JOURNAL OF BACTERIOLOGY, Aug. 1977, p. 493-498
Vol. 131, No. 2

Printed in U.S.A.

Outer Membrane Phospholipase A from *Acinetobacter* sp. HO1-N

R. E. TORREGROSSA, R. A. MAKULA, AND W. R. FINNERTY*

Departments of Microbiology and Biochemistry, University of Georgia, Athens, Georgia 30602*

Received for publication 28 February 1977

A phospholipase A_1 activity that hydrolyzed cardiolipin to triacyl- and diacyl-lysocardiolipin was localized in outer membrane preparations derived from *Acinetobacter* sp. HO1-N. The specific activity of the enzyme derived from hexadecane-grown cells was 2.5 to 3 times higher than that derived from NBYE-grown cells. An apparent K_m of 2.22 mM was determined, although inhibition kinetics resulted at the higher cardiolipin substrate concentrations. Optimal reaction conditions established no metal requirements. Enzyme activity was obligately dependent on Triton X-100 (0.5%) and was inhibited by cationic and anionic detergents. Cardiolipin-specific phospholipase D converted triacyl-lysocardiolipin to lysophosphatidylglycerol and phosphatidic acid. The specific activity of this enzyme was approximately 100 times greater than that reported for other membrane preparations derived from microorganisms.

Although information is available concerning the enzymatic synthesis of bacterial phospholipids (7, 25), phospholipid catabolism remains less well resolved. Such catabolic reactions may be involved in cellular phospholipid metabolism; however, in vivo experiments have yet to establish a role for these processes (7, 9, 25). Various phospholipases have been demonstrated in cell-free preparations from several bacteria and subjected to varying degrees of characterization. This study details the occurrence and characterization of a highly active outer membrane phospholipase A from *Acinetobacter* sp. HO1-N which hydrolyzed cardiolipin (CL) to lysocardiolipin. The occurrence of triacyl-lysocardiolipin (triacyl-LCL) as a normal phospholipid constituent of *Acinetobacter* sp. HO1-N was reported previously (22).

MATERIALS AND METHODS

The bacterial strain *Acinetobacter* sp. HO1-N and the culture conditions on hexadecane and nutrient broth-yeast extract (NBYE) have been described previously (19). Methods for the extraction and purification of cellular phospholipids as well as preparation of radioactive phospholipids by growth in the presence of [2-^{3}H]glycerol or [^{32}P]orthophosphate have been reported (22).

Thin-layer chromatography. Methods used for thin-layer chromatography (TLC) have been described (22). Development of TLC plates was in the following solvent systems: (i) solvent A, chloroform-methanol-water (65:25:4, vol/vol); (ii) solvent B, chloroform-methanol-water (95:35:5, vol/vol) used with sodium borate-impregnated Silica Gel H plates; (iii) solvent C, chloroform-methanol-5 M ammonium hydroxide (65:30:5, vol/vol); (iv) solvent D, chloroform-methanol-acetic acid (65:28:8, vol/vol). Total lipids were visualized with iodine vapors, and phospholipids were visualized with the lipid phosphate spray reagent of Dittmer and Lester (5).

Gas chromatography. Phospholipid fatty acids were transmethylated and analyzed by gas chromatography as described (12).

Pancreatic lipase treatment. The conditions for hydrolysis of CL by porcine pancreatic lipase (Sigma type VI, Sigma Chemical Co., St. Louis, Mo.) were identical to those described previously (22).

CL-specific phospholipase D treatment. Methods used for the preparation and assay of *Haemophilus parainfluenzae* CL-specific phospholipase D were described previously (22). [^{3}H]triacyl-LCL substrate was prepared by treating [^{3}H]CL with *Acinetobacter* sp. HO1-N outer membrane preparations using phospholipase A assay conditions scaled up 10-fold.

Preparation of membranes. Membranes were prepared by lysozyme treatment of washed cells as previously described (19). The separation of outer and cytoplasmic membranes was accomplished by repeated differential centrifugation. The crude membrane preparation was diluted in phosphate buffer (50 mM, pH 7.5) and centrifuged at 34,850 × *g* for 1 min to sediment the cytoplasmic membranes. The supernatant was centrifuged at 65,000 × *g* for 2 h to sediment the outer membrane. Membranes were washed two times and suspended in a small volume of phosphate buffer. This procedure yielded isolated membrane fractions with less than 10% cross-contamination as judged by chemical and enzymatic markers (19).

