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Detection of a Rapidly Metabolizing Portion of the Membrane Cardiolipin in *Haemophilus parainfluenzae*

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Heterogeneity in the metabolism of cardiolipin (CL) has been detected in *Haemophilus parainfluenzae*. Pulse-chase experiments showed that a portion of the total CL incorporated and then lost ^{32}P much more rapidly than the rest of the CL in the cells. The metabolism of each phosphate of the CL differed. The phosphate of the phosphatidyl glycerol (PG) portion of the CL had a more active metabolism than the phosphate of the phosphatidic acid portion of the molecule. Only a portion of the PG pool contributed to the formation of CL. Ethylenediaminetetraacetic acid inhibited the CL-specific phospholipase D in vitro and, when added to growing cells, resulted in more rapid PG metabolism, suggesting that CL hydrolysis contributed to the PG pool.

The membrane surrounding *Haemophilus parainfluenzae* contains the electron transport system and the lipids phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl serine (PS), phosphatidic acid (PA), and cardiolipin (CL) (21). The metabolism of these lipids has been studied extensively to determine their role in membrane formation and function. The metabolism of the phosphate of the phospholipids has been shown to change when the composition of the membrane-bound electron transport system is changed, suggesting a role in membrane formation (22). Further study has indicated that in the exponential growth phase the total phospholipid composition of the membrane does not change, but parts of the lipids undergo degradation and resynthesis (23). The most active metabolism involves the polar portions of the lipids (the alcohol esterified to the phosphate) and the 2-linked fatty acid. These data suggest that metabolism of the phospholipids is critical for some membrane functions.

The phospholipids in this organism seem to have a heterogeneous distribution in the membrane. Fragments containing lipids with larger proportions of CL and PG that were synthesized earlier and contain different proportions of fatty acids can be released from viable cells in Tris (hydroxymethyl) aminomethane buffer (Tris) containing ethylenediaminetetraacetic acid (EDTA) (18-20). This suggests that lipids of different functions might be distributed in a mosiac pattern.

Previous studies (23) indicated that CL metabolism in exponentially growing cells was complex. One or both of the diacylated glycerols accumulated radioactivity at the same time as radioactivity was lost from the middle glycerol and the phosphates during the chase portion of a pulse-chase experiment. When the highly specific CL-phospholipase D was detected in this organism with an activity equal to PS-decarboxylase (12, 13), CL metabolism was examined in detail. Studies with inhibitors indicate that the CL-specific phospholipase D is involved in the metabolism of CL in this organism (13, 14). In the present study, heterogeneity in the metabolism of CL was demonstrated.

MATERIALS AND METHODS

Materials. $H_3{}^{33}PO_4$ was supplied by New England Nuclear Corp., Boston, Mass. It contained less than 5% ^{32}P. Sources of other materials have been reported (18-23).

Growth of H. parainfluenzae. The strain, medium, methods of harvesting, and preservation were described in a previous report (19).

Lipid analysis. The lipid composition of this organism has been studied in detail (21). Samples of cultures were withdrawn onto an equal volume of crushed ice [which effectively stops ^{32}P incorporation into the phospholipids (23)] and centrifuged, and the lipid was extracted from the cells by the method of Bligh and Dyer (1). In some experiments, samples were withdrawn from the culture and added directly into the Bligh and Dyer extraction solvents without centrifugation. The lipids were separated by chromatography in two dimensions on activated silica gel-impregnated

paper (18, 24) or were deacylated quantitatively by mild alkaline methanolysis (22), and the water-soluble glycerol phosphate esters were separated by chromatography in two dimensions on acid-washed aminocellulose paper (Whatman AE-81) (22). Radioactivity was determined with a scintillation spectrophotometer (18), and phospholipid phosphate was determined colorimetrically after perchloric acid digestion (21). The glycerol phosphate esters derived from the phospholipids by mild alkaline methanolysis are designated as glycerol phosphorylglycerol (GPG) from PG, glycerol phosphorylethanolamine (GPE) from PE, glycerol phosphorylserine (GPS) from PS, L-α-glycerol phosphate (GP) from PA, and diglycerol phosphorylglycerol (GPGPG) from CL.

