JOURNAL OF BACTERIOLOGY, Nov. 1970, p. 712-718

Vol. 104, No. 2
Printed in U.S.A.

Cardiolipin-Specific Phospholipase D of *Haemophilus parainfluenzae*

II. Characteristics and Possible Significance

YOSHIE ONO AND DAVID C. WHITE

Department of Biochemistry, Albert B. Chandler Medical Center, University of Kentucky, Lexington, Kentucky 40506

Received for publication 6 August 1970

A phospholipase specific for cardiolipin (CL) was found in the membrane of *Haemophilus parainfluenzae*. The enzyme hydrolyzed CL to phosphatidic acid (PA) and phosphatidylglycerol (PG), indicating that it was a phospholipase D (an enzyme activity believed to be confined to higher plants). In addition to its substrate specificity, this enzyme was unusual in its requirement for Mg^{2+} (K_m of 1.3 mM) for maximal activity and its inhibition by chelating agents, heavy metals, some detergents, and organic solvents. When inhibitors of phospholipase activity were added to the growth medium, CL accumulated and PG disappeared in the membrane, suggesting that the phospholipase D was active in vivo. The activity of phospholipase D in cell-free homogenates was greater than expected from earlier studies of CL metabolism and greater than the other phospholipase activities detected in the homogenate. The high activity of the CL-specific phospholipase D suggests there might be a very active degradation of CL to PG and PA and an active resynthesis of CL from the hydrolysis products.

In an earlier study (8), an unusual phospholipase which hydrolyzed cardiolipin (CL) to phosphatidic acid (PA) and phosphatidylglycerol (PG) was detected in *Haemophilus parainfluenzae*. The enzyme is unusual in its specificity for CL and the fact that it is a phospholipase D (8). Phospholipase D has previously been thought to be confined to higher plants. A surprising feature of the enzyme proved to be its very high activity, considering that CL accounts for only 3 to 5% of the total phospholipid and that CL has a relatively slow metabolism in pulse-chase experiments. Previous studies have shown that the CL loses half of the ^{32}P and ^{14}C from the middle glycerol in four bacterial doublings in exponentially growing cells (14). The high enzymatic activity found in cell-free homogenates suggested that the rapid hydrolysis of CL was coupled with a rapid resynthesis from the hydrolysis products without great dilution from other pools.

MATERIALS AND METHODS

Materials. Triton X-100 (*t*-octylphenoxy polyethoxy ethanol) was supplied by Rohm and Haas, Philadelphia, Pa.; Sarcosyl NL-30 (sodium lauryl sarcosinate) was supplied by Geigy Industrial Chemicals, Ardsley, N.Y., through the kindness of H. J. McElhone; Triton N-101 (nonylphenoxy polyethoxy ethanol), Tween 20 (polyoxyethylene sorbitan monolaurate), sodium dodecyl sulfate (SDS), hexadecylpyridinium chloride, sodium deoxycholate, bis-*p*-nitrophenylphosphate (B-PNPP), and ethylenediaminetetraacetate (EDTA) were supplied by Sigma Chemical Co., St. Louis, Mo. Other materials were supplied as described (8, 10, 14).

Growth of bacteria. The growth of *H. parainfluenzae* as a source of phospholipases and *Escherichia coli* K-12 as a source of CL has been described (8).

Enzyme preparation and assay. *H. parainfluenzae* was harvested and ruptured by ultrasonic vibration to form the cell-free homogenate used in most of the experiments reported (8). The substrates were emulsified in 2% (w/v) Triton X-100 after their isolation from *E. coli* grown with ^{32}P or ^{14}C (8). The chromatographic methods for the separation and analysis of the lipids have been described (8, 12). Phospholipase D activity was based on the hydrolysis of CL to PA and PG. A typical reaction mixture contained: 100 μg of enzyme protein, 10 mM $MgCl_2$, 0.07% (w/v) Triton X-100, 20 nmoles of CL (20,000 counts/min of ^{32}P) in 50 mM potassium phosphate buffer (*p*H 7.5) in 0.5 ml. The mixture was incubated at 37 C for 30 min, and the lipids were extracted and separated by chromatography on silica gel-loaded paper, the lipids were located by autoradiography, and the radioactivity was determined (8). The activity of phospholipases A and C in *H. parainfluenzae* were assayed after ammonium sulfate precipitation by a modification of a procedure developed previously (7). Phospholipase A and C activities assayed after ammonium sulfate precipitation represented about 90% of the activity present in the

