biology

# Use of Phospholipase A to Compare Phospholipid Organization in Synaptic Membranes, Myelin, and Liposomes

M. Butler* and L.G. Abood

Center for Brain Research and Department of Biochemistry, University of Rochester Medical Center, Rochester, New York 14642

**Summary.** The pattern of fatty acid release from rat synaptic membranes in the presence of phospholipase $A_2$ (*Vipera russelli*) was compared to that from liposomes comprised of phospholipids. Phospholipase $A_2$ more readily attacked myelin and synaptic membranes than liposomes prepared from total phospholipids derived from myelin. Although hydrolysis of liposomal phospholipids occurred in the absence of added calcium, the presence of 2 mM $CaCl_2$ or 2% bovine serum albumin significantly enhanced the phospholipase attack of liposomes, but not synaptic membranes or myelin. Phospholipase exhibited a marked preference for phospholipids containing docosahexaenoic acid (22:6) in the synaptic membranes, while with liposomes the pattern of released fatty acid reflected the fatty acid composition in the two-position of the phospholipids. Although either calcium or albumin markedly increased the phospholipase hydrolysis of liposomes, neither affected the hydrolysis of synaptic membranes or the pattern of fatty acid release from liposomes. It was concluded that the nonlipid constituents, particularly the proteins, of biomembranes were responsible for the organization of the phospholipids and accounted for the observed differences between liposomes and synaptic membranes with respect to enzymic accessibility.

**Key words** phospholipase $A_2$ · phospholipid organization · myelin phospholipids · synaptic membrane phospholipids · liposome hydrolysis · phospholipase $A_2$ cofactors

## Introduction

Phospholipases $A_2$ are useful enzymes for determining membrane organization (Zwaal, Roelofsen, Domfurius & van Deenen, 1975; Sundler, Sarcione, Alberts & Vagelos, 1977) and the functional role of phospholipids in biological membranes (Abood, Salem, MacNeil & Butler, 1978; Lefkowitz, 1975). Phospholipases are also of interest because their activity is dependent on the physical characteristics of the phospholipids. Hydrolysis of artificial membranes by phospholipase $A_2$ has been extensively studied in an attempt to determine how the physiochemical properties of the lipid interface influence the enzyme. Certain conditions, such as heating a phospholipid to its phase transition temperature (Op den Kamp, de Gier & van Deenen, 1974), or additives, such as alcohols (Jain & Cordes, 1973) greatly increase the phospholipase attack of pure lipid membranes. It has been suggested that such physical treatments and agents result in structural or chemical pertubations which enhance phospholipase penetration into the lipid phase, thereby increasing hydrolysis (Verger, Mieras & de Haas, 1973). Changes in phospholipase activity or specificity induced by such additives and physical procedures may provide information on membrane organization as well as the nature of the resulting perturbation.

Since biological membranes contain numerous nonphospholipid components, the enzyme activity and substrate specificity of phospholipases differs from that observed with model membrane systems containing phospholipids (Salach, Seng, Tisdale & Singer, 1971*b*; Coles, McIlwain & Rapport, 1974). The limitations to extrapolations from artificial to natural membranes are important to consider when using phospholipases as tools in studies of biomembranes.

Phospholipase $A_2$ from *Vipera russelli* is a potent inhibitor of opiate binding in synaptic tissue, where it appears to preferentially hydrolyze polyunsaturated phospholipids (Abood, Butler & Reynolds, 1980). In the present study, this apparent specificity of phospholipase $A_2$ was investigated further by completely characterizing and comparing the hydrolysis of more homogeneous synaptic membranes and liposomes prepared from myelin phospholipids. The effects of calcium and bovine serum albumin on phospholipase activity were also investigated.

## Materials and Methods

### *Materials*

Highly purified phospholipase $A_2$ from *Vipera russelli* was obtained from Sigma Chemical Co. (St. Louis, Mo.) and purified to homogeneity on a Sephadex G-100 column according to Salach et al. (1971*a*). Fatty acid-free bovine serum albumin and *Crotalus adamanteus* venom were also purchased from Sigma. All fatty acid,

* *Present address:* University of North Carolina, Biological Sciences Research Center 220H, Chapel Hill, North Carolina 27514.

0022-2631/82/0066-0001 $01.40

methyl ester, and phospholipid standards were obtained from Supelco. All solvents were of nanograde quality or glass-distilled. Adult male Sprague-Dawley rats from Charles River were used in all experiments.