Separation of CL for enzymatic hydrolysis. A method that would allow separation and quantitative recovery of the CL was necessary for study of the various parts of the CL molecule. The CL of *H. parainfluenzae* could not be recovered intact from the silica gel-impregnated paper after chromatography with the acidic and basic solvents described previously (18). By using ascending chromatography on activated silica gel-impregnated paper (Whatman SG-81) with Marinetti's (9) solvent of chloroform, methanol, water [65:25:4 (v/v)], a good separation of CL was achieved (Fig. 1). A lipid extract was divided into three equal parts. One part was chromatographed as in Fig. 1. The second part was chromatographed in two dimensions with the acidic and basic solvents on silica gel-impregnated paper as described (18). The third portion was deacylated by mild alkaline methanolysis (17) and chromatographed in two dimensions on aminocellulose paper as described (22). The proportion of CL in the lipids was 2.8% in all three systems.

Recovery of CL. The band corresponding to CL (up to 0.5 μmole of phosphate) was cut into small pieces and soaked in 1 ml of methanol-toluene [1:1 (v/v)] for 1 hr at room temperature, and the solvent was decanted. Over 95% of the ^{32}P from the CL was recovered. Repeating the extraction with a second milliliter of solvent yielded a quantitative recovery. The CL so recovered yielded one spot corresponding to CL on two dimensional silica gel chromatography and, when deacylated by mild alkaline methanolysis, yielded exclusively GPGPG as determined by chromatography in two dimensions on aminocellulose paper.

Enzymatic hydrolysis of CL. Approximately 0.1 μmole of CL was deposited on the bottom of a culture tube (15 by 150 mm) by removing the solvent in a stream of nitrogen. The CL was suspended in 0.1 ml of 2% (v/v) Triton X-100 solution by mixing on a Vortex mixer for 2 min. Water (0.15 ml) was added, the mixing was repeated, 0.5 ml of the CL-specific phospholipase D preparation was added, and the solution was mixed. Then 0.25 ml of 20 mM $MgCl_2$ in 100 mM phosphate buffer (*p*H 7.5) was added, bringing the final concentration of $MgCl_2$ to 10 mM, the phosphate buffer to 50 mM, and the Triton X-100 to 0.2%. After mixing and incubation at 37 C for 1 hr, the reaction was stopped by extraction with the Bligh and Dyer procedure (1). The PA and PG were separated by chromatography on silica gel-impregnated paper (18). Under these conditions the CL hydrolysis was 80 to 90% complete. The CL-specific phospholipase D was prepared by sonically treating *H. parainfluenzae* in 50 mM Tris-hydrochloride buffer (*p*H 7.5) containing 0.5% Triton X-100 (10, 11). The preparation was centrifuged, and the supernatant portion was precipitated by the addition of solid ammonium sulfate to 30% (w/v). The precipitate was recovered and resuspended in 50 mM phosphate buffer (*p*H 7.5) containing 10 mM $MgCl_2$. This preparation contained 264 mg of protein per ml and hydrolyzed CL with no evidence of further hydrolysis of the products PA or PG.

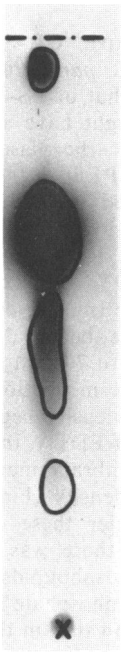

FIG. 1. *Autoradiogram after chromatography of phospholipids from Haemophilus parainfluenzae grown with $H_3{}^{32}PO_4$. Lipids were separated by ascending chromatography on activated silica gel-impregnated paper (Whatman SG-81) (24) with a solvent of chloroform, methanol, water [65:25:4 (v/v)] (9). Cardiolipin (CL) could be recovered quantitatively from the silica gel by extraction with methanol, toluene (1:1) after separation from the phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), and phosphatidic acid (PA).*

Measurement of radioactivity. Samples were assayed for radioactivity in a scintillation spectrometer (Packard model 2311). Lipids were counted on paper discs 1.5 to 2.0 cm in diameter with a scintillation fluid of 9.28 mM 2,5 bis[2(5-terbutyl benzoazol)]-thiophene (BBOT) in toluene. When ^{32}P and ^{33}P were counted simultaneously on paper discs, under conditions where ^{32}P was counted on one channel and ^{33}P plus 4% of the ^{32}P was counted on the other channel, the efficiency was 30.5% for ^{33}P and 86% for ^{32}P. Radioautograms were prepared with Kodak no-screen X-ray film as previously described (22).