total homogenate. The reaction in 1 ml was stopped by adding 0.5 ml of 20% (w/v) trichloroacetic acid and 0.5 ml of 20% (w/v) sodium deoxycholate. After mixing with a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.), the suspension was extracted with 5 ml of *n*-hexane. The mixing was then repeated and followed by centrifugation at 4,000 × *g* for 3 min. The hexane layer was recovered, and the ^{14}C and ^{32}P were determined. Reconstruction experiments indicated that 100% of added fatty acids or diglycerides and less than 2% of the phospholipids (also lysophospholipids) were recovered in the hexane in the presence of a final concentration of 5% sodium deoxycholate. The *n*-hexane-extractable products of phospholipid hydrolysis were identified as diglycerides and fatty acids by Silica Gel G thin-layer chromatography in a solvent system of petroleum ether (boiling point, 30 to 80 C)-diethyl ether-acetic acid (60:40:1, v/v) or chloroform-methanol-water (65:35:4, v/v). Reaction products formed after incubation with *Bacillus cereus* phospholipase C (diglyceride) and venom phospholipase A (fatty acids) were used as standards.

Phosphatidylserine (PS) decarboxylase activity was assayed by comparing the loss of PS to the increase in phosphatidylethanolamine (PE). The lipid extract was chromatographed in the two-dimensional, silica gel-impregnated paper system described (8). The substrate consisted of PS-*3*-^{14}C (Tracerlab, Waltham, Mass.) diluted with unlabeled PS (Applied Science Labs., State College, Pa.) suspended in 2% (w/v) Triton X-100. In the incubation mixture, 100 nmoles of PS (50,000 counts/min of ^{14}C) was added to the homogenate as in the phospholipase D assay. Phosphodiesterase was assayed with B-PNPP in the presence of 10 mM $MgCl_2$ (2). Protein and phosphate were determined colorimetrically (3, 13).

RESULTS

Phospholipase D activity. Previously, the products of the CL-specific phospholipase D activity in *H. parainfluenzae* were shown to be PA and PG (8). The rate of the reaction was linear with protein concentrations up to 100 to 150 μg of protein (Fig. 1). At higher protein concentration, PA was degraded in 30 min of incubation. The enzyme activity was linear with time for 50 min in this assay system (Fig. 2). The enzyme had a *p*H optimum between *p*H 7.5 and 8.0 (Fig. 3). The rate of hydrolysis of CL depended on the substrate concentration between 20 and 200 nmoles per ml with an apparent K_m value of 2×10^{-4} to 4×10^{-4} M and a maximal velocity (V_{max}) of 20 nmoles per min per mg of protein (Fig. 4). Higher substrate concentrations were impossible to test as emulsions formed. The enzymatic activity decreased by 98% when the preparation was heated to 60 ± 2 C for 10 min in the assay mixture containing 100 μg of protein per 0.5 ml. The crude enzyme can be stored at −20 C for several months without loss of activity.

Activity and inhibition of phospholipase D activity. The activity of phospholipase D in 1 mM $MgCl_2$ was activated by the divalent ions: $Mg^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$ (Fig. 5). Maximal activity occurred with 10 mM $MgCl_2$. The K_m for $MgCl_2$ was 1.33 mM, as measured in experiments like those illustrated in Fig. 4. Heavy metals, EDTA, and the detergents SDS, Tween 20, and hexadecylpyridinium chloride, were strongly inhibitory (Table 1). Cyanide, fluoride, ferric ions, iodoacetate, and the detergents Triton, deoxycholate, and Sarcosyl, had little effect on the enzyme activity. Treatment of the incubation mixture with diethyl ether destroyed enzyme activity.

Separation of phosphodiesterase activity. The great specificity for CL suggested the enzyme

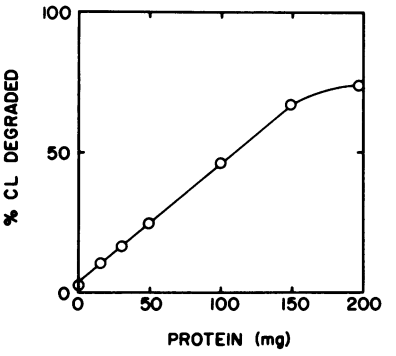

FIG. 1. *Effect of protein concentration on phospholipase D activity. The incubation mixture contained 20 nmoles of CL (20,000 counts/min of ^{32}P) added as an emulsion in 2% (v/v) Triton X-100 (8), 10 mM $MgCl_2$, 0.07% (v/v) Triton X-100, and 50 mM potassium phosphate buffer (pH 7.5) in a total volume of 50 μliters. Cell-free homogenate of H. parainfluenzae containing the phospholipase D was added to a final volume of 0.5 ml. After incubation at 37 C for 30 min, the mixture was extracted, and the decrease in CL was measured. Chromatography (8) indicated that the hydrolysis products were PG and PA.*

