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Characterization of Lysocardiolipin from *Acinetobacter* sp. HO1-N

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Triacyl-lysocardiolipin (triacyl-LCL) and diacyl-LCL were isolated from *Acinetobacter* sp. HO1-N, and their structures were determined by chemical, physical, and enzymatic procedures. Deacylation of triacyl-LCL and diacyl-LCL yielded bis-glycerylphosphorylglycerol. Periodate oxidation of both lysolipids was negative. Diglyceride and 2-monoglyceride resulted from the acetic acid hydrolysis of triacyl-LCL, whereas 2-monoglyceride was the sole product obtained from diacyl-LCL. Cardiolipin (CL)-specific phospholipase D treatment of triacyl-LCL yielded lysophosphatidylglycerol and phosphatidic acid. Pancreatic lipase treatment of CL yielded triacyl-LCL and diacyl-LCL. ^{31}P nuclear magnetic resonance spectrometry showed two resonance peaks separated by 40 Hz for CL, two overlapping peaks separated by 14 Hz for triacyl-LCL, and one peak for diacyl-LCL. The proportion of lysocardiolipin increased as a function of cell age, representing 2 to 3% of the total phospholipids in early- and mid-exponential growth, 5 to 7% in late-exponential growth, and 12% in the stationary growth phase.

Interest in the structure and enzymatic activities of biological membranes has greatly stimulated research in phospholipids. However, to date, lysophospholipids have received proportionally less attention. An increasing number of reports have detailed the occurrence of lysophospholipids in bacterial lipids for which a physiological role remains unresolved. Lysophospholipids, when documented, generally represent minor components of the phospholipid complement, with their in vivo status subject to doubt (40). Lysophospholipids have been implicated in mammalian phospholipid exchange reactions via a diacyl-monoacyl cycle not involving de novo synthesis (40). Preferential acylation of isomeric lysophosphoglycerides has been observed and related to the nonrandom distribution of saturated and unsaturated fatty acids found in natural phosphoglycerides. A similar acylation reaction has also been described in *Escherichia coli* (29). In addition, the production of phosphatidylcholine from two molecules of lysophosphatidylcholine has been observed in yeast and mammalian tissues in the absence of free fatty acids (40). It appears, therefore, that lysophospholipids play an important role in membrane physiology. This study concerns the occurrence and structural characterization of triacyl-lysocardiolipin (triacyl-LCL) and diacyl-LCL from *Acinetobacter* sp. HO1-N. Evidence is presented that triacyl-LCL is a normal phospholipid constituent of *Acinetobacter* sp. HO1-N.

MATERIALS AND METHODS

Organism and culture conditions. *Acinetobacter* sp. HO1-N was used throughout these studies. The conditions for maintenance and growth of this microorganism on hexadecane and nutrient broth-yeast extract (NBYE) have been described previously (31). Cells were obtained in kilogram quantities in a 400-liter New Brunswick fermentor.

Extraction of lipids, chromatography, and membrane isolation. The extraction of the total cellular lipids from this microorganism has been reported (22). Frozen cells (400 g, wet cell weight) were placed directly in chloroform-methanol (2:1, vol/vol) and extracted for 24 h. Procedures have been described for the preparation and enrichment of membranes from *Acinetobacter* sp. HO1-N (31, 38).

Whatman DE-23 in the acetate form was used for diethylaminoethyl-cellulose column chromatography of lipids according to the method of Rouser et al. (30). Routine purification of phospholipids (up to 150 μmol of lipid phosphorus) was accomplished on a resin bed of 2 by 20 cm. A resin bed of 5.5 by 10 cm was used for bulk purification of phospholipids.

Methods used for thin-layer chromatography (TLC) have been described (23). Plates were prepared either with Silica Gel G or Silica Gel H impregnated with 1 mM sodium tetraborate. Development of TLC plates was in the following solvent systems unless otherwise specified: (i) solvent A, chloroform-methanol-water (65:25:4, vol/vol); (ii) solvent B, chloroform-methanol-water (95:35:5, vol/

vol), used with borate 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). Lipids were visualized with the following spray reagents in the sequence specified: (i) total lipid with iodine vapors, (ii) amino nitrogen with 0.2% ninhydrin in acetone followed by heating at 110°C for 5 min, and (iii) lipid phosphorus by the phosphate spray reagent of Dittmer and Lester (10).