RESULTS

The finding of a highly active, CL-specific phospholipase D activity in *H. parainfluenzae* present at an activity equal to that of PS-decarboxylase suggested that CL might have a very rapid metabolism (11). PS-decarboxylase was responsible for the formation of PE in *H. parainfluenzae* (23). The PE represented 75 to 80% and the CL about 3 to 5% of the total phospholipids.

The most potent inhibitor of the CL-specific phospholipase D in vitro is EDTA (11). When exponentially growing cells were transferred into medium containing EDTA, the bacterial doubling time slowed from 38 min to 70 min in the first 30 min (Fig. 2a). When the incubation was continued longer, the bacterial density began to fall slowly. After the addition of EDTA, the PE, PG, and CL content of the membrane increased much faster than the bacterial density (Fig. 2a). CL increased very rapidly under these conditions. Throughout this period there was rapid incorporation of ^{32}P into the phospholipids (Fig. 2c). The specific activity of the phosphate in PG remained about 10% greater than that in the PE and 35% greater than the phosphates of CL throughout the incorporation period. In the presence of EDTA, the turnover of the phosphate of PE continued at the same rate as in exponential growth, but the loss of ^{32}P from PG was 2.4 times more rapid than during exponential growth (Fig. 2b). The CL, which showed a slow turnover during exponential growth (23), accumulated in the presence of EDTA. It appeared that EDTA inhibited the breakdown of CL, stimulated the loss of PG, and allowed the synthesis of all the lipids. During this experiment the proportion of CL in the membrane increased, the proportion of PG decreased, and the proportion of PE remained about constant.

Heterogeneity in CL metabolism. The CL that can be stripped from *H. parainfluenzae* by transfer of exponentially growing cells into Tris buffer containing EDTA was shown to have been synthesized earlier and to contain different proportions of fatty acids than the CL present in the rest of the membrane (19, 20), suggesting that at least two types of CL exist in the cell. Heterogeneity in the metabolism of CL can be shown by growing *H. parainfluenzae* for 1.1 doublings (38 min) with $H_3{}^{33}PO_4$ and then adding $H_3{}^{32}PO_4$ for 12 min, followed by transfer to nonradioactive medium (Fig. 3). In such an experiment there was little change in the ^{32}P to ^{33}P ratio in the PE (Fig. 3). There was a suggestion that a fraction of the PG was metabolized more rapidly than the remainder. The most striking changes were seen in the CL. There was a threefold change in the ^{32}P to ^{33}P ratio, reaching a maximum in 5 min followed by a second much smaller change between 12 and 20 min (Fig. 3, bottom graph).

Demonstration of the highly active CL and PG depended on the length of time the bacteria were grown with the isotope. A pulse period with $H_3{}^{33}PO_4$ for four bacterial doublings showed no evidence of heterogeneity of PE, PG, or CL metabolism (Fig. 4a). Figure 4b shows the metabolic heterogeneity of both PG and CL after a 12-min pulse with $H_3{}^{32}PO_4$.

Biosynthetic pools involved in CL metabolism. Evidence that CL metabolism involved only a portion of the PA and PG pools was suggested by incorporation data (Fig. 5). In this experiment, cells were grown for three doublings with $H_3{}^{33}PO_4$, after which $H_3{}^{32}PO_4$ was added. The ratio of ^{32}P to ^{33}P was a measure of the specific activity of the phosphate of the phospholipids. ^{32}P was most rapidly incorporated initially into PA and PG, suggesting they were the precursors of the other lipids. The ^{32}P was incorporated more slowly into CL and reached a specific activity at saturation that was lower than that of the other lipids. The lower final specific activity of CL indicated either that the portion of the PG pool that was in equilibrium with CL must have had half the specific activity of the average of all the PG, or that only a small portion of the CL pool was rapidly labeled.