### *Preparation of Synaptic Membranes*

A modification of the procedure of Whittaker, Michaelson and Kirkland (1964) was used to prepare myelin and synaptic plasma membranes from rat brain. Rats were sacrificed by cervical dislocation and the brains were quickly removed and homogenized in 10 vol 0.32 M sucrose. The homogenate was centrifuged at $1,000 \times g$ for 10 min, and the supernatant was removed and centrifuged at $17,500 \times g$ for 20 min. After the pellet was suspended in 0.32 M sucrose, it was layered over a discontinuous sucrose gradient of 0.8 and 1.2 M sucrose and the tube centrifuged for 1 hr at $100,000 \times g$ in a Beckman SW 40 rotor. The band of material at the 0.32 M–0.8 M interface was collected, homogenized in 20 vol distilled water, and then centrifuged for 30 min at $70,000 \times g$. This pellet was used as a source of myelin membranes. The material at the 0.8 M–1.2 M interface was also removed and pelleted, and then homogenized in 5 vol distilled water and left on ice for 1 hr. The homogenate was then layered over a 0.6, 0.8, 1.0, and 1.2 M sucrose gradient and centrifuged for 1 hr at $100,000 \times g$. The bands of material at the 0.6 M–0.8 M sucrose and 0.8 M–1.0 M sucrose interfaces were collected, diluted with 10 vol water and centrifuged at $70,000 \times g$ for 30 min. Electron microscopy revealed that the resulting pellet consisted largely of fragments of synaptic plasma membranes, partially disrupted synaptosomes, some small vesicles, and a few unidentified membrane fragments.

### *Treatment of Membranes with Phospholipase $A_2$*

Synaptic membranes were suspended in 20 mM Tris-HCl, pH 7.5, to a concentration of 2 mg protein/ml. Protein was determined according to Lowry, Rosenborough, Farr and Randall (1951), using bovine serum albumin as a standard. Aliquots of the membrane suspension were incubated at 37° for different times with the same concentration of phospholipase $A_2$ (g/mg protein), or for 10 min with different concentrations of phospholipase. Controls were membranes incubated in Tris without the enzyme and were used to determine background levels of free fatty acids. $CaCl_2$ and bovine serum albumin were added to a final concentration of 2 mM or 2%, respectively.

### *Preparation of Liposomes and Incubation with Phospholipase $A_2$*

Lipids were extracted from synaptic membranes according to Bligh and Dyer (1959). The total lipid extract was applied to a silicic acid column, and the total phospholipids were eluted in methanol (after the elution of neutral lipids and cerebrosides) according to Rouser, Kritchevsky and Yamamoto (1976). Phosphate determinations were performed according to Bartlett (1959). Aliquots of the total phospholipid fraction were transferred to homogenizers, and the solvent was evaporated in a stream of argon. Buffer with or without 2 mM $CaCl_2$ or 2% albumin was added followed by phospholipase $A_2$, so that the enzyme/phospholipid ratio was the same as that for the synaptic membrane preparations (520 nmol total phospholipid/mg protein). Samples were homogenized until a cloudy suspension formed and then were incubated as for synaptic membranes.

### *Determination of the Fatty Acids Present in the Two-Position of Phospholipids*

The composition of the total fatty acids in the two-position of substrate phospholipids was determined by exposing synaptic membranes to high concentrations of phospholipase $A_2$ (10 μg/mg protein) or by hydrolyzing purified lipids with *Crotalus adamanteus* venom in a diethyl ether buffer system (Yabuchi & O'Brien, 1968).

### *Fatty Acid Analysis*

After incubation with phospholipase $A_2$, samples were placed on ice and 20 μg pentanoic acid were added as an internal standard for quantification of total fatty acids released. Lipids were extracted as described above and then applied as a 3–4 cm band to silica gel G plates. The plates were developed in a heptane/diethyl ether/acetic acid 90:10:1 system, which separated free fatty acids from all other lipids. After the lipids were exposed to iodine for 10 sec and the fatty acid bands were marked, the iodine was volitized by heating the plates to 50 °C in the presence of argon. The fatty acid bands were scraped into glass stoppered tubes containing 4 ml of 5% HCl in methanol (which had been prepared by bubbling HCl gas into anhydrous methanol). The tubes were flushed with argon and refluxed at 65 °C for 90 min. After the addition of 2 ml water, the methyl esters were extracted twice with 6 ml petroleum ether and the ether extracts pooled and washed with 3 ml of 2% sodium bicarbonate. The samples were centrifuged and the ether phase was removed and concentrated by a stream of argon.

The methyl esters were analyzed on a Hewlett-Packard 5830A digital gas chromatograph with flame ionization detectors, using SP-2330, 10% Degs and OV-1 columns obtained from Supelco. Methyl esters were identified by comparing their retention times to those of authentic standards. Peak data were expressed as area % and were converted to molar quantities for individual fatty acid species by comparison with the internal standard. Except where indicated, all data points represent the mean of at least three separate determinations.