In hexadecane-grown cells, membrane fractionation was, in some cases, accomplished by discontinuous sucrose gradient density centrifugation of crude membrane preparations (19).

Phospholipase assay. The standard incubation mixture for the detection of phospholipase A activity against CL contained, in a final volume of 100 μl: 50 nmol of [^{3}H]CL (300 to 350 cpm per nmol), 0.5% Triton X-100, 0.1 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer, and membrane suspension (15 to 20 μg of protein). The pH of the Tris-maleate buffer was 6.0 with membrane from NBYE-grown cells and 7.0 with membrane from hexadecane-grown cells. The reaction was initiated by the addition of membrane suspension. Blanks contained no membrane or heat-treated (100°C, 2 min) membrane. All assays were done in duplicate and incubated at 37°C for 10 min with shaking. The reaction was stopped by the addition of 1.5 ml of chloroform-methanol (2:1, vol/vol) with rapid mixing. After 5 min at room temperature, the extract was washed with 0.4 ml of 0.9% NaCl, and the mixture was chilled in an ice bath. Separation of phases was accomplished by centrifugation (2,000 × *g* for 5 min). The aqueous layer was carefully removed and discarded. The chloroform layer was washed a second time and evaporated to dryness. Unlabeled triacyl-LCL and diacyl-LCL carriers were added, and the entire sample was analyzed on a Silica Gel G thin-layer plate in solvent system C. Lipids were visualized with iodine vapors, and those areas corresponding to the lysocardiolipins were transferred to scintillation vials, followed by the addition of 1 ml of methanol-water (2:1, vol/vol) and 10 ml of scintillation fluid containing 33% Triton X-100 (1). Radioactivity was determined in a liquid scintillation spectrophotometer (Nuclear-Chicago Mark II). The recovery of triacyl-LCL and diacyl-LCL under these conditions was 91 and 87%, respectively, as determined by the addition of radioactive triacyl-LCL and diacyl-LCL to duplicate assays containing unlabeled CL and heat-inactivated membranes. Specific activity of the phospholipase A activity against CL (CL phospholipase) was defined as the total nanomoles of lysocardiolipin formed per minute per milligram of membrane protein. Total lysocardiolipin is the sum of triacyl-LCL and diacyl-LCL.

Protein determination. Protein was estimated by the method of Lowry et al. (10), using bovine serum albumin as the standard.

RESULTS

The enzymatic origin of lysocardiolipin was demonstrated initially in outer membrane preparations of *Acinetobacter* sp. HO1-N. [^{3}H]CL was degraded to two products during the phospholipase reaction, with conditions as described in Materials and Methods. When the products from large-scale reactions were purified from silica gel TLC plates, the major product was identified as triacyl-LCL by co-chromatography with authentic triacyl-LCL derived from *Acinetobacter* (22) in solvent systems A, B, C, and D. The minor product was identified as diacyl-LCL (22). The conversion to water-soluble products was negligible even when the assay period was extended to 60 min.

Characteristics of CL phospholipase. CL phospholipase was localized in the outer membrane of *Acinetobacter* sp. HO1-N (Table 1). The activity observed in the cytoplasmic membrane and in band 1 represented cross-contamination with the outer membrane. The product generated by the action of CL phospholipase on CL was, however, distributed in all membrane fractions (22).

The optimal conditions for the assay of CL phospholipase were determined with outer membrane preparations derived from hexadecane- and NBYE-grown cells. A pH optimum of 7 for CL phospholipase was obtained from hexadecane-grown cells as contrasted to pH 6.0 for NBYE-grown cells (Fig. 1). Triton X-100 concentration was essentially identical for CL phospholipase derived from hexadecane- and NBYE-grown cells (Fig. 2). The respective en-

TABLE 1. *Distribution of CL phospholipase activity in membrane fractions derived from Acinetobacter sp. HO1-N*

Membrane fraction[a]	nmol of lysocardiolipin formed/min per mg	
	NBYE	Hexadecane
Band 1		0.46
Cytoplasmic membrane	2.6	6.4
Outer membrane	27.8	70.68

[a] Band 1 is a low-density membrane isolated from hexadecane-grown cells (19).