Differences in the metabolism of the two phosphates of CL. Hydrolysis of the CL from cells grown with ^{32}P for a short time with the CL-specific phospholipase D from *H. parainfluenzae* indicated that the enzyme specifically attacked one of the phosphates of CL, as the specific activity of the two phosphates from CL was different (Fig. 6). This experiment also indicated a difference in the kinetics of incorporation of ^{32}P into each of the two phosphates of CL. Incorporation of ^{32}P into the PA phosphate appeared to be slower than incorporation into the PG phosphate, but the PA phosphate came from a pool with a higher specific activity (Fig. 6). The experiment also indicated that the phosphates of PA and PG of the CL were formed from a portion of the total cellular PA and PG phosphate pools with about half the specific activities of the total PA and PG phosphate pools.

DISCUSSION

Although CL was originally thought to be a stable lipid in gram-negative bacteria (6, 8), recent studies have shown that CL metabolism appears to be affected by changes in the growth temperature (4, 11), inhibition of cell division or growth by antibiotics (17) by the application of colicines (2), or by deprivation of glycerol (9)

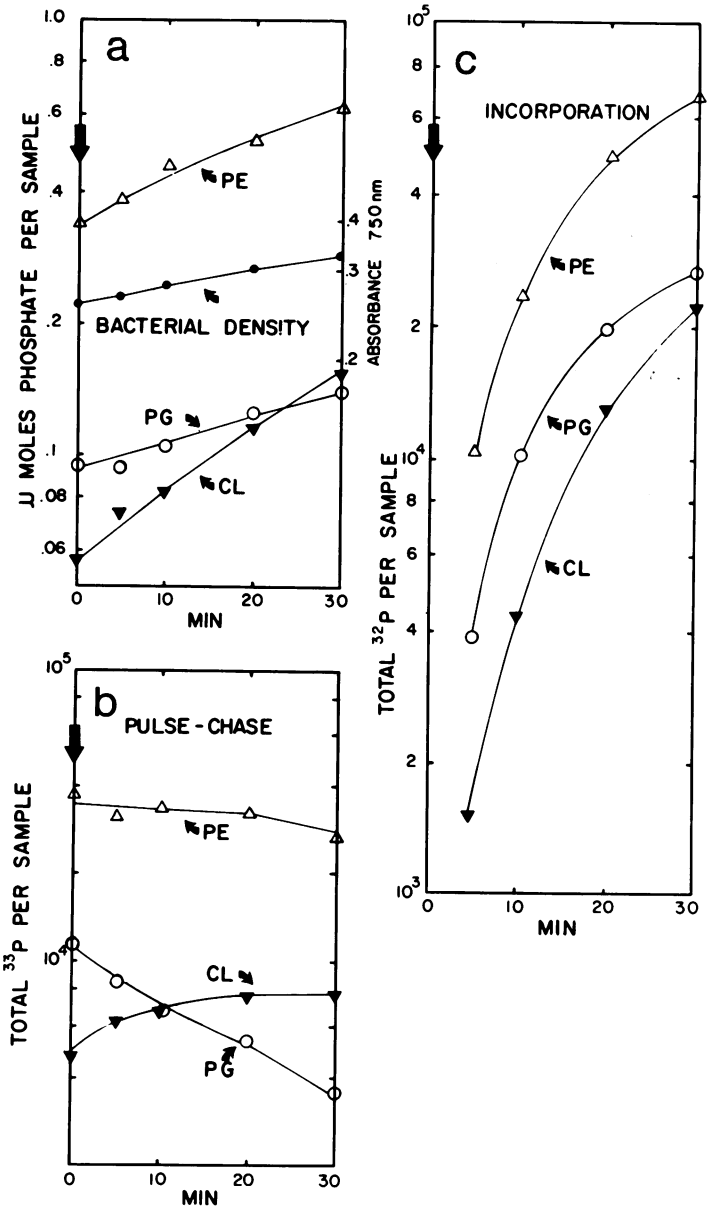

FIG. 2. *Effect of 1 mM EDTA on cardiolipin metabolism. Haemophilus parainfluenzae was grown with 150 μCi of $H_3{}^{33}PO_4$ per 300 ml for 70 min (38-min doubling time), and the cells were washed with 50 mM phosphate buffer (pH 7.6) at 37 C. Cells were resuspended in 300 ml of medium containing 1 mM EDTA at 37 C. A 50-ml sample was withdrawn; 750 μCi of $H_3{}^{32}PO_4$ was added, and further samples