### *Separation of Phospholipid Classes by Two-Dimensional Thin-Layer Chromatography*

In order to determine the fatty acid composition of individual phospholipids, lipid extracts (0.5 μmol phospholipid) were subjected to silica gel H thin-layer chromatography using chloroform/methanol/water/acetic acid, 65:43:3:1 in the first, and chloroform/methanol/water, 60:35:8 in the second dimension. After the plates were developed in iodine vapor and lipid, spots were scraped and transesterified as described above. In order to determine whether the procedure of exposing of lipids to iodine vapor and heating the TLC plates resulted in an alteration of the fatty acids present, duplicate samples of brain phospholipids not exposed to iodine were compared to those developed in iodine. No significant alterations occurred in even the polyunsaturated acids.

## Results

### *Fatty Acid Composition of Synaptic Membranes*

Myelin membranes prepared by subcellular fractionation contained a larger phospholipid-to-protein ratio than synaptic membranes: 814 nmol/mg as compared to 520 nmol/mg. Synaptic membranes contained more polyunsaturated fatty acids than myelin, although palmitic (16:0), oleic (18:1), arachidonic (20:4), and docosahexaenoic (22:6) acids comprised about 90% of the total two-position fatty acids in both membranes (Table 1). The surprisingly large amount of 16:0 released by phospholipase $A_2$ was due to the

**Table 1.** The composition of two-position fatty acids of phosphoglycerides from myelin and synaptic membranes

| Fatty acid | Myelin | Nerve endings |
|---|---|---|
| 16:0 | 14.5±0.6 | 20.5±2.9 |
| 18:1 | 45.7±2.4 | 21.7±1.2 |
| 20:4 | 13.4±0.8 | 19.0±1.5 |
| 22:6 | 15.0±0.8 | 30.8±1.2 |
| Other | 11.4±1.2 | 8.5±0.9 |

Phospholipids from myelin and synaptic membranes were hydrolyzed completely by phospholipase $A_2$. Free fatty acids were separated from lyso-phospholipids, and analyzed by gas chromatography. Individual fatty acids are expressed as the mean standard deviation of their molar percentages of the total. "Other" fatty acids include 16:1, 18:2, 20:1, 20:3, 22:4 (*n*-6) and 22:5 (*n*-6).

**Table 2.** Fatty acid composition of the major phospholipids in myelin membranes

| Fatty acid | Phosphoglycerides | | |
|---|---|---|---|
| | Ethanolamine | Choline | Serine |
| 16:0 | 7.6±0.8 | 39.1±1.1 | 2.8±1.0 |
| 18:0[a] | 23.2±1.0 | 15.8±3.0 | 43.3±2.3 |
| 18:1 | 31.8±2.9 | 36.1±1.8 | 31.5±3.2 |
| 20:4 | 8.6±0.8 | 3.3±0.5 | 5.6±0.4 |
| 22:6 | 9.9±0.6 | 1.9±0.2 | 10.8±0.5 |
| Other | 18.9±2.7 | 3.8±0.3 | 6.0±1.4 |

The phospholipids from myelin were separated by two-dimensional thin-layer chromatography and analyzed for their fatty acid composition by gas chromatography. Values represent molar % of the total fatty acids and are expressed as mean standard deviation. "Other" includes 16:0, 18:0 and 18:1 fatty aldehydes, and 16:1, 18:1, 20:0, 20:1, 20:3, 22:4 (*n*-6) and 22:5 (*n*-6) fatty acids. The total molar % of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine was 45.0±2.0, 31.5±2.9 and 14.1±0.5 of the total extracted phospholipid.

[a] Includes 18:1 fatty aldehyde.

**Table 3.** Fatty acid composition of the major phospholipids in control and phospholipase $A_2$-treated synaptic membranes

| Fatty acid | Phosphatidylethanolamine | | |
|---|---|---|---|
| | Control | 0.1 μg | 1.0 μg |
| 16:0 | 9.4 | 8.7 | 10.9 |
| 18:0 | 29.9 | 23.6 | 21.7 |
| 18:1 | 10.7 | 11.0 | 14.1 |
| 20:4 | 12.8 | 13.1 | 11.7 |
| 22:6 | 23.5 | 21.6 | 15.4 |
| Other | 13.7 | 21.9 | 26.2 |
| Fatty acid | Phosphatidylcholine | | |
| | Control | 0.1 μg $PLA_2$ | 1.0 μg $PLA_2$ |
| 16:0 | 56.7 | 60.5 | 65.3 |
| 18:0 | 10.9 | 10.6 | 9.9 |
| 18:1 | 22.1 | 21.7 | 20.1 |
| 20:4 | 4.4 | 2.8 | 1.4 |
| 22:6 | 2.6 | 1.2 | 0.4 |
| Other | 2.4 | 3.2 | 2.8 |
| Fatty acid | Phosphatidylserine | | |
| | Control | 0.1 μg $PLA_2$ | 1.0 μg $PLA_2$ |
| 16:0 | 3.2 | 4.1 | 10.0 |
| 18:0 | 44.8 | 45.8 | 43.4 |
| 18:1 | 14.0 | 20.0 | 28.3 |
| 20:4 | 2.5 | 1.9 | 1.9 |
| 22:6 | 29.7 | 21.9 | 11.0 |
| Other | 5.8 | 6.2 | 5.4 |