Hydrolytic procedures. Phospholipids were deacylated by mild alkaline methanolysis at 0°C (42). The water-soluble glycerylphosphoryl esters were analyzed by TLC on precoated cellulose thin-layer plates (Eastman 13255, Eastman Kodak Co., Rochester, N.Y.) in solvent system E, 3.8 mM ethylenediaminetetraacetic acid (EDTA)-0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 67% ethanol (by volume) (42), or solvent system F, ethanol-0.5 M ammonium acetate, pH 7.5 (7:3, vol/vol) (25). Compounds were visualized with the phosphate spray reagent of Hanes and Isherwood (16).

Phospholipids were hydrolyzed at 100°C for 90 min in 90% acetic acid (20). The diethyl ether-soluble products were analyzed on Silica Gel G thin-layer plates in solvent system G, petroleum ether-diethyl ether-acetic acid (70:30:1, vol/vol).

Cardiolipin (CL), purified from *Acinetobacter* HO1-N, was treated with porcine pancreatic lipase (Sigma type VI, Sigma Chemical Co., St. Louis, Mo.) by using the method of DeHass et al. (9). The standard reaction mixture consisted of 1 ml of 0.1 M borate buffer (pH 8.0) containing 5 mM $CaCl_2$, 7 mg of sodium deoxycholate, 4.5 mg of bovine serum albumin, 0.55 μmol of CL, and 50 U of lipase (14,000 U/mg). The reaction mixture was incubated at 30°C for 1, 2, and 4 h. The reaction was stopped by the addition of chloroform-methanol using the proportions of Bligh and Dyer (6). The chloroform-soluble products were analyzed by TLC in solvent systems A, B, and C.

CL-specific phospholipase D treatment. CL-specific phospholipase D was prepared from *Haemophilus parainfluenzae* by the method of Tucker and White (39). Phospholipase D treatment was performed essentially as described by Tucker and White (39) except that the reaction mixture was extracted with *n*-butanol to increase the recovery of lysophospholipids (5). The substrate, [^{3}H]triacyl-LCL, was purified from *Acinetobacter* sp. HO1-N grown in the presence of [2-^{3}H]glycerol as described below. The butanol-soluble phospholipase D products were separated by TLC in solvent system C. Phosphatidylglycerol, lysophosphatidylglycerol, phosphatidic acid, and lysophosphatidic acid were added as unlabeled carriers. Radioactivity of each component was determined as described by Torregrossa et al. (38). Hydrolysis of [^{3}H]CL by phospholipase D was performed similarly except that products were separated on borate-impregnated Silica Gel H thin-layer plates with the solvent system chloroform-methanol-5 M ammonium hydroxide (95:35:5, vol/vol). Phosphatidic acid and lysophosphatidic acid were purchased from Serdary Research Laboratories (London, Ontario, Canada). Phosphatidylglycerol was purified from cellular lipid extracts of *Acinetobacter* sp. HO1-N. Lysophosphatidylglycerol was prepared by treatment of phosphatidylglycerol with pancreatic lipase as described above.

Preparation of radioactive phospholipids. *Acinetobacter* HO1-N was grown to the early-exponential growth phase in NBYE medium. Either 1 mCi of [^{32}P]orthophosphate or 1 mCi of [2-^{3}H]glycerol (New England Nuclear Corp., Boston, Mass.) was added to a 1-liter culture, and the cells were grown to the early-stationary growth phase. The washed cell pellet was extracted for total lipids, and the radioactive phospholipids were purified as described. The purity of each phospholipid was determined by TLC in solvent systems B and C. [^{3}H]glycerol-labeled CL was deacylated, and less than 1% of the radioactivity was recovered in the fatty acyl moieties.