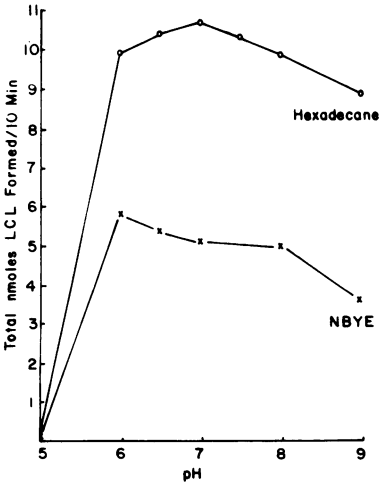

FIG. 1. *Hydrolysis of CL by outer membrane preparations from hexadecane- and NBYE-grown cells as a function of pH. The amounts of membrane protein were 15 and 14.8 μg, respectively. Buffers (0.1 M final concentration) were sodium acetate, pH 5.0; Tris-maleate, pH 6.0, 6.5 and 7.0; Tris-hydrochloride, pH 8.0; glycine-sodium hydroxide, pH 9.0.*

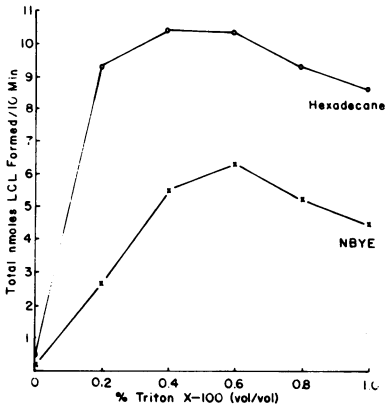

FIG. 2. *Effect of Triton X-100 concentration on the hydrolysis of CL by outer membrane preparations from hexadecane- and NBYE-grown cells (16 and 14.8 μg, respectively). Assays were performed under optimum conditions for each membrane preparation except that the Triton X-100 concentration was varied.*

zyme preparations were obligately dependent upon Triton X-100, showing maximal activity at 0.5% final concentration. The sonication of CL into aqueous suspension did not alter the requirement for detergent. Enzyme activity was linear to 15 μg of membrane protein (Fig. 3A). In time course studies, enzyme activity decreased approximately 12% after 5 min of incubation (Fig. 3B). Incubations were routinely timed for 10 min to obtain significant radioactivity in the products. The specific activity of CL phospholipase derived from hexadecane-grown cells was routinely 2.5 to 3 times higher than that derived from NBYE-grown cells. A Lineweaver-Burk plot for activity with varying CL concentrations as well as the Michaelis-Menten plot showed that CL at high concentrations inhibited CL phospholipase (Fig. 4). The apparent K_m value for CL obtained from extrapolation of the Lineweaver-Burk plot was 2.22 mM.

Table 2 shows the effect of various treatments on the specific activity of CL phospholipase. The addition of calcium ion (5 mM) routinely inhibited the enzyme by 8 to 10%. Ethylenediaminetetraacetic acid exhibited no effect on the enzyme at concentrations up to 1 mM. Assays performed at 25 and 45°C significantly decreased the enzyme activity, whereas preincubation at 100°C for 2 min completely inactivated the enzyme. In addition, CL phospholipase was inactive in the presence of 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 0.1% hexadecyltrimethylammonium bromide, or 10% methanol.

Specificity of CL phospholipase. The ratio of $C_{16:0}$ to $C_{16:1}$ fatty acids in CL purified from hexadecane-grown cells was 1.063. This CL was treated separately with pancreatic lipase (specific for the 1-acyl chain of phospholipids [4]) and with CL phospholipase. The free fatty acids released were extracted and purified by TLC in solvent system F (22), methylated, and analyzed by gas chromatography. The ratios of $C_{16:0}$ to $C_{16:1}$ fatty acids released by pancreatic lipase and CL phospholipase were identical (Table 3), indicating phospholipase A_1 activity. Triolein (0.1 μmol) was not hydrolyzed in 1 h under standard assay conditions as judged by TLC.