Synaptic membranes were incubated with different concentrations of phospholipase $A_2$ (μg/mg protein) for 10 min at 37 °C. Lipids were extracted and total phospholipds were separated by two-dimensional thin-layer chromatography. Individual phospholipids were transesterified and the methyl esters analyzed by gas chromatography. Results are in molar % and represent the mean of two determinations agreeing within 10%. (For "other" fatty acids, *see* Table 2.)

high content of dipalmitoylphosphatidylcholine in brain (Yabuchi & O'Brien, 1968). Ethanolamine and serine phosphoglycerides contained most of the 22:6 in both myelin and synaptic membranes, while phosphatidylcholine contained more saturated fatty acids (Tables 2, 3).

### *Hydrolysis of Synaptic Membranes and Liposomes by Phospholipase $A_2$*

The phospholipids of both myelin and synaptic membranes were readily hydrolyzed by phospholipase $A_2$ from *Vipera russelli* (Table 4), and could be completely hydrolyzed at high concentrations of phospholipase (10 μg/mg protein). The decrease in hydrolysis with time (Fig. 1) may have been due to the accumulation of inhibitory by-products, since the presence of 1% albumin enhanced hydrolysis significantly at high concentrations of phospholipase $A_2$. The addition of 2 mM $CaCl_2$ had no effect on phospholipase activity towards synaptic membranes.

A study of the hydrolysis of multilammelar liposomes of the total phospholipids from myelin was also performed. Such liposomes were attacked to a much lesser extent than membranes containing an equal amount of substrate (Figs. 1 and 2). Although the addition of 2 mM $CaCl_2$ significantly increased the hydrolysis of liposomes, phospholipase $A_2$ was active even in its absence, and the presence of 2% bovine serum albumin had an even greater effect. The addition of albumin actually increased the hydrolysis of liposomes to the level obtained with myelin membranes (Fig. 2). The addition of such neutral lipids as cholesterol or cerebrosides to the phospholipids

**Table 4.** Comparison of the hydrolysis of synaptic and myelin membranes by Phospholipase $A_2$

| Synaptic endings | | | Myelin | | |
|---|---|---|---|---|---|
| μg phopholipase mg protein | nmol fatty acid mg protein | % membrane phospholipid hydrolyzed | μg phospholipase mg protein | nmol mg protein | % membrane phospholipid hydrolyzed |
| 0.01 | 36.2±4.3 | 7.0 | 0.02 | 37.8±1.6 | 4.6 |
| 0.1 | 139.6±21.3 | 26.9 | 0.1 | 152.4±6.8 | 18.7 |
| 1.0 | 365.2±30.7 | 70.2 | 1.0 | 373.4±52.3 | 45.8 |

Membranes were incubated with different concentrations of phospholipase $A_2$ for 10 min at 37 °C. Lipids were extracted and analyzed for free fatty acids, which are expressed as mean±standard deviation. The percentage of the membrane phospholipid hydrolyzed was derived from the fatty acid data and the total lipid phosphorous measurements in each membrane type (*see* text).

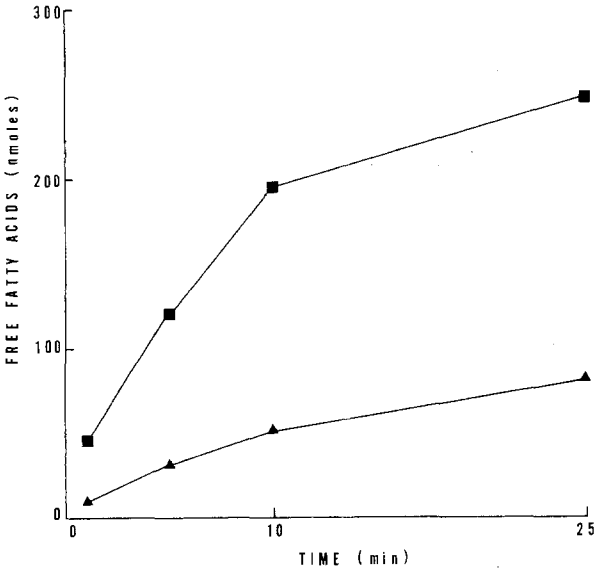


**Fig. 1.** The hydrolysis of myelin membranes and liposomes by phospholipase $A_2$ as a function of time. Myelin membranes (■) and liposomes of the total phospholipids from myelin (▲) were incubated with 0.2 μg phospholipase $A_2$ per mg membrane protein or its phospholipid equivalent (*see* Methods). The total free fatty acids were corrected for background levels in controls