^{31}P nuclear magnetic resonance (NMR) spectrometry. ^{31}P NMR spectra were obtained on a JOEL PFT-100 spectrometer interfaced to the JOEL EC-100 data system. Data were obtained by using 8,000 data points with a spectral band width of 4 kHz. External 85% H_3PO_4 was used as a reference.

Analytical methods. Ester groups were determined by the method of Snyder and Stephens (37), with the modification that hydroxylaminolysis was carried out at 65°C for 10 min with tripalmitin as the standard. Glycerol was determined in the deacylated phospholipids by a modification of the method of Burton (7). Phospholipids were deacylated by alkaline methanolysis in 0.1 M methanolic KOH (30 min, 37°C), which was shown to give quantitative deacylation of CL. Tripalmitin (Sigma Chemical Co., St. Louis, Mo.) was used as the standard in the range of 0.1 to 0.5 μmol. The presence of vicinal diols in the intact phospholipids was determined by the method of Ames (2). Lipid phosphorus was measured by the method of Bartlett (11). Individual phospholipid species were quantitated from silica gel thin-layer plates as previously described (23). Protein was determined by the method of Lowry et al. (19) using bovine serum albumin as a standard.

RESULTS

Characterization of lysocardiolipins. Lipid extracts of *Acinetobacter* sp. HO1-N contained two minor acidic phospholipids that were recovered in fraction VI by diethylaminoethyl-cellulose fractionation (Table 1). The major phospholipids eluting in fractions I to V have been characterized previously (22). The two phospholipids recovered in fraction VI were purified by preparative TLC in solvent system C and judged pure by TLC in solvent systems A, B, C, and D. Characterization for each phospholipid follows.

Triacyl-LCL. Triacyl-LCL was the major phospholipid recovered in fraction VI and migrated below CL in all TLC solvent systems

TABLE 1. *Fractionation of Acinetobacter HO1-N phospholipids by diethylaminoethyl-cellulose chromatography*

Fraction	Elution solvents[a]	Lipids eluted[b]
I	C	Neutral lipids
II	CM (1:1, vol/vol)	PE
III	M	Trace PE
IV	CM (4:1), 5 mM NH_4Ac, 0.2% NH_3	PG + FFA
V	CM (4:1), 10 mM NH_4Ac, 0.4% NH_3	CL
VI	CM (4:1), 25 mM NH_4Ac, 1% NH_3	3-LCL + 2-LCL
VII	CM (4:1), 50 mM NH_4Ac, 2% NH_3	Uncharacterized lipids
VIII	M	Salts

[a] Lipids were applied to the column in chloroform. Elution volumes were equal to 10 column volumes. Solvent abbreviations: C, chloroform; M, methanol; NH_4Ac, ammonium acetate; NH_3, concentrated ammonium hydroxide.

[b] Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; FFA, free fatty acid; CL, cardiolipin; 3-LCL, triacyl-lysocardiolipin; 2-LCL, diacyl-lysocardiolipin.

tested (Fig. 1). Mild alkaline methanolysis yielded a water-soluble product that was chromatographically identical to bis-glycerophosphorylglycerol (GPGPG) obtained by deacylation of authentic CL in solvent systems E and F, as well as by two-dimensional cellulose-TLC in the same solvent systems. When [^{3}H]triacyl-LCL was deacylated together with non-radioactive CL and the water-soluble products were analyzed by cellulose-TLC, radioactivity was recovered in GPGPG. The molar ratio of ester to glycerol to phosphate in triacyl-LCL was 1.65:1.50:1.00 (theoretical, 1.5:1.5:1.0).

