Hydrolysis of Phosphatidylethanolamine Induced by Nominally Synthetic Lysophosphoglycerides: Methodological Implications†

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ABSTRACT: Synthetic lysophosphatidylethanolamine (LPE) obtained from commercial sources augmented the apparent activity of phospholipase A_2 (PLA_2) in cardiac mitochondrial and microsomal fractions. For elucidation of this phenomenon, 2-[1-^{14}C]linoleylphosphatidylethanolamine was incubated in the absence of cell protein with selected concentrations of LPE, lysophosphatidylcholine (LPC), Ca^{2+}, ionic and nonionic detergents, and phospholipids with functional groups similar to those of LPE. Hydrolysis of phosphatidylethanolamine (PE) was evaluated by measurement of ^{14}C-labeled free fatty acid released and confirmed by quantification of [^{14}C]ethanolamine-labeled LPE formed. The reaction was dependent on the concentrations of Ca^{2+}, PE, and LPE, exceeding 1.5 nmol/h with 20 μM LPE and 30 μM PE. Hydrolysis occurred in the presence of as little as 1 μM LPE. PE was not hydrolyzed by comparable concentrations of ionic or nonionic detergents or by several closely related phosphatides, including LPC. Purification of synthetic LPE by high-performance LC to remove contaminating PLA_2 eliminated the effect. LPE-induced hydrolysis of PE was found to depend on contamination of the LPE by PLA_2 from *Crotalus atrox*, employed in the commerical synthesis of the lysophosphatide from the precursor used, phosphatidylethanolamine. Contamination of commercially obtained lysophosphoglycerides by PLA_2 constitutes a technical pitfall which may cloud interpretation of experiments performed with inadequately purified material.

Many investigators employing commerically available, nominally synthetic lysophosphoglycerides have concluded that these moieties alter the properties of cell membranes. For example, they have been reputed to promote fusion of somatic and gametic cells (Koprowski & Croce, 1973), lyse erythrocytes and bacteria (Lucy et al., 1969), restore beating to cultured cardiac myocytes (Goshima, 1971), and alter electrophysiological properties of Purkinje fibers (Sobel et al., 1978). Moreover, altered activities of several enzymes such as Na^+,K^+-ATPase (Karli et al., 1979), adenylate and guanylate cyclases (Shier et al., 1976; Ahumada et al., 1979), and CTP:phosphocholine cytidylyltransferase (Choy & Vance, 1978) have been attributed to the effects of synthetic lysophosphoglycerides. In the course of an investigation of modulators of PLA_2 activity we noted apparent activation by synthetic LPE obtained from commerical sources. Characterization of this phenomenon indicated that the lysophosphoglyceride was contaminated with PLA_2 used in the synthetic process and incompletely removed during commerical purification.

Experimental Procedures

Materials.[1] PC, PE, LPC, LPE, GPC, GPE, LPG, and *Crotalus adamanteus* PLA_2 were obtained from Sigma Chemical Co. [1-^{14}C]Linoleic acid, [1,2-^{14}C]ethanolamine hydrochloride, and 1-palmitoyl[^{14}C]PC, -PE, and -LPE were obtained from New England Nuclear Corp. Purity of reagents exceeded 95% with respect to lipids based on two-dimensional thin-layer chromatography on silica gel OF plates from New England Nuclear Corp. (Sobel et al., 1978). LPE was purified by high-performance liquid chromatography (high-performance LC) with a Waters Association system equipped with a Model 450 variable absorbance UV meter. LPE was isocratically eluted from a Whatman PXS-10/25 SCX column with acetonitrile–methanol–water (225:150:45) at a flow rate of 2.2 mL/min. Absorbance was monitored at 203 nm.

LPA was prepared from LPE incubated with Savoy cabbage phospholipase D at pH 5.8 with 125 mM Ca^{2+}. Products were separated by solvent fractionation (Long et al., 1967).