Hydrolysis of triacyl-LCL by phospholipase D. CL phospholipase was incubated with CL under optimal conditions and the product, triacyl-LCL, was isolated and purified by TLC.

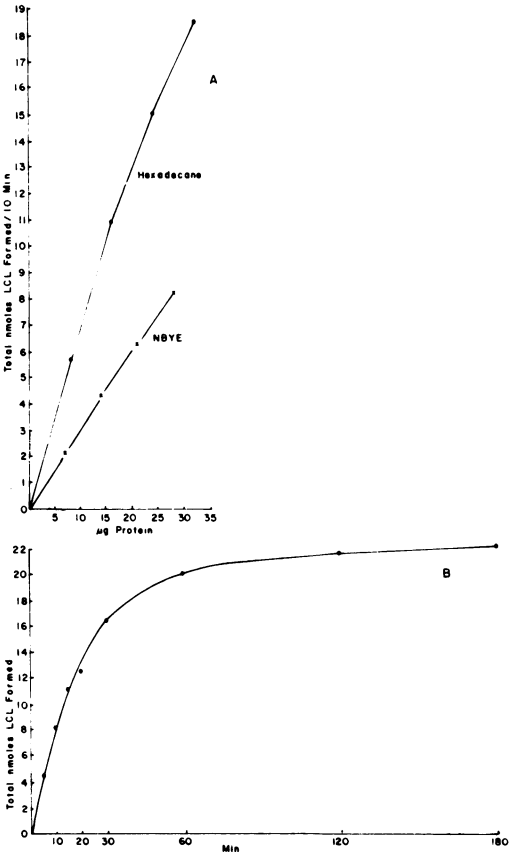

FIG. 3. *Rate of CL hydrolysis as a function of protein concentration (A) and time (B). (A) Effect of increasing outer membrane protein concentrations on the rate of CL hydrolysis by outer membrane derived from both hexadecane- and NBYE-grown cells. (B) Time course for the hydrolysis of CL by outer membrane from hexadecane-grown cells (16 μg of membrane protein).*

Incubation of this triacyl-LCL with CL-specific phospholipase D yielded lysophosphatidylglycerol and phosphatidic acid.

DISCUSSION

Phospholipase A activity associated with the outer membrane of *Acinetobacter* sp. HO1-N formed triacyl-LCL and, to a lesser extent, diacyl-LCL from CL. Optimal reaction conditions were similar for CL phospholipase derived from NBYE-grown cells and hexadecane-grown cells, although the latter exhibited 2.5 to 3 times greater specific activities. The CL phospholipase of *Acinetobacter* was similar to the phospholipase A_1 purified from *Bacillus megaterium* spores (16) with respect to pH optimum, heat inactivation, and response to calcium, ethylenediaminetetraacetic acid, and sodium dodecyl sulfate. However, the spore phospholipase was obtained in soluble form using carbonate buffer (pH 10.9). CL phospholipase was dissimilar to the membrane-bound phospholipase previously described for *E. coli* (1, 6, 18), *B. subtilis* (8), and *Mycobacterium phlei* (13, 14), all of which required calcium and were heat stable. *Acinetobacter* sp. HO1-N CL phospholipase was unusual in that a high concentration of detergent (0.5% Triton X-100) was required for optimal activity. Optically clear suspensions of substrate were obtained at much lower concentrations of detergent. Higher detergent concentrations may be partially solubilizing the phospholipase and thus increasing its activity.

Positional specificity has not been definitively determined owing to difficulties in obtaining specifically labeled CL. However, comparison of the fatty acids released by CL phospholipase A with those released by pancreatic

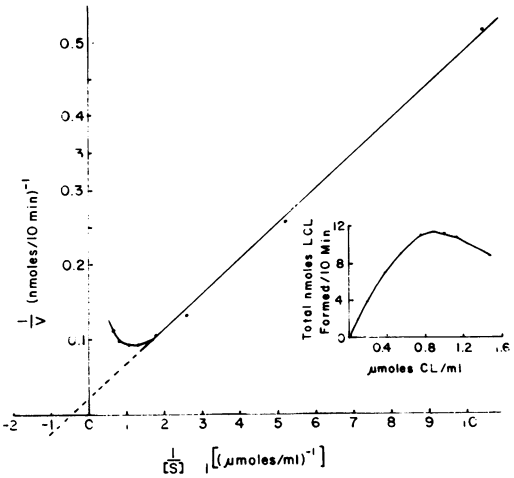

FIG. 4. *Lineweaver-Burk plot of CL phospholipase as a function of varying CL concentrations. (Insert) Michaelis-Menten plot for these data. Outer membrane (11.6 μg) from hexadecane-grown cells was used.*