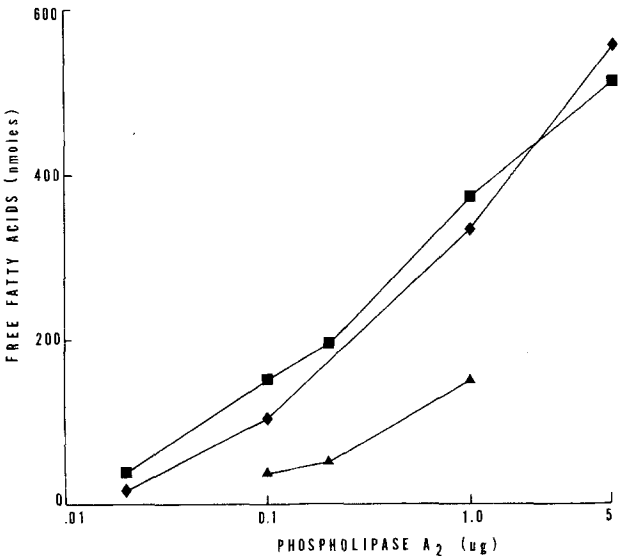


**Fig. 2.** Total free fatty acids released from myelin membranes and liposomes at various concentrations of phospholipase $A_2$ (fatty acids named in figure). Myelin membranes (■) and liposomes with (◆) or without (▲) 2% bovine serum albumin were incubated with different concentrations of phospholipase $A_2$ for 10 min at 37 °C

mixture did not alter the rate and pattern of fatty acid release by phospholipase $A_2$. A similar study using liposomes prepared from total myelin lipids also yielded comparable results.

### *Specificity in Phospholipase $A_2$ Attack of Membranes and Liposomes*

The substrate specificity of phospholipase $A_2$ was investigated in synaptic membranes and lipsosomes. When phospholipase $A_2$ was incubated with synaptic or myelin membranes, there was a disproportionate release of fatty acid species relative to the amounts present in the two-position of the substrate phospholipids. Although 22:6 and 20:4 comprised 30 and 19% of the total 2-position fatty acids in synaptic membrane phospholipids, respectively, they comprised 50 and 25% of the free fatty acids released by 0.01 μg phospholipase $A_2$. Only 11 and 10% of the fatty acids released were 18:1 and 16:0, respectively, even though they comprised 22 and 21% of the total two-position fatty acid present (Table 1). This disproportionate release of fatty acids at different concentrations of phospholipase $A_2$ was reflected in the fatty acid composition of the intact phospholipids in which the relative amounts of 22:6 decreased while 18:1 increased in phosphatidylethanolamine and phosphatidylserine (Table 3).

The fatty acids released from myelin membranes were even less proportionate to their relative distribution in membrane phospholipid. Although only 15% of the total fatty acids in the two-position of myelin

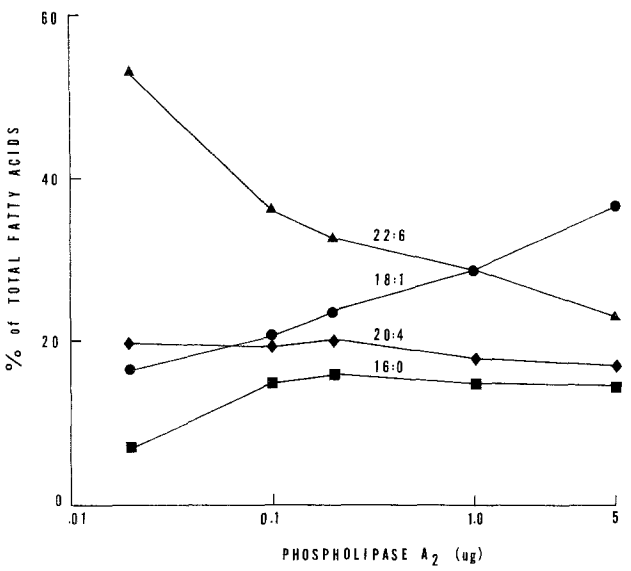


**Fig. 3.** The pattern of fatty acid release from myelin membranes at various concentrations of phospholipase $A_2$. Myelin membranes were incubated with phospholipase $A_2$ (μg/mg protein) for 10 min at 37°. Data points represent the mean molar percentage of the total fatty acids for each of the major, individual fatty acids released

