

# Phospholipid metabolism during bacterial growth

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**ABSTRACT** *Haemophilus parainfluenzae* incorporates glycerol and phosphate into the membrane phospholipids without lag during logarithmic growth. In phosphatidyl glycerol (PG), the phosphate and unacylated glycerol moieties turn over and incorporate radioactivity much more rapidly than does the diacylated glycerol. At least half the radioactivity is lost from the phosphate and unacylated glycerol in about 1 doubling. The total fatty acids turn over slightly faster than the diacyl glycerol.

In phosphatidyl ethanolamine (PE), which is the major lipid of the bacterium, ethanolamine and phosphate turn over and incorporate radioactivity at least half as fast as the phosphate in PG. The glycerol of PE did not turn over in 4 bacterial doublings. In phosphatidic acid the glycerol turns over at one-third the rate of phosphate turnover. By means of a modified method for the quantitative recovery of 1,3-glycerol diphosphate from cardiolipin, the phosphates and middle glycerol of cardiolipin were shown to turn over more rapidly than the acylated glycerols during bacterial growth. There is no randomization of the radioactivity in the 1- and 3-positions of the glycerol in the course of 1 doubling.

The fatty acids of PG turn over faster than those in PE. In both lipids the 2-fatty acids turn over much faster than the 1-fatty acids. At both positions the individual fatty acids have their own rates of turnover. The distribution of fatty acids between the 1- and 2-positions is the same as in other organisms, with more monoenoic and long-chain fatty acids at the 2-position.

The different rates of turnover and incorporation of radioactivity into different parts of the lipids suggest that exchange reactions may be important to phospholipid metabolism.

**SUPPLEMENTARY KEY WORDS** turnover · incorporation · fatty acids · phospholipase A and C · phosphatidyl ethanolamine · phosphatidyl glycerol · cardiolipin · phosphatidic acid

Abbreviations: PE, 3-*sn*-phosphatidyl ethanolamine; GPE, *sn*-glycerol 3-phosphoryl ethanolamine; PG, 3-*sn*-phosphatidyl 1'-*sn*-glycerol; GPG, *sn*-glycerol 3-phosphoryl-1'-*sn*-glycerol; PA, phosphatidic acid;  $\alpha$ -GP, *sn*-glycerol 3-(dihydrogen phosphate); PS, 3-*sn*-phosphatidyl serine; GPS, *sn*-glycerol 3-phosphoryl-1'-serine; CL, cardiolipin(di-3-*sn*-phosphatidyl 1',3'-*sn*-glycerol); GPGPG, bis[*sn*-glycerol-3-phosphoryl]1',3'-*sn*-glycerol; PGP, *sn*-glycerol 1,3-diphosphate; P<sub>i</sub>, inorganic phosphate; and BBOT, 2,5-bis[2(5-*tert*-butyl benzoxazolyl)]-thiophene. Fatty acids are designated by the number of carbon atoms: number of double bonds, with the addition, where necessary, of cyc (cyclopropane) or br (branched).

**T**HE METABOLISM of the membrane lipids changes when the membrane-bound electron transport system in bacteria is modified (1-3). Since structural interrelationships of these membrane components are essential to the function of the electron transport system (4), we hoped that more detailed understanding of lipid metabolism could lead to understanding of the process by which the bacterial membrane is organized.

*Haemophilus parainfluenzae* was utilized as it requires a functioning membrane-bound electron transport system for growth and metabolism (5). Radioactive glycerol and phosphate are incorporated without detectable lag into the membrane lipids of this organism. During changes in the composition of the membrane-bound electron transport system there are changes in the metabolism of the lipids (3, 6, 7). The lipids of the membrane consist of the phospholipids PE, PG, PA, PS, CL, and phosphatidyl choline (7), which account for 98% of the extractable fatty acids (8).

Published studies of the metabolism of bacterial phospholipids described the incorporation and turnover of <sup>32</sup>P. The different phospholipids have been shown to have different metabolic activities. Cardiolipin is the most active lipid in *Mycobacteria* (9). In most eubacteria PG has the most active metabolism (3, 10-12). In this study, examination of the metabolism of the carbon-containing parts of the lipids reveals that the metabolism is much more complicated than predicted from studies of <sup>32</sup>P. Use of <sup>14</sup>C- and <sup>32</sup>P-labeled phospholipids allows the prediction of the properties of some of the enzymes involved in phospholipid metabolism during bacterial growth.

## MATERIALS AND METHODS

Compounds labeled with <sup>14</sup>C were supplied by New England Nuclear Corp., Boston, Mass. Carrier-free H<sub>3</sub><sup>32</sup>PO<sub>4</sub> was supplied in plastic bottles by Tracerlabs, Inc., Waltham, Mass. Other materials were supplied as described previously (4-7).