Radiolabeled phosphatidylethanolamine was prepared by acylation of 1-palmitoylglycerophosphorylethanolamine (Waite & VanDeenen, 1967). Sonicates of 50 μmol of LPE and 60 μmol of [^{14}C]linoleic acid (1 mCi) were incubated at 37 °C for 2 h in 21 mL of a reaction medium containing 450 μmol of disodium ATP, 11 μmol of disodium CoA, 0.1 M phosphate buffer, pH 7.4, 5 mM $MgCl_2$, and 125 mg of rabbit liver microsomes prepared from a 25% w/v homogenate in 0.25 M sucrose and 0.1 M Tris, pH 7.4, and centrifuged at 110000*g* for 1 h after removal of the 10000*g* sediment. 1-Palmitoyl-[2-^{14}C]linoleylphosphatidylethanolamine and other phospholipids were extracted in chloroform–methanol (Bligh & Dyer, 1959). Radiolabeled products of the reaction were separated by silica gel chromatography on a 2.4 × 30 cm column with 50 g of silica gel by elution with $CHCl_3$–CH_3OH–NH_4OH (520:200:40) after elution of less polar lipids with 10% CH_3OH in $CHCl_3$. Specific radioactivity of the products exceeded 5 μCi/μmol.

[^{14}C]PC was prepared similarly but separated by column chromatography on silicic acid (40 g) rather than silica gel with initial elution with 10% methanol in $CHCl_3$ to remove less polar lipids and with subsequent elution with 40% methanol in $CHCl_3$.

For experiments requiring phosphatidylethanolamine labeled in the polar head group rather than in the fatty acid moieties, labeling was performed in vivo by injecting 200-g male guinea pigs intraperitoneally with 500 μCi of [^{14}C]ethanolamine. Twenty-four hours later, the liver was excised, homogenized

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[1] Abbreviations used: PC, phosphatidylcholine (dipalmitoyl); PE, phosphatidylethanolamine (dipalmitoyl); LPC (lysophosphatidylcholine), 1-palmitoylglycerophosphorylcholine; LPE (lysophosphatidylethanolamine), 1-palmitoylglycerophosphorylethanolamine; GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; LPG (lysophosphatidylglycerol), 1-acylglycerophosphorylglycerol from *E. coli*; LPA (lysophosphatidic acid), L-1-palmitoyl[1-^{14}C]glycerophosphoric acid; PLA_2, phospholipase A_2; LC, liquid chromatography.

0006-2960/80/0419-1934$01.00/0

at 0–4 °C with a Polytron homogenizer for two 15-s bursts at a speed setting of 5, extracted with $CHCl_3$–CH_3OH (1:2), and filtered through Whatman No. 1 paper. Phospholipids in the extract were separated by silica gel column chromatography. The isolated radiolabeled product was >90% pure with respect to lipids with <4% contamination with labeled PC and <3% contamination with labeled cardiolipin on the basis of two-dimensional TLC.

Assays of Phospholipase A_2 Activity. Rabbit mitochondria and microsomes were prepared from 25% w/v homogenates in 0.25 M sucrose and 0.1 M Tris, pH 7.4. After removal of the 1000-g pellet, mitochrondria were obtained by centrifugation of the supernate at 10000*g* for 30 min, and microsomes were obtained by centrifugation of the mitochondrial supernate at 100000*g* for 60 min. PLA_2 activity was assayed with sonicates of [^{14}C]PE (30–125 μM), mitochondria or microsomes containing 0.025–0.15 mg of protein or *Crotalus adamanteus* phospholipase A_2, 1 unit, employed as a standard, $CaCl_2$ (0–5 mM), and 0.1 M Tris buffer at the selected pH in a final volume of 0.25–0.4 mL with incubation for selected intervals (5 min to 2 h) at 37 °C. Reactions were terminated with methanol, and media were extracted conventionally (Bligh & Dyer, 1959). The labeled fatty acid product of hydrolysis was separated on thin-layer chromatograms developed in 10% methanol in $CHCl_3$ with radioactivity assayed without elution by liquid scintillation spectrometry of scraped regions of the plates.