were taken. The lipids were extracted and separated as in Fig. 1. Bacterial density was measured as the absorbance at 750 nm in 13-mm round test tubes. An absorbance at 750 nm of 0.5 corresponds to a bacterial density of 0.28 mg (dry wt) per ml. Total lipid phosphate in the phosphatidyl ethanolamine (PE, △) phosphatidyl glycerol (PG, ○), and cardiolipin (CL, ▼) of each 50-ml sample and the bacterial density are illustrated in a. Turnover of ^{33}P in the phospholipids is illustrated in b. Incorporation of ^{32}P into the phospholipids is illustrated in c. Time at which the culture was transferred to the EDTA-containing medium is indicated by the arrow. Radioactivity was determined in a scintillation spectrometer.*

or oleate (5) in auxotrophs. CL accumulates during the stationary growth phase in some gram-negative bacteria (3, 15). The metabolism of CL also indicated metabolic heterogeneity, as the

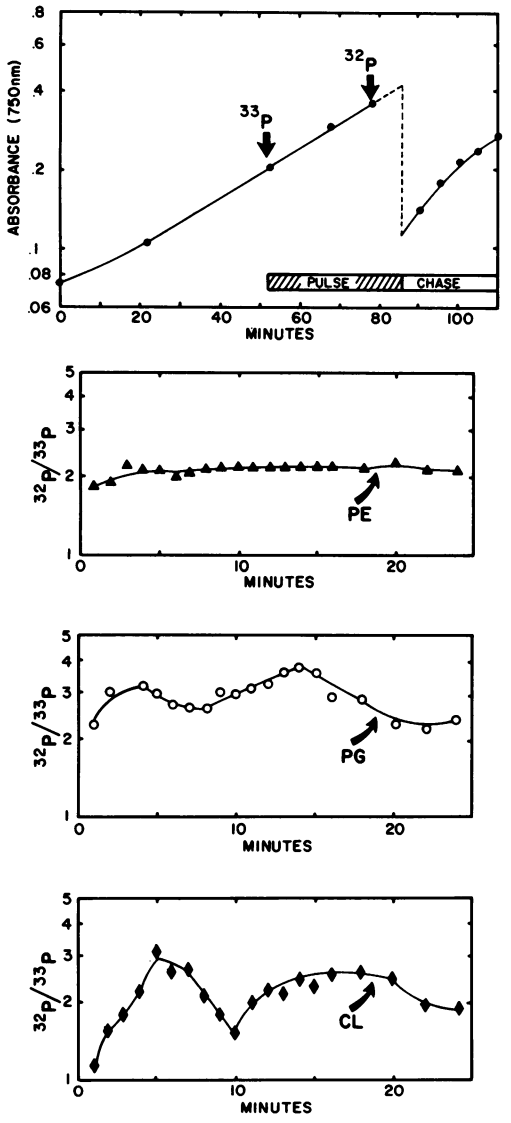

FIG. 3. *Turnover of ^{32}P and ^{33}P in the phospholipids of exponentially growing Haemophilus parainfluenzae. Cells were grown in 100 ml of medium as illustrated in the upper graph. After 53 min, 58 μCi of $H_3{}^{33}PO_4$ was added (first arrow); at the second arrow, 509 μCi of $H_3{}^{32}PO_4$ was added, and the cells were centrifuged at 37 C. Cells were resuspended in 1 ml of the radioactive medium used for the pulse and then immediately diluted into 200 ml of nonradioactive medium at 37 C (chase). Samples were removed and added directly to a separatory funnel containing chloroform and methanol and extracted. Lipids were analyzed as in Fig. 2. Cells remained in exponential growth throughout this experiment.*