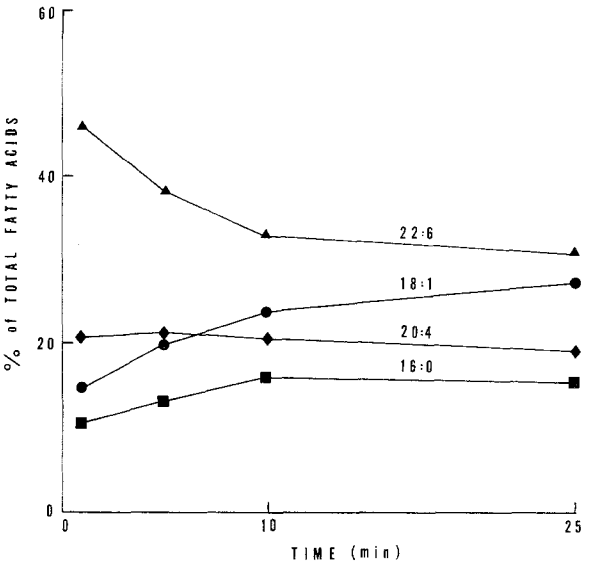


**Fig. 4.** The pattern of the fatty acid release from myelin membranes as a function of time. Myelin membranes were incubated with 0.2 μg phospholipase per mg membrane protein, and the free fatty acids were analyzed at different time points

phospholipids was 22:6 (Table 1), more than 50% of the free fatty acids released by 0.02 μg enzyme was 22:6, while only 17% was 18:1, even though it comprised over 45% of the 2-position fatty acids (Fig. 3). The relative proportions of fatty acids released by phospholipase $A_2$ became more representative of their actual distribution in the membranes as the degree of hydrolysis increased. Although the percentages of 16:0 and 20:4 in the released free fatty acid pool varied significantly from their actual distribution in substrate phospholipids, the greatest differences between the amount released and remaining in the membrane occurred with 22:6 and 18:1 (Fig. 3). After incubation of myelin membranes with 1.0 μg phospholipase $A_2$ for 10 min, more than 90% of the 22:6 present in the two-position of the membrane phospholipids had been released, while only 32% of the available 18:1 was hydrolyzed.

The release of fatty acids in the presence of phospholipase $A_2$ was also studied as a function of time (Fig. 4). The results indicate that, relative to their concentrations in the membrane, phospholipids containing 22:6 were hydrolyzed fastest and 18:1 phospholipids were hydrolyzed slowest. After the first 10 min 150 nmol of 22:6 and about 50 nmol of 18:1 per mg protein was hydrolyzed (data not shown). The relative percentages of 20:4 in the free fatty acid pool did not vary significantly with time, as determined by analysis of variance.

In liposomes comprised of total myelin phospholipids, the relative amounts of the major fatty acids

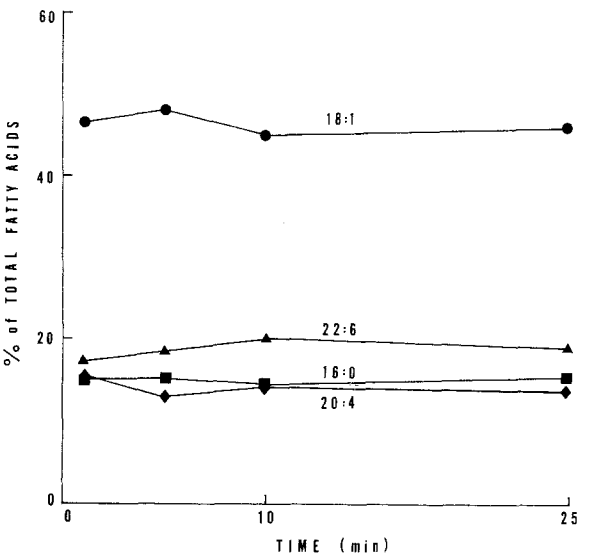


**Fig. 5.** The pattern of the fatty acid release from liposomes as a function of time. Liposomes of the total phospholipids from myelin membranes containing 1 mg protein were incubated with 0.2 μg phospholipase $A_2$ for 10 min at 37°

released by phospholipase $A_2$ did not vary significantly with time and were representative of their proportions in the substrate phospholipids (Fig. 5). When the release of fatty acids from liposomes formed in the presence of 2% albumin was studied as a function of enzyme concentration, there were significant variations in the relative amounts of fatty acids released at different concentrations, while more 22:6 was hy-

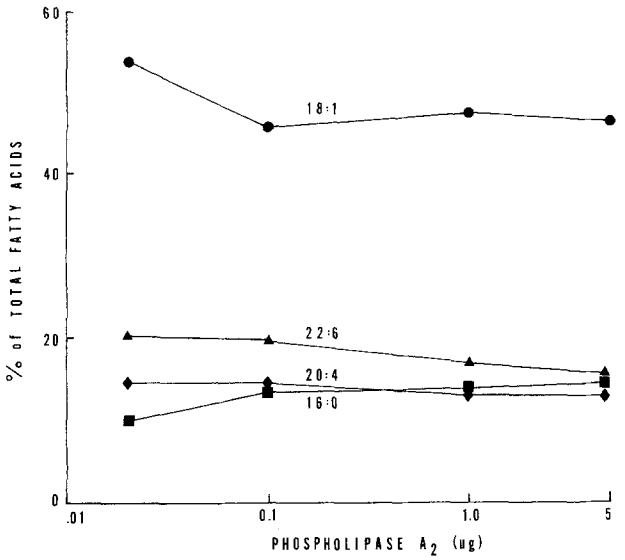


**Fig. 6.** The pattern of fatty acid release from liposomes containing 2% bovine serum albumin. Liposomes of total phospholipids from myelin were formed in the presence of 2% albumin and were incubated with different concentrations of phospholipase $A_2$ (μg/phospholipid equivalent of 1 mg membrane protein)

drolyzed than 16:0 at lower concentrations (Fig. 6). However, the relative percentages of the major fatty acids were always fairly representative of their proportions in the phospholipids from myelin.