Phospholipolysis occurring in the presence of synthetic lysophosphoglycerides was assayed in media without aliquots of myocardial subcellular fractions in which 30 μM [^{14}C]PE, 0.1 mM Tris, pH 7.4, selected concentrations of $CaCl_2$ (generally 5 mM), and selected concentrations of LPE, LPC, stearylamine, Triton X-100, or deoxycholate (0–400 μM) were incubated at 37 °C for 5–60 min in a final volume of 250 μL. Products were extracted (Bligh & Dyer, 1959) and radiolabeled fatty acid was quantified by scintillation spectrometry. Ethanolamine-labeled LPE was separated by two-dimensional TLC with the following two solvent systems: (1) $CHCl_3$–CH_3OH–NH_4OH–pyridine (130:58:15:4) and (2) $CHCl_3$–acetone–CH_3OH–CH_3COOH–H_2O (60:80:20:20:10). Verification of products was accomplished by assaying radioactivity of ethanolamine-labeled LPE in fractions separated by high-performance LC with nonradioactive standards employed to localize peaks.

Results

PLA_2 Activity. Rabbit heart mitochondria (n = 9 preparations), microsomes (n = 4), and rabbit liver microsomes (n = 3) exhibited PLA_2 activity with an average of 0.038 ± 0.004 (SD), 0.15 ± 0.03, and 3.2 ± 1.1 nmol of fatty acid liberated/(mg of protein·min). As shown in Figure 1, addition of synthetic lysophosphatidylethanolamine apparently augmented mitochondrial phospholipase A_2 activity by more than tenfold even when the exogenous LPE concentration was within the range that would result from accumulation of LPE generated in the PLA_2 reaction system in the absence of reacylation and lysophospholipase activity. Equivalent concentrations of synthetic LPC did not augment apparent PLA_2 activity comparably. Although modest augmentation of activity was evident when very large concentrations of LPC were included in the reaction medium, concentrations of 10 μM did not significantly increase apparent PLA_2 activity in mitochondria in experiments in which augmentation by LPE at the same concentrations was striking (Figure 1).

PLA_2 activity in heart mitochondria and microsomes with [^{14}C]PC as substrate was consistently less than 10% of that

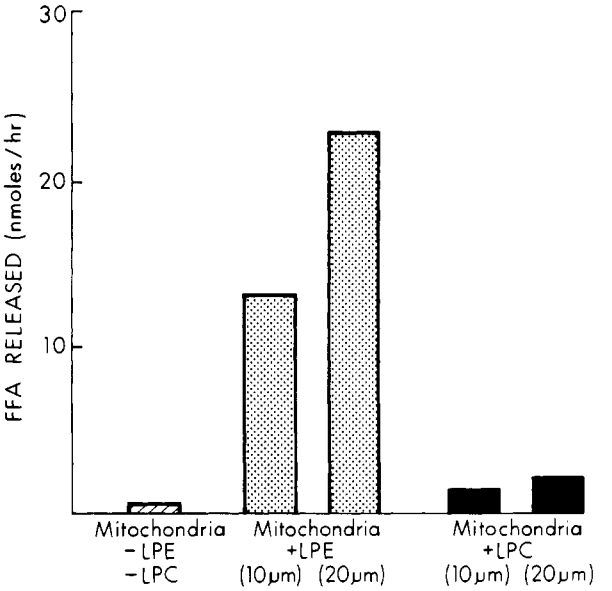

FIGURE 1: Differential effects of LPE and LPC on release of ^{14}C-labeled fatty acid from PE in the presence of cardiac mitochondria. Incubations were performed for 90 min in a final volume of 0.25 mL with media containing 0.15 mg of rabbit myocardial mitochondrial protein, 5 mM Ca^{2+}, 0.1 mM 2-[^{14}C]acyl-PE, and 0.1 M Tris, pH 7.4, with or without LPE or LPC at the concentrations indicated. Results expressed are means ± SE from six assays of each type with each determination in duplicate.

seen with equivalent concentrations of [^{14}C]PE (n = 6 determinations). Under these conditions, addition of 10 μM LPC did not alter PLA_2 activity, whereas 10 μM LPE appeared to increase activity by 260% (averages of two determinations in each case).