TABLE 3. *Distribution of fatty acids released from CL[a] by pancreatic lipase and CL phospholipase*

Enzyme	Fatty acid (μg)	$C_{16:0}/C_{16:1}$
CL phospholipase		
$C_{16:0}$	2:00	1.775
$C_{16:1}$	1.55	
Pancreatic lipase		
$C_{16:0}$	2.33	1.8095
$C_{16:1}$	1.755	

[a] CL was purified from hexadecane-grown cells and found to have a $C_{16:0}/C_{16:1}$ ratio of 1.0625.

TABLE 2. *Effect of various treatments on the specific activity of outer membrane CL phospholipase derived from hexadecane- and NBYE-grown cells*

Treatment	Hexadecane (nmol/min per mg)	%	NBYE (nmol/min per mg)	%
Complete[a]	82.00	100	30.36	100
Minus Triton X-100	0.43	0.52	0.17	0.56
Plus 5 mM $CaCl_2$	76.07	92.77	27.23	89.7
Plus 1 mM ethylenediaminetetraacetic acid	80.69	98.40	ND[b]	
25°C	20.17	24.6	ND	
45°C	39.85	48.6	ND	
Preincubation at 100°C for 2 min	0.00	0.00	0.00	0.00
Sodium dodecyl sulfate (0.1%)	0.00	0.00	ND	
Sodium deoxychoblate (0.25%)	0.00	0.00	ND	
Hexadecyltrimethylammonium bromide (0.1%)	0.00	0.00	ND	
Methanol (10%)	0.00	0.00	0.00	0.00

[a] [^{32}P]CL, 80 nmol; Triton X-100, 0.5%; Tris-maleate, pH 7.0 (hexadecane), or Tris-maleate, pH 6.0 (NBYE), 100 mM; outer membrane protein, 10 μg. Incubation was at 37°C for 10 min.

[b] ND, Not determined.

lipase (Table 3) indicate specificity for the 1-acyl chain. The structure of cellular triacyl-LCL (22) also indicated 1-acyl hydrolysis. CL phospholipase is thus identified as a phospholipase A_1.

The two "ends" of the CL molecule are reported as nonequivalent (15) and exhibit different metabolic turnover rates in *H. parainfluenzae* (23), *E. coli* (3), and *Staphylococcus aureus* (21). The specificity of CL-specific phospholipase D for the bond between phosphate and C3' of the central glycerol of CL (2) was extended to include triacyl-LCL. The hydrolysis of triacyl-LCL by CL-specific phospholipase D yielded only lysophosphatidylglycerol and phosphatidic acid, indicating that triacyl-LCL was formed by the preferential hydrolysis of the 1-acyl chain of CL, as indicated in Fig. 5. The preferential hydrolysis exhibited by CL phospholipase A_1 further substantiates the nonequivalent nature of the two "ends" of CL. CL phospholipase A_1 does not appear to be absolutely specific for the 1-acyl chain since diacyl-LCL is also formed in vitro, although to a much lesser extent. It is possible that diacyl-LCL was formed by a lysophospholipase in the outer membrane.

In general, CL is not rapidly degraded by membrane-associated phospholipase A. Ono and Nojima (14) reported a CL-hydrolyzing activity in the membrane fraction of *Mycobacterium phlei* which yielded 1,3-di-*O*-(glycerylphosphoryl)glycerol in extended (20-h) incubation periods. A stepwise removal of fatty acids was postulated. In a latter study (13), phospholipase A was purified approximately 500-fold from *M. phlei* membranes which deacylated neutral and acidic phospholipids at comparable rates. This preparation also contained a nonspecific lysophospholipase. A phospholipase A activity in the outer membrane of *Neisseria gonorrhoeae* hydrolyzed CL to LCL at a rate that was 50% that of phosphatidylethanolamine hydrolysis (20). CL was hydrolyzed at a low rate by particulate preparations from *E. coli* (1, 6, 18); however, purified *E. coli* phospholipase A_1 (18) hydrolyzed phosphatidylethanolamine, phosphatidylglycerol, and CL at comparable rates in the presence of Triton X-100. The specific activity for CL hydrolysis by *Acineto-*