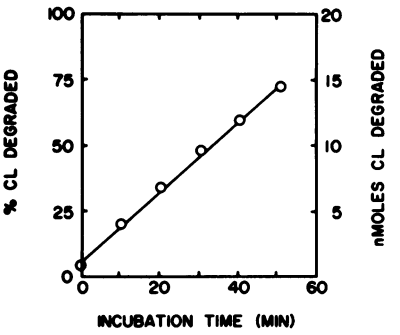

FIG. 2. *Time course of CL hydrolysis by phospholipase D from H. parainfluenzae. The reaction mixture contained 100 μg of protein and was analyzed as in Fig. 1.*

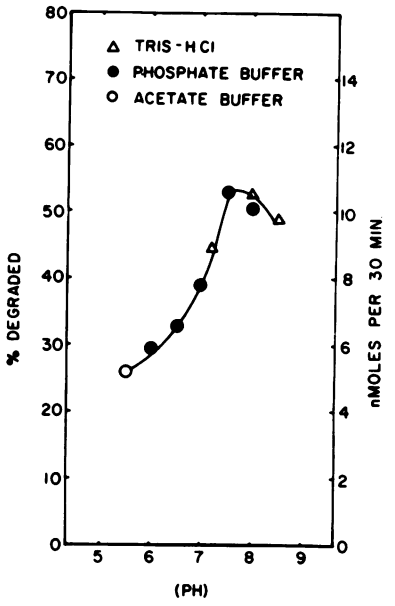

FIG. 3. *Optimal pH level of phospholipase D from H. parainfluenzae. CL hydrolysis was measured as in Fig. 1 except that the pH was changed. The incubation mixture contained 100 μg of protein (cell-free homogenate of H. parainfluenzae). Symbols: ○, 50 mM acetate buffer; ●, indicates 50 mM phosphate buffer; △, 50 mM Tris-hydrochloride buffer.*

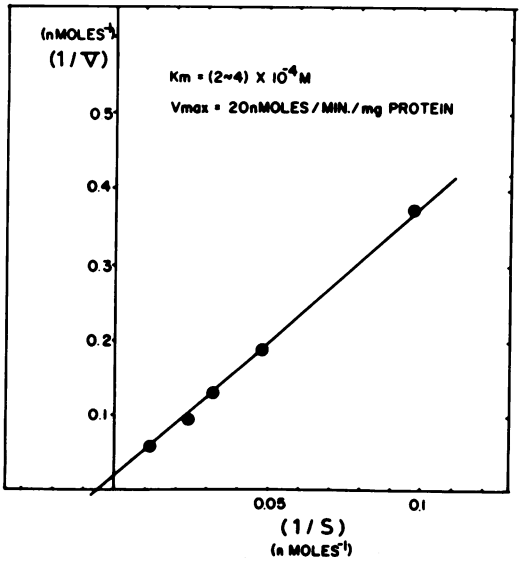

FIG. 4. *Effect of CL concentration on the rate of hydrolysis by H. parainfluenzae phospholipase D. Assay conditions were as in Fig. 1 except that the CL concentration was varied. The incubation mixture contained 100 μg of protein (cell-free preparation).*

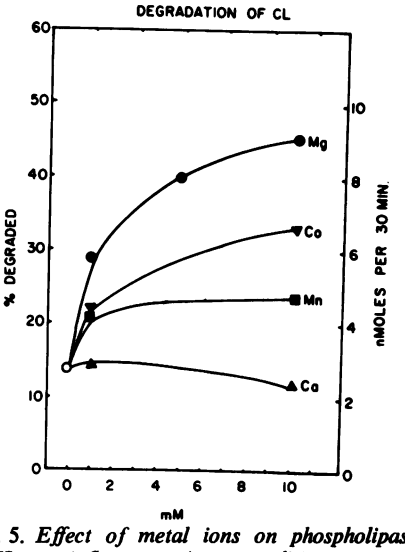

FIG. 5. *Effect of metal ions on phospholipase D from H. parainfluenzae. Assay conditions were as in Fig. 1 with 100 μg of protein of the cell-free homogenate. Symbols: ○, enzymatic activity in the presence of 1 mM $MgCl_2$; ●, activity in 1 mM $MgCl_2$ plus higher concentrations of $MgCl_2$; ▼, activity in 1 mM $MgCl_2$ plus higher $CoCl_2$ concentrations; ■, activity in 1 mM $MgCl_2$ plus higher $MnCl_2$ concentrations; ▲, activity in 1 mM $MgCl_2$ plus higher $CaCl_2$ concentrations.*