Although effects of synthetic lysophosphoglycerides on PLA_2 activity of myocardial subcellular fractions might have been due in part to direct effects on phospholipase or its immediate environment and access to substrate, we considered the possibility that the apparent "activity" did not require the presence of myocardial tissue. When 20 μM LPE was incubated for 1 h with 30 μM [^{14}C]PE and 5 mM $CaCl_2$ in 0.1 M Tris, pH 7.4, ^{14}C-labeled fatty acid release totaled 1.9 nmol/h. Corresponding values for hydrolysis in 0.1 M phosphate, pH 7.4, and in unbuffered water were 1.6 and 1.5 nmol/h, respectively. In contrast, in control assays performed in the absence of LPE, <0.1 nmol of ^{14}C-labeled fatty acid was released. No hydrolysis occurred in the absence of LPE with or without addition of LPC or a long-chain aliphatic amine, stearylamine. As shown in Figure 2, hydrolysis of PE in the presence of commerical LPE was dependent upon the time of incubation, the concentration of radiolabeled substrate, and the concentration of synthetic LPE. Concentrations of synthetic LPE as low as 1 μM elicited consistent increases in FFA liberated from radiolabeled PE. The hydrolysis of PE was not paralleled by equivalent hydrolysis of radiolabeled PC in the presence of exogenous LPE or LPC.

Hydrolysis induced by synthetic LPE was pH dependent. Maximum hydrolysis occurred at neutral pH. At pH 5 and 9 only 23 and 60% of [^{14}C]PE was hydrolyzed compared to hydrolysis at pH 7.3. In the absence of added Ca^{2+}, hydrolysis induced by 10 μM LPE averaged 0.6 nmol/h with 30 μM [^{14}C]PE as substrate. A concentration-dependent increase in hydrolysis was observed with additions of Ca^{2+} with maximum hydrolysis in response to 6 mM Ca^{2+} proceeding at a rate of 1.9 nmol/h. No further augmentation of hydrolysis occurred with additional calcium. Mg^{2+} was without apparent effect on the rate of reaction when results were compared to those

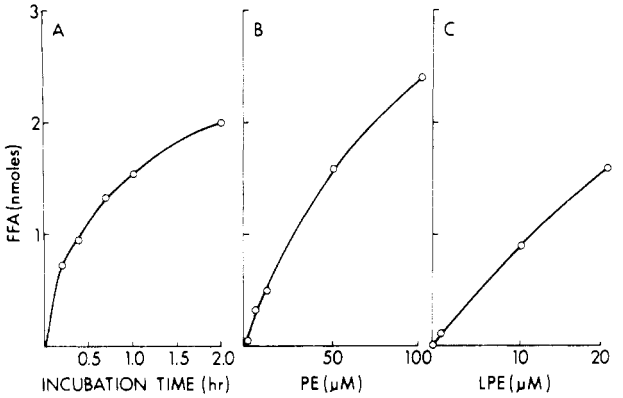

FIGURE 2: The dependence of radiolabeled fatty acid release from PE on time (A) and concentrations of PE (B) and LPE (C). Incubations were performed for times indicated in the graph (A) or for 1 h (B and C) in a final volume of 0.25 mL containing 0.1 M Tris buffer, pH 7.4, and 5 mM $CaCl_2$. Concentrations of lysophosphoglycerides were as follows: (A) LPE, 20 μM, PE, 30 μM; (B) LPE, 10 μM; (C) PE, 30 μM.