Periodate oxidation of the intact phospholipid was negative. Mild acid hydrolysis in 90% acetic acid preferentially cleaves phosphodiester bonds adjacent to a free hydroxyl group (8). Acetic acid hydrolysis of triacyl-LCL yielded two ether-soluble products identified as diglyceride and monoglyceride by TLC. Acetic acid hydrolysis of CL resulted in the formation of diglyceride as the only detectable ether-soluble product. Acyl migration occurred since both 1,2-diglycerides and 1,3-diglycerides were detected by TLC in the hydrolysis products of both CL and triacyl-LCL. The periodate oxidation of the ether-soluble products resulting from acetic acid hydrolysis of 93 nmol of CL phosphorus yielded 8.8 nmol of glycerol. Periodate oxidation of triacyl-LCL (122 nmol of lipid phosphorus) resulted in 14.8 nmol of glycerol as ether-soluble product. The theoretical amount of periodate-positive material expected from CL and triacyl-LCL would be 0, assuming diglyceride and 2-monoglyceride, respectively, as products. The periodate-positive material resulting from CL can be attributed to some deacylation yielding monoglyceride (20). Glyceride products obtained from the acetic acid hydrolysis of triacyl-LCL indicated that the monoglyceride formed was predominantly 2-monoglyceride. The small amount of periodate-positive material represented deacylation and some acyl migration from the 2-position.

Treatment of CL with porcine pancreatic lipase—a lipase known to specifically hydrolyze the 1-acyl fatty acyl ester bond of phospholipids (9)—resulted in the production of two products migrating below CL in solvent system C. Similar results have been obtained from the hydrolysis of CL by snake venom phospholipase A (24). The major product was purified by TLC and was found to co-chromatograph in solvent systems A, B, and C with triacyl-LCL purified from *Acinetobacter* sp. HO1-N.

Diacyl-LCL. The minor phospholipid eluting in fraction VI was identified as diacyl-LCL by

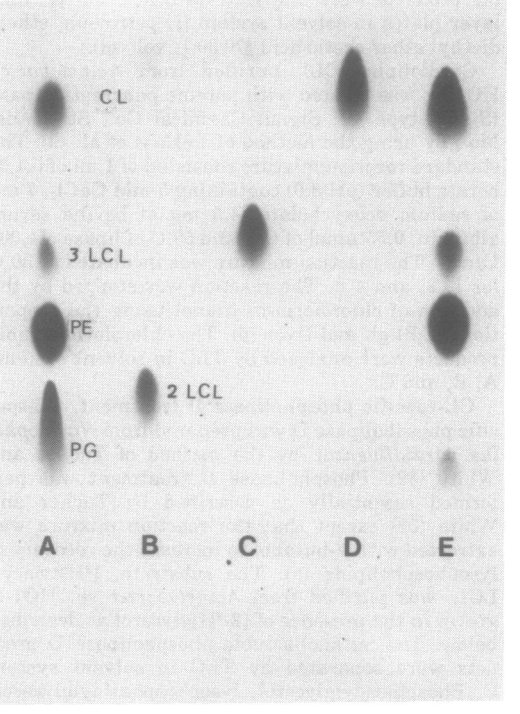

FIG. 1. *Thin-layer chromatograms of Acinetobacter sp. HO1-N phospholipids and purified CL derivatives on a borate-impregnated Silica Gel H plate in solvent system C. (A) Phospholipids from mid-exponential-growth-phase cells; (B) diacyl-LCL; (C) triacyl-LCL; (D) CL; (E) phospholipids from stationary-growth-phase cells. Abbreviations as in Table 1. Samples (A) and (E) contained 100 nmol of lipid phosphorus.*

similar methods. This lipid represented a trace component of the phospholipid composition of *Acinetobacter* sp. HO1-N and was isolated in significant amounts only from cells extracted in large quantity. The pure compound migrated below triacyl-LCL in all TLC solvent systems (Fig. 1). Mild alkaline hydrolysis produced a phosphoryl ester chromatographically identical to bis-glycerylphosphorylglycerol in solvent systems E and F. In addition, a single component (GPGPG) was observed when the deacylated products of CL, triacyl-LCL, and diacyl-LCL were co-chromatographed by two-dimensional cellulose-TLC.

The ester-to-glycerol-to-phosphate molar ratio for diacyl-LCL was 1.10:1.45:1.00 (theoretical, 1:1.5:1). No vicinal diols were detected in the intact lipid by periodate oxidation, indicating that the two fatty acyl groups are positioned on opposite sides of the molecule.