could be a relatively nonspecific phosphodiesterase. However, gel filtration column chromatography clearly separated the phospholipase D activity from the phosphodiesterase activity (Fig. 6). The CL-specific phospholipase D activity eluted in the same volume as Blue Dextran of molecular weight about 2,000,000. B-PNPP did not inhibit the CL-specific phospholipase D activity (Table 1).

Subcellular localization of phospholipase D. After sonic oscillation, the phospholipase D activity was much enriched in small membrane fragments which sedimented at 25,000 × *g* (Table 2). Some activity remained in the supernatant, possibly attached to smaller membrane fragments since some cytochrome *b* can be detected in this fraction after prolonged sonic oscillation.

PS decarboxylase and phospholipases A and C activities. PS decarboxylase was active in the cell-free homogenate (Table 3). Diglyceride and free fatty acids were formed when phospholipids were incubated with a partially purified homogenate (Table 3). After the addition of 10 mM $MgCl_2$, the CL-specific phospholipase D activity was readily demonstrated in this preparation. The total activities of the phospholipases A and C were not increased by adding detergents, calcium ions, diethyl ether, or changing the *p*H.

TABLE 1. *Inhibitors of H. parainfluenzae phospholipase D*

Inhibitor	Concn	Relative activity[a]
	mM	
$MgCl_2$	1	100
$BaCl_2$	1	120
$ZnCl_2$	1	20
$HgCl_2$	1	16
$CuCl_2$	1	13
$FeCl_3$[b]	1	117
Ethylenediaminetetra-acetate	1	0
KCN	10	108
NaF	10	93
Iodoacetate	10	86
Bio-*p*-nitrophenylphosphate	2	122
Sodium deoxycholate	10	121
Sodium dodecyl sulfate	5	0
Hexadecylpyridinium chloride	0.1[d] (w/v)	0
Tween 20	0.1[d] (w/v)	15
Sarcosyl NL-30	0.1[d] (w/v)	108
Triton N-101	0.1[d] (w/v)	156
Diethyl ether treated[c]		30

[a] Incubation mixture contained ^{32}P-cardiolipin (20 nmoles), 1 mM $MgCl_2$, 0.07% (w/v) Triton X-100, 50 mM potassium phosphate buffer (*p*H 7.5), and 100 μg of protein in the cell-free homogenate to which the components listed here were added. Incubation was carried out at 37 C for 30 min, and the amount of cardiolipin, phosphatidic acid, and phosphatidylglycerol was determined after chromatographic separation.

[b] Assay as above except that 50 mM Tris-hydrochloride buffer was used.

[c] To 0.5 ml of the reaction mixture, 0.5 ml of diethyl ether was added, the supension was mixed vigorously, and the ether was removed in a stream of nitrogen before the incubation.

[d] Expressed as per cent.

Phospholipase D activity in vivo. It was possible to inhibit phospholipase D activity in the cell-free homogenate (Table 1). Radioactivity was incorporated into the fatty acids, glycerols, and phosphate of the phospholipids of *H. parainfluenzae* during 3.4 doublings, after which the cells were washed and suspended into nonradioactive medium; thus, the replacement of the radioactivity with unlabeled isotopes could be studied (Fig. 7). During growth in nonradioactive medium, the CL lost about 5% and the PG about 45% of their radioactivities in 1 hr. The doubling time was 48 min. In the presence of 1 mM $CuCl_2$, which inhibited phospholipase D activity in vitro by 87% the doubling time of the cells increased to 54 min (110% of normal), the CL accumulated and the