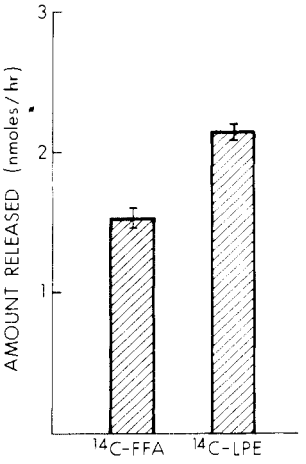

FIGURE 3: Correspondence between ^{14}C-labeled fatty acid released from 2-[^{14}C]acyl-PE and [^{14}C]ethanolamine formed from [^{14}C]-ethanolamine-labeled PE. Experiments with substrate labeled in the two loci were performed separately. Incubations were performed for 1 h in a final volume of 0.25 mL containing 0.1 M Tris, pH 7.4, 30 μM PE, 20 μM LPE, and 5 mM $CaCl_2$ in the reaction medium. Results expressed are means ± SE from two sets of experiments with all determinations performed in duplicate. ^{14}C-Labeled fatty acid was assayed by thin-layer radiochromatography and [^{14}C]ethanolamine-labeled PLE by TLC and high-performance LC as described in the text.

obtained under conditions in which no divalent cation was added to the reaction medium.

Experiments were performed in which PE was radiolabeled in the polar head group in order to determine whether the liberation of fatty acid from [^{14}C]PE induced by LPE was accompanied by accumulation of LPE from the radiolabeled PE as well. When [^{14}C]ethanolamine-labeled PE was incubated with synthetic LPE, [^{14}C]ethanolamine-labeled LPE was formed in quantities comparable to those anticipated on the basis of liberation of ^{14}C-labeled fatty acid from PE radiolabeled in the 2-acyl group as can be seen in Figure 3. Quantification of [^{14}C]ethanolamine-labeled LPE was obtained by separation of the phospholipids with the use of high-performance liquid chromatography (Figure 4).

Experiments were performed to verify that accumulation of LPE was accompanied by an equivalent decline in PE; 40 μM LPE was incubated with 30 μM PE for 1 h in 3.75 mL of Tris buffer, pH 7.4, with 5 mM $CaCl_2$. Extracted products were separated by two-dimensional TLC, and lipid phosphate

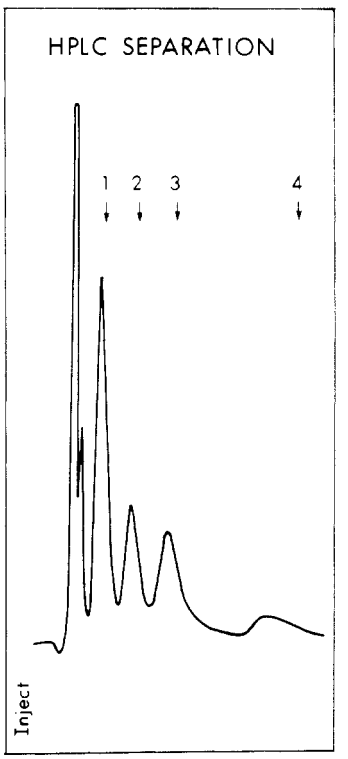

FIGURE 4: Representative chromatogram obtained by high-performance LC with an injection of 20 μL, a Whatman PXS-10/25 SCX column, elution with acetonitrile–methanol–H_2O (225:150:45), a flow rate of 2.2 mL/min, and peaks detected with a variable absorbance UV monitor at 203 nm. Peaks were authenticated with standards whose purity was verified by two-dimensional thin-layer radiochromatography. Peaks designated 1, 2, 3, and 4 represent PE, LPE, PC, and LPC, each containing 3–100 nmol of unsaturated fatty acid.