TLC analysis of the ether-soluble products obtained from acetic acid hydrolysis indicated only monoglyceride. Periodate oxidation of the ether-soluble acetic acid hydrolysis products yielded 17 nmol of glycerol from 76 nmol of diacyl-LCL (as lipid phosphorus). Acid-catalyzed deacylation and acyl migration account for the periodate-positive material.

Diacyl-LCL purified from *Acinetobacter* sp. HO1-N co-chromatographed with the minor product produced by pancreatic lipase treatment of CL in solvent systems A, B, and C.

^{31}P NMR spectrometry. CL yields two distinct resonance signals by ^{31}P NMR spectrometry (17), indicating nonequivalence of the two phosphorus atoms (28). ^{31}P NMR spectrometry was used to investigate the extent to which fatty acyl groups contributed to the nonequivalence of phosphorus in CL and related lysophospholipids. CL showed two distinct peaks separated by 40 Hz, triacyl-LCL gave two overlapping peaks separated by 14 Hz, and diacyl-LCL gave a single peak (Fig. 2). A reduction in resolution of the two phosphorus atoms of triacyl-LCL was concomitant with the loss of the fatty acid. The loss of the second fatty acid, in the case of diacyl-LCL, generated a molecule with indistinguishable resonance signals, indicating equivalence of the phosphorus atoms. An alternate possibility for explaining the equivalence of the phosphorus atoms in diacyl-LCL was possible by assuming the loss of fatty acids to be at the 2 and 2′ positions (Fig. 3). However, this explanation conflicted with the experimental data obtained from acetic acid hydrolysis of diacyl-LCL.

Hydrolysis of triacyl-LCL by CL-specific phospholipase D. CL-specific phospholipase D

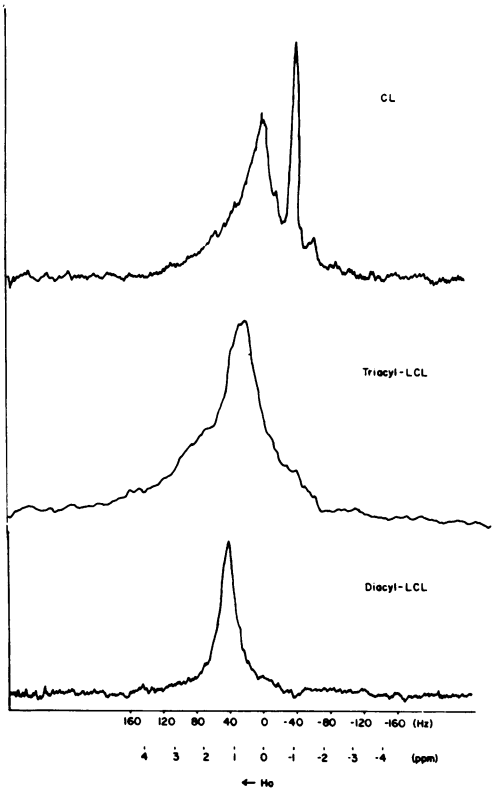

FIG. 2. *^{31}P NMR spectra obtained from CL and its lyso derivatives in chloroform-methanol (9:1, vol/vol). Abbreviations are as in Table 1.*

obtained from *H. parainfluenzae* was used to distinguish the fatty acyl chain positioning in triacyl-LCL. This phospholipase D hydrolyzed freshly prepared [^{3}H]triacyl-LCL to [^{3}H]lysophosphatidylglycerol and [^{3}H]phosphatidic acid and [^{3}H]CL to [^{3}H]phosphatidylglycerol and [^{3}H]phosphatidic acid. These reaction conditions resulted in greater than 70% hydrolysis of triacyl-LCL and CL.