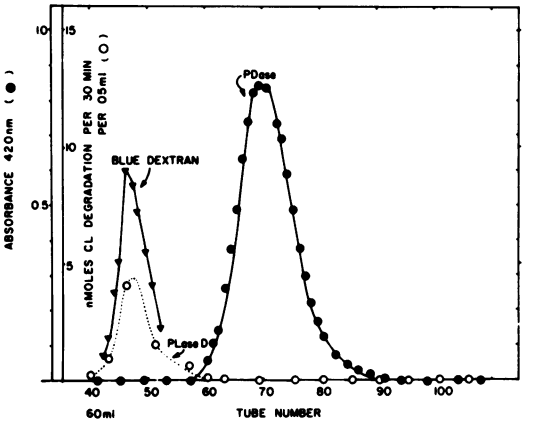

FIG. 6. *Column chromatography of phospholipase D from H. parainfluenzae. A column (2.5 by 60 cm) of Sephadex-G-100 was equilibrated with 50 mM Tris-hydrochloride (pH 7.5) containing 0.1 M KCl, and 5 ml (9.75 mg of protein) of the cell-free homogenate of H. parainfluenzae suspended in 50 mM potassium phosphate buffer (pH 7.5) was added. Activities were eluted from the column with the Tris-hydrochloride buffer at a flow rate of 0.33 ml per min, and 1.65-ml fractions were collected. A 0.5-ml portion was removed from each tube and assayed for phospholipase D activity as in Fig. 1 (○), and phosphodiesterase was assayed with 1.1 mM B-PNPP in the assay mixture (●). The elution volume of Blue Dextran (molecular weight, 2,000,000) was also determined by its absorption at 700 nm (▼).*

TABLE 2. *Localization of phospholipase D activity in H. parainfluenzae*

Fraction	Total protein	Specific phospholipase D activity[a]
	mg	
Total cell-free homogenate[b]	485	0.84
Fraction I[c]	267	0.62
Fraction II[d]	48	2.96
Supernatant[e]	184	0.49

[a] Values expressed as nanomoles hydrolyzed per minute per milligram.

[b] *H. parainfluenzae* suspended in 50 mM phosphate buffer (*p*H 7.5) with 10 mM $MgCl_2$ were treated with ultrasonic vibration at the maximal level of the Branson LS-75 Sonifier at intervals for a total of 5 min (8). After the treatment, no intact cells were detected by phase-contrast microscopy.

[c] Fraction I indicated large membrane-wall fragments in the pellet after centrifugation at 4,030 × *g* for 10 min.

[d] Fraction II indicated smaller membrane fragments in the pellet after centrifugation at 25,000 × *g* for 30 min.

[e] Supernatant of fraction II.

TABLE 3. *Phospholipase and phosphatidylserine decarboxylase activities of H. parainfluenzae*

Substrate	Cell-free homogenate[a]	Partially purified preparation[b]
PE	ND	0.043
PG	ND	0.005
CL	3.29	0.006 (3.40)
PS[c]	1.88	

[a] Values expressed as nanomoles degraded per minute per milligram of protein. Assay conditions: 50 μliters containing 100 nmoles of a substrate either phosphatidylethanolamine (PE), phosphatidylglycerol (PG), or (cardiolipin CL; specific activity, about 1,000 counts/min of ^{32}P per nmole) or PS (phosphatidylserine; specific activity, 1,500 counts/min of ^{14}C per nmole, serine-*3*-^{14}C labeled) that had been suspended in 2% (w/v) Triton X-100 was added to 10 mM potassium phosphate buffer (*p*H 7.5) containing 10 mM $MgCl_2$ and 0.85 mg of protein of the cell-free homogenate of *H. parainfluenzae* in 1 ml. The reaction was assayed after 15 min at 37 C, and the rate of ^{32}P loss was calculated. ND, not detected.

[b] Values expressed as nanomoles degraded per minute per milligram of protein. Assay conditions: 50 μliters containing 50 nmoles of either PE, PG, or CL labeled in the fatty acids by growth with acetate-*1* ^{14}C at specific activities of 1,250, 1,250, and 285 counts per min per μmole, respectively, and suspended in 2% Triton X-100 was added to 50 mM glycylglycine-NaOH buffer (*p*H 7.0) with 2.25 mg of protein in a final volume of 1 ml. The enzymes were prepared by sonic oscillation in the presence of 0.5% (w/v) sodium deoxycholate, centrifugation at 30,500 × *g* for 30 min, and precipitation of the supernatant with 22.8% ammonium sulfate. After a second centrifugation, the precipitate was utilized for enzyme assays. Samples were removed at intervals, trichloroacetic acid and deoxycholate were added, and the fatty acids and diglycerides were extracted with hexane. Thin-layer chromatography with Silica Gel G with a solvent of chloroform-methanol-water (65:35:4,v/v) was used to separate the fatty acids (R_F 0.7) and the diglycerides (R_F 1.0). CL-specific phospholipase D was assayed in the preparation after adding 10 mM $MgCl_2$ (number in parentheses).