Table I: Effects of Detergents Compared to LPE[a]

effector (μM)		% increase in hydrolysis
LPE:	10	1900
	20	2800
	2 (mM)	4200
deoxycholate:	75	45
	400	69
Triton X-100:	50	0
	100	17

[a] Reaction conditions were the same as those described in Figure 1, with 0.15 mg of rabbit heart mitochondria, 100 μM PE, Tris, pH 7.4, 5 mM $CaCl_2$, and deoxycholate, Triton, or LPE in the concentrations indicated. Results expressed (averages of duplicate experiments) are percentage increases in fatty acid released from [^{14}C]PE [in nmol/(mg of protein·h)] under conditions with the detergent or LPE present compared to the average control value of 2.5 nmol/(mg of protein·h) with no exogenous LPE or detergent added.

was measured by a modification of the Bartlett procedure (Sobel et al., 1978). Under these conditions, the decline in PE (38 nmol) was equivalent to the rise in LPE (41 nmol).

Experiments were performed in order to determine whether the observed hydrolysis reflected a nonspecific detergent effect of LPE; [^{14}C]PE was incubated with deoxycholate and Triton X-100. As can be seen in Table I, concentrations of deoxycholate (DOC) and Triton, even in marked molar excess of the effective concentrations of LPE, were without apparent effect. Furthermore, stimulation of apparent PLA_2 activity in mitochondria by DOC and by Triton X-100 was considerably less than that induced by LPE (Table I). Augmented hydrolysis induced by synthetic LPE was not inhibited by detergent added to the reaction medium. Other related sub-

stances including GPC, GPE, LPE, or LPA in concentrations as high as 20 μM did not induce any detectable hydrolysis of [^{14}C]PE in the presence or absence of 5 mM $CaCl_2$.

Results after Additional Purification. In order to exclude potential effects of trace but enzymatically significant amounts of PLA_2, commerically obtained synthetic LPE was purified further by high-performance LC. Aliquots of 20 μL of LPE in chloroform–methanol (2:1) were separated on a PXS-10/25 SCX column with acetonitrile–methanol–H_2O (225:150:45) at a flow rate of 2.2 mL. Under these conditions retention times of PE, LPE, PC, and LPC were shown to be 3.5, 5.5, 9.0, and 15.0 min (Figure 4). Purity of pooled products was verified by two-dimensional TLC. Incubation of PE with purified LPE, equivalent to LPE phosphorus eliciting hydrolysis prior to purification, consistently led to no hydrolysis. Thus hydrolysis prior to purification was attributable to a contaminant in the commerically available preparation, rather than LPE itself. Since the effect observed in the presence of unpurified LPE was not obtained with other phosphatides or detergents and since the degree of hydrolysis of PE was dependent upon incubation time as well as on concentrations of PE, unpurified LPE, and Ca^{2+}, we concluded that the commerically obtained LPE, but not LPC, was contaminated with the snake venom PLA_2 used to synthesize the lysophosphoglyceride from its parent compound. The method of purification used by the supplier, filtration over Whatman filter paper in hot chloroform, had not completely removed PLA_2 activitiy from LPE. Similar difficulties were encountered with material from other suppliers.

Discussion

Because phospholipolysis is a concomitant of ischemic injury in liver (Chien et al., 1978) and in myocardium (Sobel et al., 1978) and because local accumulation of lysophosphoglycerides may contribute to derangement of membrane structure and function, elucidation of the effects of these amphipathic moieties on tissues constitutes a focus of research. Although many investigators (Poole et al., 1970) synthesized lysophosphoglycerides by using venom phospholipase and may have been aware of the possibility of contamination, evaluation of lysophophatides for residual PLA_2 activity has not been reported nor are methods of separation of the product from the enzyme described in detail. Recently, investigators frequently have obtained lysophosphoglycerides commerically, from sources that do not emphasize that the lysophosphoglycerides are synthesized enzymatically.