The effect of 2 mM calcium or 2% albumin in the presence of phospholipase was investigated on the fatty acid release from myelin and liposomes (data not shown). Calcium alone produced no enhancement of hydrolysis of myelin while 2% albumin resulted in a 38% increase in the presence of 0.2 μg phospholipase $A_2$. In the presence of 0.1 g phospholipase $A_2$, calcium produced a 74% and albumin a 167% increase in total fatty acids released from liposomes. Neither calcium nor albumin altered the pattern of fatty acids released from either myelin or liposomes.

## Discussion

In order to determine the activity of phospholipase $A_2$ towards pure and phospholipid membranes, it was essential that both sides of the phospholipid bilayers in the artificial membrane be exposed to the enzyme in order to ensure that any differences in activities were not due to an inability of the enzyme to reach the substrate. Unilammelar lipid vesicles formed by sonication could not be used as the model membranes, since phospholipase $A_2$, which would have to be added after sonication, would only have access to the outer layer of the vesicle. Misleading results might have been obtained since lipids distribute asymmetrically in mixed vesicles because of their small size and high degree of curvature (Litman, 1973; Lee & Forte, 1978). Multilammelar liposomes, however, could be formed in the presence of phospholipase, which would then be trapped in between and have access to both sides of the bilayers.

When the phospholipase hydrolysis of membranes and liposomes was compared, several major differences were observed. Firstly, less total phospholipid was hydrolyzed in liposomes than in synaptic membranes containing an equal amount of substrate; secondly, hydrolysis of lipsomes, but not membranes, was greatly increased by the addition of calcium or albumin; and lastly, even after the addition of calcium or albumin to liposomes, phospholipase $A_2$ did not exhibit the preference for phospholipids containing 22:6 as it did towards synaptic membranes.

The net increase in phospholipase hydrolysis of liposomal phospholipid produced by calcium or albumin is reminiscent of the stimulation caused by interfacial activators, which appear to enhance enzyme activity by perturbing the phospholipid phase (Verger et al., 1973). Although enhancement of phospholipase activity by the addition of calcium could be due to a requirement of the enzyme for calcium, hydrolysis did occur in its absence, and the addition of albumin alone increased hydrolysis of liposomes to an even greater extent. In a manner similar to other activating agents, calcium binds to phospholipid interfaces to alter such physiochemical parameters as phase transitions and surface pressure and charge (Toko & Yamafuji, 1980). Bovine serum albumin may have increased hydrolysis in synaptic membranes by removing the reaction by-products, since its effect was greater at high concentrations of phospholipase $A_2$. In liposomes, however, the enhancing effect of albumin was greater at low concentrations of phospholipase, where it increased hydrolysis threefold. Albumin has been shown to bind electrostatically to phospholipids and to increase the surface pressure in monolayers (Quinn & Dawson, 1970). It is possible, therefore, that the addition of calcium and bovine serum albumin to liposomes stimulated phospholipase activity by interacting with and perturbing the lipid phase in a way that made it more susceptible to enzymic attack. In synaptic membranes, however, the addition of calcium or albumin had relatively little effect. The inability of additives such as calcium and anesthetics (which active hydrolysis of pure lipid membranes) to stimulate phospholipase attack of biological membranes has been observed by other investigators (Salach et al., 1971*b*; Scherphof & Westenberg, 1975). Apparently nonlipid components of natural membranes help promote phospholipid hydrolysis.

Since the phospholipids in multilammelar liposomes were readily accessible to phospholipase $A_2$,

any inherent specificity of the enzyme for a particular molecular species of phospholipid should have been exhibited in studies with liposomes. Under all the conditions used, however, the relative amounts of fatty acids released from liposomes by phospholipase $A_2$ were representative of their proportions in the two-position of the substrate phospholipids, and all the molecular species of phospholipids were hydrolyzed at a comparable rate. In myelin membranes, however, the preference of phospholipase $A_2$ for phospholipids containing 22:6 was especially great, while phospholipids containing 18:1 were hydrolyzed the least relative to their amounts in the membrane. The preference for 22:6 was not an intrinsic property of the enzyme because it was not manifest in liposomal phospholipids. It seems most likely that it resulted from the greater accessibility of phospholipids in synaptic membranes, particularly the 22:6 phospholipids (predominantly serine and ethanolamine phosphoglycerides). The differential accessibility was not induced by random interactions among the phospholipids themselves, since it did not occur in liposomes. Although calcium and albumin increased the total hydrolysis of liposomes, neither affected the specificity of phospholipase $A_2$. It is possible, however, that membrane proteins having more specific interactions with phospholipids are responsible for the organization of phospholipids accounting for the observed differences in enzymic accessibility. Another possibility is that the synaptic membranes resealed into vesicles and only the outer leaflet containing predominantly 22:6 phospholipid was accessible to $PLA_2$.