[c] PS decarboxylase was assayed as the rate of PS disappearence and PE appearence after separation by chromatography on silicic acid-impregnated paper (8).

PG turnover remained about the same. In the presence of 1 mM $ZnCl_2$, which inhibited phospholipase D activity 80% in vitro, the doubling time slowed to 150 min and CL accumulated about as rapidly as with $CuCl_2$; the PG turnover, however, slowed to half its maximal rate. EDTA, which completely inhibited phospholipase D activity in vitro, stopped growth and caused an increase in CL from 5 to 15% and a decrease in PG from 11 to 3.5% of the total lipids in 1 hr.

DISCUSSION

The CL-specific phospholipase D activity of *H. parainfluenzae* required divalent metal ions for activity. The unusual substrate specificity (8) suggested the phospholipase D activity represented a nonspecific phosphodiesterase. Since B-PNPP did not inhibit phospholipase D activity (Table 1) and the phospholipase D and B-PNPP phosphodies-

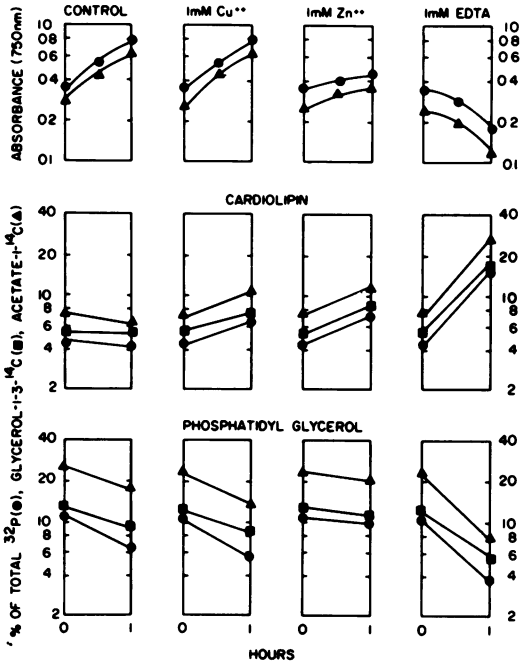

FIG. 7. *Activity of H. parainfluenzae phospholipase D in vivo. Cells were grown with 200 μCi of* $H_3{}^{32}PO_4$ *and 100 μCi of glycerol-1,3-*^{14}C (■) *or 100 μCi of* $H_3{}^{32}PO_4$ (●) *and 100 μCi of acetate-1-*^{14}C (▲) *per 300 ml for 3.4 doublings. The acetate-1-*^{14}C *labels the fatty acids of the phospholipids and the glycerol-1,3-*^{14}C *labels both the fatty acids and the glycerols (14). After growth with* ^{32}P *and* ^{14}C, *the cells were washed and suspended in nonradioactive medium and allowed to grow for 1 hr. The nonradioactive medium was supplemented with 1 mM* $CuCl_2$, *1 mM* $ZnCl_2$, *or 1 mM EDTA as indicated at the top of the figure. Samples were withdrawn at the beginning and end of the incubation in nonradioactive medium; the lipids were extracted, separated by two-dimensional chromatography on silica gel-loaded paper (8), and located by autoradiography; and the proportions of* ^{32}P *and* ^{14}C *in the CL (middle curves) and PG (lower curves) were determined. The lipids contained between 2 ×* 10^5 *and 9 ×* 10^5 *counts/min of* ^{32}P *and 3 ×* 10^4 *and 15 ×* 10^4 *counts/min of* ^{14}C. *The upper curves represent the changes in bacterial density measured as the changes in absorbance at 750 nm (13).*

terase activities were clearly separated by gel filtration chromatography (Fig. 6), the phospholipase D activity was characteristic of a true phospholipase.