Lysophosphoglycerides synthesized commerically or in the laboratory have been implicated in a variety of membrane-related phenomena. In high concentrations LPC lyses cells and bacteria (Lucy et al., 1969), and in lower concentrations LPC promotes fusion of fibroblasts and BHK cells (Poste & Papahadjopoulos, 1976), as well as erythrocytes (Poole et al., 1970). In contrast, LPC inhibits the usual fusion of cultured skeletal myocytes (Reporter & Raveed, 1973).

In addition to altering properties of cell fusion, LPC alters electrophysiological properties of membranes. Goshima (1971) reported that the lysophosphatide restored beating cultured cardiac myocytes which had been treated with phospholipase C. Sobel et al. (1978) demonstrated that both LPC and LPE altered several electrophysiological parameters of canine Purkinje fibers, perhaps accounting in part for dysrhythmias occurring during myocardial ischemia.

Other investigators have suggested that lysophosphoglycerides alter activities of enzymes, particularly those associated with membranes. Thus, LPC may augment activity of guanylate cyclase (Shier et al., 1976) and appears to have a biphasic effect on adenylate cyclase (Ahumada et al., 1979). Karli et al. (1979) have suggested that LPC may inhibit myocardial Na^+,K^+-ATPase. LPC also may inhibit the activity of hepatic CTP:phosphocholine cytidylyltransferase (Choy & Vance, 1978), although LPE appears to enhance activity; since activity was augmented by treatment with PLA_2 as well, it is possible that the LPE employed may have been contaminated with phospholipase A_2.

We repeatedly found that the lots of LPC available to us did not have PLA_2 activity under conditions of these experiments, although LPE clearly was contaminated. These results suggest that either phospholipase from *Crotalus atrox* has a greater affinity for LPE or coincidental lot-to-lot variation in the contamination of the lysophosphoglycerides leads to the disparity. Since acquisition of valid data in investigations employing exogenous lysolipids presupposes that these compounds are not contaminated with substances which might independently simulate potential effects of the lysophosphoglycerides, it will be helpful not only to take into account the method of synthesis of commercial lysophosphoglycerides but also to evaluate potential PLA_2 activity and to purify rigorously all compounds which prove to be contaminated.

References

Ahumada, G. G., Bergmann, S. R., Carlson, E., Corr, P. B., & Sobel, B. E. (1979) *Cardiovasc. Res. 13*, 377.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.

Chien, K. R., Abrams, J., Serroni, A., Martin, J. T., & Farber, J. L. (1978) *J. Biol. Chem. 253*, 4809.

Choy, P. D., & Vance, D. E. (1978) *J. Biol. Chem. 253*, 5163.

Goshima, K. (1971) *Exp. Cell Res. 67*, 352.

Karli, J. N., Karikas, G. A., Hatzipavlou, P. K., Levis, G., M., & Moulopoulos, S. N. (1979) *Life Sci. 24*, 1869.

Koprowski, H., & Croce, C. M. (1973) *Methods Cell Biol. 7*, 251.

Long, C., Odavic, R., & Sargent, E. J. (1967) *Biochem. J. 102*, 221.

Lucy, J. A., Dingle, J. T., & Fell, H. B., Eds. (1969) *Lysosomes in Biology and Pathology*, North-Holland, Amsterdam.

Poole, A. R., Howell, J. I., & Lucy, J. A. (1970) *Nature (London) 227*, 810.

Poste, G., & Papahadjopoulos, D. (1976) *Methods Cell Biol. 14*, 23.

Reporter, M., & Raveed, O. (1973) *Science 181*, 863.

Shier, W. T., Baldwin, J. H., Nilsen-Hamilton, M., Hamilton, R. T., & Thanassi, N. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1586.

Sobel, B. E., Corr, P. B., Robison, A. K., Goldstein, R. A., Witkowski, F. X., & Klein, M. S. (1978) *J. Clin. Invest. 62*, 546.

Waite, M., & VanDeenen, L. L. M. (1967) *Biochim. Biophys. Acta 137*, 498.