This research was supported by U.S. Public Health Service grants DA00464 and NS 15345.

## References

Abood, L.G., Butler, M.B., Reynolds, D. 1980. Effect of calcium and physical state of synaptic membranes on phosphatidylserine requirement for opiate binding. *Mol. Pharmacol.* **17**:290–294

Abood, L.G., Salem, N., MacNeil, M., Butler, M. 1978. Phospholipid changes in synaptic membranes by lipolytic enzymes and subsequent restoration of opiate binding with phosphatidyl serine. *Biochim. Biophys. Acta* **530**:35–46

Bartlett, G.R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* **234**:466–468

Blight, E.G., Dyer, W.J. 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**:911–917

Coles, E., McIlwain, D.L., Rapport, M.M. 1974. The activity of pure phospholipase $A_2$ from *Crotalus atrox* venom on myelin and on pure phospholipids. *Biochim. Biophys. Acta* **337**:68–78

Jain, M.K., Cordes, E.H. 1973. Phospholipases: I. Effect of *n*-alkanols on the rate of enzymatic hydrolysis of egg phosphatidylcholine. *J. Membrane Biol.* **14**:101–118

Lee, H.C., Forte, J.G. 1978. Asymmetric labeling of amino lipids in liposomes. *Biochim. Biophys. Acta* **554**:375–387

Lefkowitz, R. 1975. Catecholamine stimulated myocardial adenylate cyclase: Effects of phospholipase digestion and the role of membrane lipids. *J. Mol. Cell. Cardiol.* **7**:27–37

Litman, B.J. 1973. Lipid model membranes. Characterization of mixed phospholipid vesicles. *Biochemistry* **12**:2545–2554

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**:256–275

Op den Kamp, J.A.F., Gier, J. de, Deenen, L.L.M. van 1974. Hydrolysis of phosphatidyl choline liposomes by pancreatic phospholipase at the transition temperature. *Biochim. Biophys. Acta* **345**:253–256

Quinn, P., Dawson, R.M.C. 1970. The penetration of serum albumin into phospholipid monolayers of different fatty acid chain length and interfacial charge. *Biochem. J.* **119**:21–25

Rouser, G., Kritchevsky, G., Yamamoto, A. 1967. Column chromatographic and associated procedures for separation and determination of phospholipids and glycolipids. *In:* Lipid Chromatographic Analysis. G.V. Marinetti, editor. Vol. 1, pp 99–163. Marcel Dekker, New York

Salach, J.I., Seng, R., Tisdale, H., Singer, T.P. 1971 *b*. Phospholipase A of snake venoms. II. Catalytic properties of the enzyme from *Naja naja*. *J. Biol. Chem.* **246**:340–346

Salach, J.I., Turini, R., Seng, R., Hauber, S., Singer, T.P. 1971 *a*. Phospholipase A of snake venoms. I. Isolation and molecular properties of isoenzymes from *Naja naja* and *Vipera russelli* venoms. *J. Biol. Chem.* **246**:331–339

Scherphof, G., Westenberg, H. 1975. Stimulation and inhibition of pancreatic phospholipase by local anesthetics as a result of their interaction with the substrate. *Biochim. Biophys. Acta* **398**:442–451

Sundler, R., Sarcione, S.L., Alberts, A.W., Vagelos, P.R. 1977. Evidence against phospholipid asymmetry in intracellular membranes from liver. *Proc. Natl. Acad. Sci. USA* **74**:3350–3354

Toko, K., Yamafuji, K. 1980. Influence of monovalent and divalent cations on the surface area of phosphatidyl glycerol monolayers. *Chem. Phys. Lipids* **26**:79–99

Verger, R., Mieras, M.C.E., Haas, G.H. de 1973. Action of phospholipase A at interfaces. *J. Biol. Chem.* **248**:4023–4034

Whittaker, V.P., Michaelson, I.A., Kirkland, R.J.A. 1964. The separation of synaptic vesicles from nerve-ending particles ("synaptosomes"). *Biochem. J.* **90**:293–303

Yabuchi, H., O'Brien, J.S. 1968. Positional distribution of fatty acids in glycerophosphatides of bovine gray matter. *J. Lipid Res.* **9**:65–67

Zwaal, R.F.A., Roelofsen, B., Domfurius, P., L.L.M. van 1975. Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. *Biochim. Biophys. Acta* **406**:83–96

Received 30 March 1981; revised 19 June 1981 and 23 October 1981