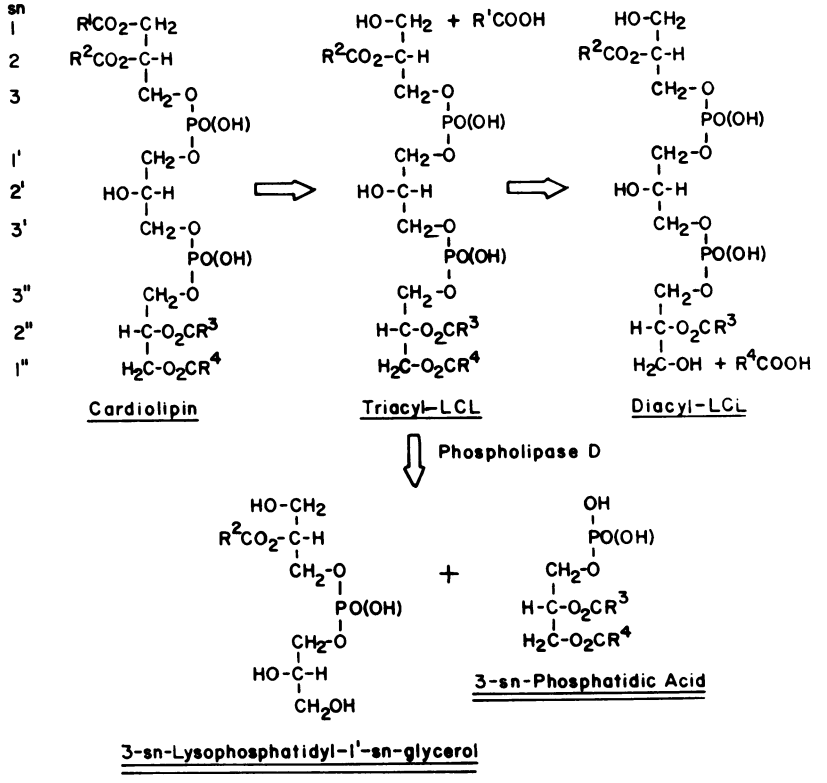

FIG. 5. *Proposed scheme for the hydrolysis of CL by Acinetobacter sp. HO1-N outer membrane phospholipase A_1. The position of fatty acyl groups in triacyl-LCL was determined by identification of the indicated phospholipase D hydrolysis products.*

bacter sp. HO1-N outer membrane preparations was at least 100 times greater than that reported in membrane preparations from *M. phlei* or *E. coli*. The highly active soluble phospholipase A_1 purified from *B. megaterium* spores (16) hydrolyzed CL at a rate that was only 2% of that for phosphatidylglycerol hydrolysis.

The in vivo role of phospholipases remains unclear. Phospholipid degradation in bacteria appears to be exceedingly complex with respect to the number of enzymes involved, their regulation, and their localization within the cell (1, 7). The fatty acyl chains of *Acinetobacter* sp. HO1-N phospholipids appear to exhibit rapid turnover (11, 17). The phospholipase A_1 revealed in these studies could thus be involved in phospholipid turnover via a deacylation-reacylation cycle. It has been proposed that a monoacyl-diacyl cycle plays an as yet unknown role in membranes (9, 24). Further studies are necessary to elucidate the role of phospholipase A_1 in *Acinetobacter* phospholipid metabolism.