Quantitation of triacyl-LCL. Triacyl-LCL represented 5 to 7% of the total lipid phosphorus obtained from NBYE- or hexadecane-grown cells (late-exponential growth phase), although higher values were obtained from specific membrane fractions (Table 2). The amount of triacyl-LCL increased to 12% of the total phospholipids in stationary-growth-phase cells. Triacyl-LCL was not localized exclusively in the outer membrane but rather was distributed throughout all membrane fractions (Table 2). The following experiment was performed to determine whether triacyl-LCL was produced during the manipulation of cells before lipid extraction. A

TABLE 2. *Distribution of triacyl-LCL in membrane fractions of Acinetobacter sp. HO1-N*

Membrane fraction[a]	% of triacyl-LCL[b]	
	NBYE	Hexadecane
Band 1		11
Cytoplasmic membrane	10	3
Outer membrane	15	19
Inclusion membrane		6

[a] Band 1 is a low-density membrane present in hexadecane-grown cells (31). Inclusion membrane is an intracellular structure present in hexadecane-grown cells (32).

[b] Relative to the total phospholipid in the respective membrane preparations.

1-liter NBYE culture was grown to the mid-exponential growth phase and divided into two 400-ml samples. One sample was immediately extracted with 1,500 ml of chloroform-methanol (1:2, vol/vol) chilled to −20°C. The extraction was continued at room temperature for 6 h. The chloroform-methanol extract was washed using the proportions of Bligh and Dyer (6), and the triacyl-LCL was quantitated from the chloroform layer by TLC. The second 400-ml sample was centrifuged, and the cell pellet was washed twice before lipid extraction using the method of Bligh and Dyer (6). No significant differences were found to exist between the direct extraction and extraction of washed cells. Triacyl-LCL represented an average 2.3% of the total lipid phosphorus in both extraction procedures in three separate experiments. The range for the direct extraction was 1.3 to 3.3%, whereas the range for the extraction of washed cells was 1.4 to 3.2%. These values are lower than those normally observed, possibly a result of the use of younger cells and/or the method of extraction. The recovery of triacyl-LCL was 81% using the proportions of Bligh and Dyer and 94% by the Folch procedure (22), respectively, as determined by the addition of [^{3}H]triacyl-LCL. These results, however, indicate that triacyl-LCL was produced in vivo rather than formed by physical manipulation of the cells. In addition, the proportion of lysocardiolipin increased as a function of cell age, representing 2 to 3% of the total phospholipids in early- and mid-exponential growth, 5 to 7% in late-exponential growth, and 12% in stationary-growth-phase cultures.

DISCUSSION

A lysophospholipid that represents a significant portion of the normal phospholipid complement of a bacterium becomes of interest in determining the in vivo role of phospholipases (1, 14). We characterized the occurrence and structure of lysocardiolipin in *Acinetobacter* sp. HO1-N.

Triacyl-LCL was routinely observed in whole-cell lipid extracts of *Acinetobacter* sp. HO1-N, representing from 2 to 12% of the total phospholipids, depending on the age of the culture and the method of extraction. Diacyl-LCL was observed only in trace amounts. The occurrence of triacyl-LCL does not appear to be an artifact of physical manipulations of cells before lipid extraction. Triacyl-LCL was observed when cellular lipids were extracted directly from actively growing cells in amounts identical to that extracted from washed cells. The amount of lysophosphatidylethanolamine in *Neisseria gonorrhoeae* was greatly reduced when growing cells were extracted directly (33). Lysophosphatidylethanolamine was not observed in cellular lipid extracts of *Acinetobacter* sp. HO1-N, even though phosphatidylethanolamine was the major phospholipid of this organism (22). Further evidence for the in vivo occurrence of triacyl-LCL was its presence in significant amounts throughout all membrane fractions (Table 2). In contrast, the level of lysophosphatidylethanolamine in *E. coli* was 10 times greater in the cell wall complex (outer membrane plus peptidoglycan) than in the cytoplasmic membrane, with commensurate differences in the level of phosphatidylethanolamine in the two membrane fractions (41). These differences were not believed to occur in vivo owing to the presence of phospholipase A in the outer membrane.