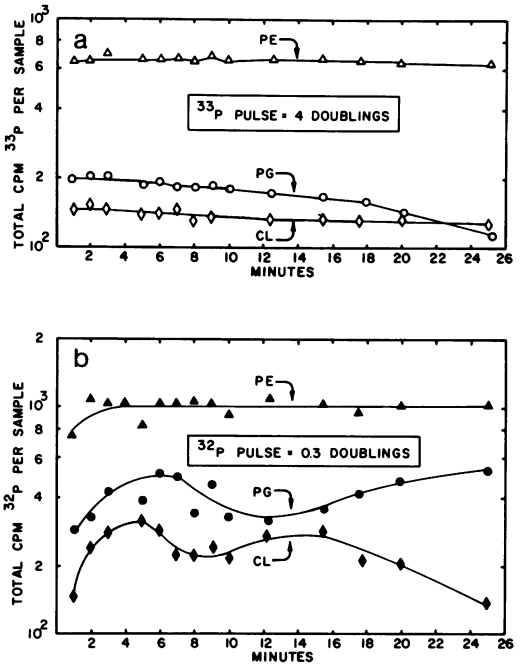

FIG. 4. *Turnover of ^{32}P and ^{33}P in the phospholipids of Haemophilus parainfluenzae after growth with $H_3{}^{33}PO_4$ for four bacterial doublings and $H_3{}^{32}PO_4$ for 12 min. Growth conditions and lipid analysis were as in the experiment illustrated in Fig. 3. The total ^{33}P (a) and ^{32}P (b) in each sample were determined as in Fig. 3.*

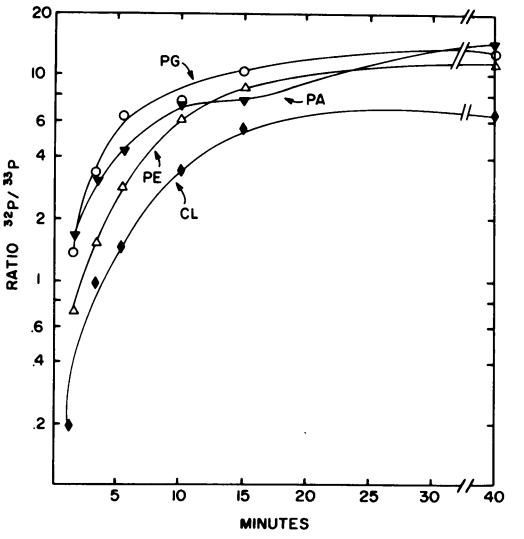

FIG. 5. *Incorporation of ^{32}P and ^{33}P into exponentially growing Haemophilus parainfluenzae. Cells were grown with 250 μCi of $H_3{}^{33}PO_4$ per 2,200 ml for 2 hr, and 1.9 mCi of $H_3{}^{32}PO_4$ was added. Samples were withdrawn onto ice, and the lipids were separated as in Fig. 2. During the experiment the cell density increased from 0.18 to 0.35 mg (dry wt) per ml.*

loss of ^{32}P from CL after transfer to nonradioactive medium in both *Escherichia coli* (6) and *Staphylococcus aureus* (16) was biphasic with a fast and slow phase. Membrane fragments from *H. parainfluenzae* are released into dilute buffers containing EDTA (18). The CL in these membrane fragments was formed much earlier than the CL remaining in the cells, suggesting a nonuniform distribution of CL (19). A nonuniform distribution of CL has been detected in *Micrococcus lysodeikticus* (10). When the CL-specific phospholipase D was detected in *H. parainfluenzae* in activities much in excess of that predicted from the concentration of CL in the membrane (21) or the metabolism of CL as detected in simple pulse-chase experiments (23), an intensive study of CL metabolism in this organism was begun.

EDTA is a powerful inhibitor of the CL-specific phospholipase D (12, 13). The addition of EDTA to exponentially growing cells stopped the breakdown of CL and allowed it to accumulate (Fig. 2). When CL was accumulating, PG loss was much faster, suggesting that the continual liberation of PG from CL hydrolysis normally adds to the PG pool. The addition of EDTA had little effect on the loss of ^{32}P from PE, again showing the interrelationship between the metabolism of PG and CL in this organism. EDTA had little effect on the incorporation of ^{32}P into the lipids. In the presence of EDTA, the lipid content increased about 15%.

Even more striking evidence for the rapid metabolism of CL and for the involvement of the CL-specific phospholipase D in CL hydrolysis comes from studies with inhibitors of CL metabolism (14). Pentachlorophenol and *p*-hydroxymercuribenzoate block CL synthesis but allow CL hydrolysis. This results in the quantitative conversion of CL into equimolar amounts of PG and PA within 3 min (14). Apparently, all the membrane CL can be hydrolyzed by the phospholipase in the presence of these inhibitors. PG not only is formed from CL but also serves as a precursor of CL in this bacterium. The inhibitors carbonyl cyanide m-chlorophenol hydrazone and tetrachlorosalicylanilide block CL hydrolysis but allow synthesis. This results in the loss of 23% of the PG with its quantitative conversion into CL within 1 min (14).