The *H. parainfluenzae* phospholipase D had additional properties that were not typical for other phospholipases. The specificity for CL was unusual. PE, PG, PA, and PS from either *E. coli* or *H. parainfluenzae;* phosphatidylcholine, phosphomono- and dimethylethanolamine from yeast; and ceramide phosphoryl glycerol, ceramide phosphoryl glycerol phosphate, and ceramide phosphoryl ethanolamine from *Bacteriodes melaninogenicus* were not hydrolyzed. Although the phospholipase D activity was inhibited by some heavy metals, cyanide, fluoride, ferric ions or iodoacetate had no effect on its activity (Table 1). Some detergents, SDS, hexadecyl-pyridinium chloride, and polyoxyethylene sorbitan monolaurate, were inhibitory although alkyl phenoxypolyethoxyethanols or sodium lauryl sarcosinate were stimulatory. Diethyl ether, which is usually required or at least stimulatory to phospholipase activity, inhibited the *H. parainfluenzae* phospholipase D activity as utilized in this study (Table 1). A butanol treatment of this enzyme preparation also completely inactivated the enzyme. Addition of either a total lipid preparation from *H. parainfluenzae* or PE or PG that were suspended in Triton X-100 to the solvent inactivated enzyme did not restore activity. Calcium ions which activate many phospholipase activities were not stimulatory in the presence of 1 mM $MgCl_2$ (Fig. 5).

The phospholipase D activity has a requirement for a high Mg^{2+} ion concentration (K_m of 1.3 mM). This high concentration is reasonable in vivo, since the calculated soluble Mg^{2+} concentration in *E. coli* is 4 mM (4) and other enzymes like DNA polymerase (9) or the amino acid-activating enzymes (1) require at least 1 mM Mg^{2+} for activity.

Phospholipase A has been shown to be membrane-bound in *Mycobacterium phlei* (6), suggesting its involvement in membrane function. The *H. parainfluenzae* phospholipase D activity was also enriched in membrane fragments after cell rupture (Table 2), and a high Mg^{2+} ion concentration seems necessary for membrane stability (5). In *H. parainfluenzae*, the chelating agent EDTA inhibited CL-specific phospholipase D activity (Table 1) caused an accumulation of CL and a loss of PG (Fig. 7), and, when added to cells from the exponential growth phase suspended in tris(hydroxymethyl)aminomethane (Tris) buffer, caused a release of membrane fragments from viable cells (10). The membrane fragments released from the cells in EDTA-Tris are particularly enriched in CL and to a lesser extent in PG when compared to the rest of the membrane (11).

In pulse-chase experiments with exponentially growing cells, CL has a slow metabolism and is a minor lipid (3 to 5% of the total) in *H. parainfluenzae* (12). The CL-specific phospholipase D has an unexpectedly high activity. This phospholipase D was more active than PS decarboxylase in homogenates (Table 3), and PS decarboxylase forms the PE which comprises 78 to 85% of the total phospholipid (12). At the V_{max}, the CL-specific phospholipase D activity could degrade all of the CL in 1 or 2 min. Both phospholipase A and C activities were predicted from the turnover of PG and PE during exponential growth (14). Phospholipase C activity with PG was expected to be four times more active than CL-hydrolyzing activity (14). In the partially purified preparations necessary to demonstrate phospholipase A and C activities, the phospholipase D activity was a hundred times the maximal activities of phospholipase A and C thus far demonstrated (Table 3).

The CL-specific phospholipase D was active in vivo, as demonstrated by pulse-chase experiment with exponentially growing cells performed in the presence of enzyme inhibitors (Fig. 7). As in previous experiments, ^{14}C and ^{32}P in the fatty acids and glycerols were lost faster from PG than from CL in the chase period. If 1 mM $CuCl_2$ were present, the growth rate was decreased by 10%, rate of loss of glycerol from PG was decreased by 10%, and CL was accumulated. In the presence of 1 mM $ZnCl_2$, there was a doubling of the generation time, a doubling of the turnover time for PG, and, again, an accumulation of CL. In the presence of 1 mM EDTA, growth stopped, CL accumulated (an increase of 10% in the ^{14}C-glycerol) accompanied by an almost equal loss of PG (8% loss of ^{14}C-glycerol) (Fig. 7). In vitro, 1 mM $CuCl_2$, $ZnCl_2$, and EDTA inhibited phospholipase D