Lyso derivatives of CL have been reported in several species of mycoplasma (15, 26, 27, 35, 36) and bacteria (12, 13, 18, 33). Triacyl-LCL was reported to represent as much as 20% of the phospholipids of *Spiroplasma citri* (15). Exterkate et al. (13) reported lysocardiolipins in 9 of 10 strains of bifidobacteria of human intestinal origin. The inhibition of cell wall synthesis in *Bifidobacterium bifidum* var. *pennsylvanicus* by growth in the absence of human milk increased the amount of triacyl-LCL, although total lipid phosphorus tended to decrease (12). These reports generally lacked evidence for the in vivo occurrence of lysocardiolipin. The methodologies employed did not preclude the artifactual formation of lysocardiolipin by chemical or enzymatic degradation of CL during extraction and analysis. In addition, these reports lacked detailed characterization of the formation and localization of lysocardiolipins, and the existence of a phospholipase activity yielding lysocardiolipin from CL was demonstrated only in *N. gonorrhoeae* (33). A particulate CL phospho-

lipase A with unusually high specific activity is characterized in the accompanying paper (38).

The structures of triacyl-LCL and diacyl-LCL given in Fig. 3 are based on evidence obtained through chemical and enzymatic degradative procedures. That these phospholipids are derivatives of CL was demonstrated by the formation of GPGPG upon mild alkaline methanolysis and by co-chromatography with lyso-derivatives of CL produced by hydrolysis of CL with pancreatic lipase. Acetic acid hydrolysis of triacyl-LCL produced di- and monoglyceride, as expected from the previous data. Identification of 2-monoglyceride indicated that C1 or C1″ was not acylated in the intact lysophospholipid. The production of 2-monoglyceride by acetic acid hydrolysis of diacyl-LCL indicated that, in the intact lipid, the two fatty acyl chains were positioned on C2 and C2″. This arrangement of fatty acids was further supported by the lack of vicinal diols in the intact lipid.

^{31}P NMR spectrometry of CL yielded two distinct resonance signals in agreement with previous findings (17), indicating nonequivalence of the two phosphorus atoms in CL (28). The ^{31}P NMR spectra of the lysocardiolipins indicated that fatty acids contribute to the resolution of the phosphorus atoms. The nonequivalent nature of the two "ends" of the CL molecule is further substantiated by the apparent differential metabolism of CL in *H. parainfluenzae* (25, 39), *E. coli* (4), and *Staphylococcus aureus* (34).

Astrachan (3) demonstrated that CL-specific phospholipase D of *H. parainfluenzae* exhibits absolute specificity for the bond between phosphate and C3′ of cardiolipin (Fig. 3). This allows the use of this enzyme as a tool in structure analysis. We observed that CL-specific phospholipase D hydrolyzed triacyl-LCL to lysophosphatidylglycerol and phosphatidic acid, indicating the fatty acid positioning shown in Fig. 3. These findings bear on the stereospecificity of CL-specific phospholipase D. It appears the enzyme does not recognize CL specifically but rather the phosphatidyl-3′-*sn*-glycerol bond as suggested by Astrachan (3).

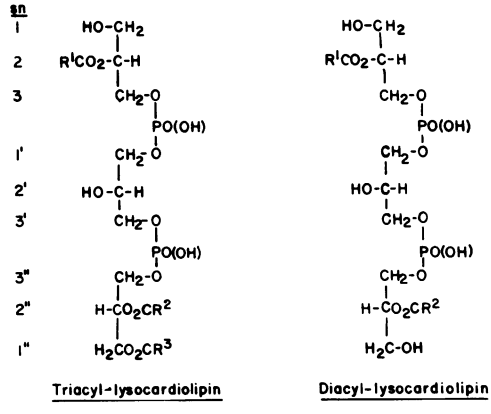

FIG. 3. *Proposed structures for triacyl-LCL and diacyl-LCL isolated from Acinetobacter sp. HO1-N.*

The assignment of a specific structural and/or functional role of a particular phospholipid has, with few exceptions, been conjecture. The present study indicates that triacyl-LCL was present in vivo. Further study is required to determine a role, if any, for triacyl-LCL in the membrane of *Acinetobacter* sp. HO1-N.

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