In the present study, heterogeneity in the metabolism of CL has been demonstrated. A portion of the cellular CL with higher metabolic activity than the rest can be detected (Fig. 3 and 4). The detection of this active portion required both extensive sampling and a true zero time sample. In previous experiments (23), the resuspension of the cells at the initiation of the chase period was done in nonradioactive medium,

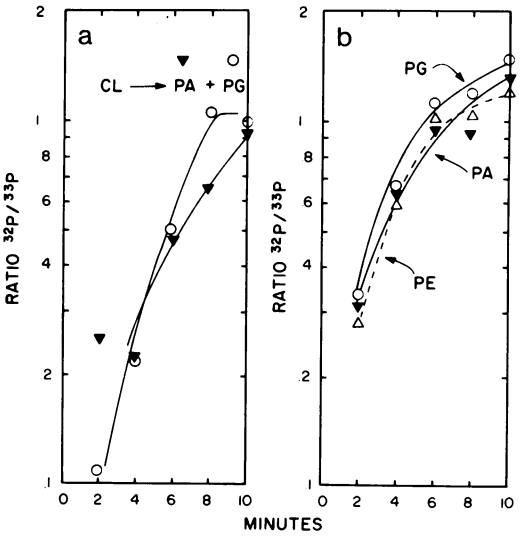

FIG. 6. *Incorporation of ^{32}P and ^{33}P into the two phosphates of cardiolipin. Haemophilus parainfluenzae was grown with 440 μCi of $H_3{}^{33}PO_4$ per 2,200 ml for three doublings, and, at a bacterial density of 0.16 mg (dry wt) per ml, 2.8 mCi of $H_3{}^{32}PO_4$ was added. The culture was sampled and the lipids were extracted and separated chromatographically as in Fig. 1. Cardiolipin (CL) was recovered and hydrolyzed with phospholipase D. The hydrolytic products PA (▼) and PG (○) were separated on silica gel-impregnated paper with a solvent of diisobutylketone, chloroform, methanol, pyridine, 0.5 M ammonium chloride, pH 10.6 (25:30:17.5:35:6) (24). a, PA and PG derived from the enzymatic hydrolysis of CL; b, PA (▼), PE (△), and PG (○) in the cells.*

which took about 5 min. When the resuspension was done in radioactive medium followed by a rapid 1 to 200 dilution into nonradioactive medium, the detection of these earlier events was possible. The striking change in the ratio of ^{32}P to ^{33}P occurred as the rapidly metabolizing CL first incorporated and then lost ^{32}P (Fig. 3). The maximum difference in the ratio occurred at about 17 min after addition of the ^{32}P. The second maximum occurring at about 27 min after the pulse with ^{32}P may represent the reutilization of ^{32}PG derived from the highly active CL that was responsible for the first change in ratio. When the incorporation period was too long, the heterogeneity of phosphate metabolism of PG and CL was not seen (Fig. 4). Metabolic heterogeneity in the metabolism of the phosphate can also be detected in PG but not in PE (Fig. 3, 4).

Heterogeneity in the localization of CL has been demonstrated in *H. parainfluenzae*. Membrane fragments with higher proportions of CL are released by viable cells when they are incubated in EDTA-Tris (18–20). Incorporation studies showed that the total cellular PA and PG had twice the specific activity of the CL (Fig. 5).

This suggested that either a small portion of the total PG and PA pools was involved in CL metabolism, or that the bulk of the CL was very slowly metabolized and thus greatly reduced the average specific activity.

Heterogeneity in the metabolism of both of the phosphates of CL can be detected. By isolating the CL and using the CL-specific phospholipase D, it was shown that the enzyme was specific for one of the phosphates of CL (Fig. 6). The PA portion of the CL molecule had a slower incorporation of ^{32}P from a pool with a higher specific activity than the PG portion of the CL molecule (Fig. 6).

ACKNOWLEDGMENT

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