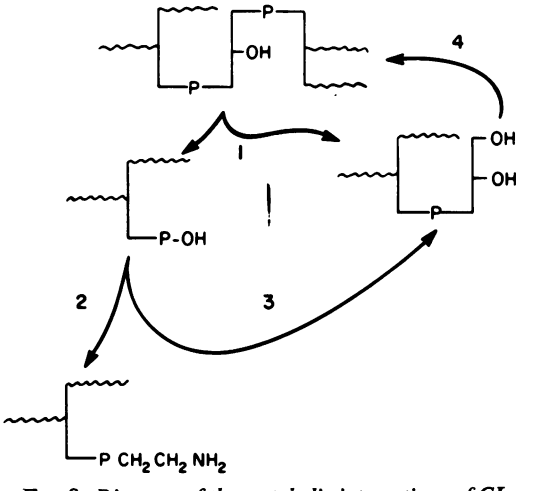

FIG. 8. *Diagram of the metabolic interactions of CL.*

activity by 87, 80, and 100%, respectively, and each of these caused an accumulation of radioactivity in CL in exponentially growing *H. parainfluenzae*. $CuCl_2$ and $ZnCl_2$, which slow the growth rate, also slow the turnover of PG. EDTA, which completely inhibited growth and phospholipase D activity, caused a rapid accumulation of CL, matched by a similar decrease in PG. This evidence strongly suggests that the CL-specific phospholipase D is functioning in vivo and that the rate of turnover of CL determined in a classic pulse-chase experiment is a gross underestimation of the true rate. The underestimation is because of recycling activity between PG and CL. CL must have a high rate of metabolism and is both formed from and contributes to the PG pool. Since the PG pool is so large, the rapid metabolism of CL cannot be detected in classical pulse-chase experiments. Possible reactions are indicated in Fig. 8. The phospholipase D forms PA and PG from CL (reaction 1, Fig. 8). The PA can be transformed into PE, PG, or CL, and the PG into CL via cytidine diphosphate diglyceride (reactions 2 to 4, Fig. 8).

The membrane localization of the CL-specific enzyme, the apparent high rate of CL metabolism, and the fact that the CL apparently is concentrated in the 50*S* portion of the ribosome (K. F. Hedden and E. Cundliffe, Bacteriol. Proc., 1970, p. 66) makes further understanding of the function of this enzyme imperative.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant GM 10285 from the National Institutes of General Medical Sciences.

LITERATURE CITED

1. Berg, P., F. H. Bergman, E. J. Offngand, and M. Dieckmann. 1963. The enzymatic synthesis of amino acyl derivatives of ribonucleic acid. I. The mechanism of leucyl-valyl-isoleucyl- and methionyl ribonucleic acid formation. J. Biol. Chem. **236**:1726–1734.
2. Futai, M., and Mizuno. 1967. A new phosphodiesterase forming nucleoside 5′-monophosphate from rat liver. J. Biol. Chem. **242**:5301–5307.
3. 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.
4. Lusk, J. E., R. J. P. Williams, and E. P. Kennedy. 1968. Magnesium and the growth of *Escherichia coli*. J. Biol. Chem. **243**:2618–2624.
5. McQuillen, K. 1960. Bacterial protoplasts, p. 288–289. *In* I. C. Gunsalus and R. Y. Stainer (ed.), The bacteria, vol. 1. Academic Press Inc., New York.
6. Ono, Y., and S. Nojima. 1969. Phospholipase of the membrane fraction of *Mycobacterium phlei*. Biochim. Biophys. Acta **176**:111–119.
7. Ono, Y., and S. Nojima. 1969. Phospholipase A of *Mycobacterium phlei*: a regulatory membrane enzyme with ferric iron as effector. J. Biochem. (Tokyo) **65**:979–981.
8. Ono, Y., and D. C. White. 1970. Cardiolipin-specific phospholipase D activity in *Haemophilus parainfluenzae*. J. Bacteriol. **103**:111–115.
9. Richardson, C. C., C. L. Shildkraut, H. V. Aposhian, and A. Kornberg. 1964. Enzymatic synthesis of deoxyribonucleic acid. XIV. Further purification and properties of deoxyribonucleic acid polymerase of *Escherichia coli*. J. Biol. Chem. **239**:222–232.
10. Tucker, A. N., and D. C. White. 1970. Release of membrane components from viable *Haemophilus parainfluenzae*. J. Bacteriol. **101**:498–507.
11. Tucker, A. N., and D. C. White. 1970. Heterogeneity of the phospholipid composition in the bacterial membrane. J. Bacteriol. **101**:508–513.
12. White, D. C. 1968. Lipid composition of the electron transport membrane of *Haemophilus parainfluenzae*. J. Bacteriol. **96**:1159–1170.
13. White, D. C., and F. E. Frerman. 1967. Extraction, characterization and cellular localization of the lipids of *Staphylococcus aureus*. J. Bacteriol. **94**:1854–1867.
14. White, D. C., and A. N. Tucker. 1969. Phospholipid metabolism during bacterial growth. J. Lipid Res. **10**:220–233.