LITERATURE CITED

1. **Albright, F. R., D. A. White, and W. J. Lennarz.** 1973. Studies on enzymes involved in the catabolism of phospholipids in *Escherichia coli*. J. Biol. Chem. 248:3968–3977.
2. **Astrachan, L.** 1973. The bond hydrolyzed by cardiolipin-specific phospholipase D. Biochim. Biophys. Acta 296:79–88.
3. **Audet, A., R. Cole, and P. Proulx.** 1975. Polyglycerophosphatide metabolism in *Escherichia coli*. Biochim. Biophys. Acta 380:414–420.
4. **DeHass, G. H. L. Sarda, and J. Rogen.** 1965. Positional specific hydrolysis of phospholipids by pancreatic lipase. Biochim. Biophys. Acta 106:638–640.
5. **Dittmer, J. C., and R. L. Lester.** 1964. A simple specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. 5:126–127.
6. **Doi, O., M. Ohki, and S. Nojima.** 1972. Two kinds of phospholipase A and lysophospholipase in *Escherichia coli*. Biochim. Biophys. Acta 260:244–258.
7. **Finnerty, W. R., and R. A. Makula.** 1975. Microbial lipid metabolism. Crit. Rev. Microbiol. 4:1–40.
8. **Kent, C., and W. J. Lennarz.** 1972. An osmotically fragile mutant of *Bacillus subtilis* with an active membrane-associated phospholipase A_1. Proc. Natl. Acad. Sci. U.S.A. 69:2793–2797.
9. **Lands, W. E. M.** 1965. Lipid metabolism. Annu. Rev. Biochem. 34:313–346.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
11. **Makula, R. A., and W. R. Finnerty.** 1971. Microbial assimilation of hydrocarbons: phospholipid metabolism. J. Bacteriol. 107:806–814.
12. **Makula, R. A., and W. R. Finnerty.** 1972. Microbial assimilation of hydrocarbons: cellular distribution of fatty acids. J. Bacteriol. 112:398–407.
13. **Nishijima, M., Y. Akamatsu, and S. Nojima.** 1974. Purification and properties of a membrane-bound phospholipase A_1 from *Mycobacterium phlei*. J. Biol. Chem. 249:5658–5667.
14. **Ono, Y., and S. Nojima.** 1969. Phospholipases of the membrane fraction of *Mycobacterium phlei*. Biochim. Biophys. Acta 176:111–119.
15. **Powell, G. L., and J. Jacobus.** 1974. The non-equivalence of the phosphorus atoms in cardiolipin. Biochemistry 13:4024–4026.
16. **Raybin, D. M., L. L. Bertsch, and A. Kornberg.** 1972. A phospholipase in *Bacillus megaterium* unique to spores and sporangia. Biochemistry 11:1754–1760.
17. **Sampson, K. L., and W. R. Finnerty.** 1974. Regulation of fatty acid biosynthesis in the hydrocarbon-oxidizing microorganism, *Acinetobacter* sp. Arch. Microbiol. 99:203–220.
18. **Scandella, C. J., and A. Kornberg.** 1971. A membrane-bound phospholipase A_1 purified from *Escherichia coli*. Biochemistry 10:4447–4456.
19. **Scott, C. C. L., R. A. Makula, and W. R. Finnerty.** 1976. Isolation and purification of membranes from a hydrocarbon-oxidizing *Acinetobacter* sp. J. Bacteriol. 127:469–480.
20. **Senff, L. M., W. S. Wegener, G. F. Brooks, W. R. Finnerty, and R. A. Makula.** 1976. Phospholipid composition and phospholipase A activity of *Neisseria gonorrhoeae*. J. Bacteriol. 127:874–880.
21. **Short, S. A., and D. C. White.** 1970. Metabolism of the glucosyl diglycerides and phosphatidylglucose of *Staphylococcus aureus*. J. Bacteriol. 104:126–132.
22. **Torregrossa, R. E., R. A. Makula, and W. R. Finnerty.** 1977. Characterization of lysocardiolipin from the hydrocarbon-oxidizing *Acinetobacter* sp. HO1-N. J. Bacteriol. 131:486–492.
23. **Tucker, A. N., and D. C. White.** 1971. Detection of a rapidly metabolizing portion of the membrane cardiolipin in *Haemophilus parainfluenzae*. J. Bacteriol. 108:1058–1064.
24. **van Deenen, L. L. M.** 1966. Some structural and dynamic aspects of lipids in biological membranes. Ann. N.Y. Acad. Sci. 137:717–730.
25. **van Deenen, L. L. M., and G. H. de Hass.** 1966. Phosphoglycerides and phospholipases. Annu. Rev. Biochem. 35:157–194.