The medium, culture conditions, bacterial strain, and harvesting conditions have been described previously (3). The dry weight was measured as described (5).

### Isolation and Deacylation of the Lipids

In experiments on the turnover or incorporation of radioisotopes, samples of 100–200 ml were withdrawn from culture and added to an equal volume of ice. The ice effectively stops  $^{32}\text{P}$  incorporation into the lipids (3). The bacteria were centrifuged, resuspended in 50 mM phosphate buffer pH 7.6, and extracted by the Bligh and Dyer procedure described previously (7). In “pulse-chase” experiments the bacteria were grown in the presence of isotopes for 1–2 hr, centrifuged at 37°C, and resuspended in nonradioactive medium as described previously (3).

Lipids were deacylated by mild alkaline methanolysis. The methanolysis was stopped with Biorex 70 (an acrylic polymer with carboxylic acid exchange groups, from Bio-Rad Laboratories, Richmond, Calif.) and the glycerol 3-phosphate esters were separated from the methyl esters of the fatty acids by extraction with three portions of diethyl ether (7). The glycerol phosphate esters were separated by paper chromatography (7).

### Thin-Layer Chromatography

The three major phospholipids of *H. parainfluenzae* can be separated by thin-layer chromatography. Silica Gel G plates were prepared as described previously (13) and the lipids were separated in chloroform–methanol–glacial acetic acid 7:3:1. If a spot containing less than 0.5  $\mu\text{mole}$  of lipid phosphate is applied at the origin, the lipids separate as indicated in the radioautogram of lipids labeled with  $^{32}\text{P}$  in Fig. 1. In this system  $R_f$  values are CL, 0.97; PG, 0.76; PS, 0.16; and PE, 0.44. The proportions of the phosphate in the lipids determined after separation in this system were CL, 2.38%; PG, 16.97%; PE, 78.05%; and PS with other trace lipids, 2.5%. These proportions are close to those reported with other separation techniques (3, 7).

The three major lipids were recovered from the silica gel as described (13) and subjected to mild alkaline methanolysis. The resulting glycerol phosphate esters were identified by chromatography on aminocellulose in two solvent systems (7). The glycerol phosphate esters were located by radioautography. The CL contained 94.3% GPGPG with 5.7% GPG,  $\text{P}_i$ , and  $\alpha\text{-GP}$ . The PE contained 98% GPE, and 2% GPG which represents 1.6% of the total PG in the bacteria. The PG contained 1.6% PA. The PA was quantitatively removed from the PG by thin-layer chromatography in chloroform–methanol–6.7 M ammonium hydroxide 33:18.2:2.5. After separation of the PG and PA, the PA, PG, and PE migrated as single spots during two-dimensional thin-

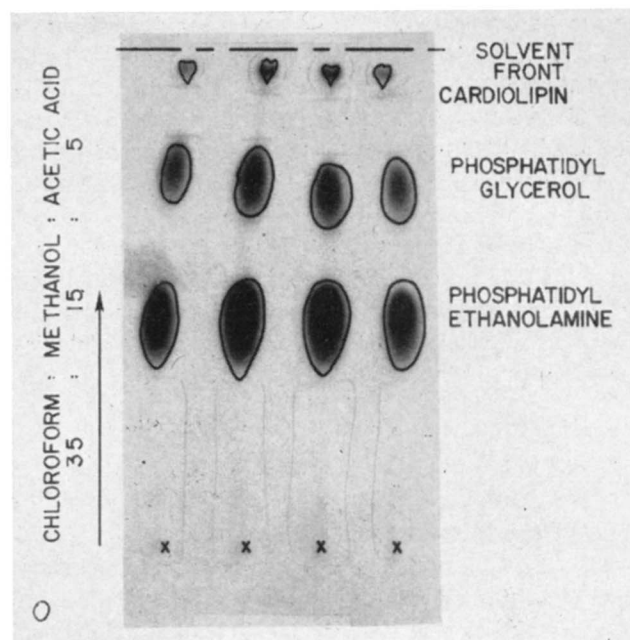


FIG. 1. Radioautogram of the lipids of *H. parainfluenzae* grown with  $^{32}\text{P}$  after separation by thin-layer chromatography on Silica Gel G.

layer chromatography (chloroform–methanol–acetic acid–water 45:7:3.15:0.5 in the first dimension and the chloroform–methanol–ammonium hydroxide solvent in the second dimension).

### Periodate Treatment

Labeled PG was treated with periodic acid by a method derived from that reported by MacFarlane (14). The PG, with sufficient unlabeled carrier to make 1  $\mu\text{mole}$  of lipid, was dissolved in a small volume of chloroform, 1 ml of 71.5 mM ethanolic periodic acid was added, and the mixture was incubated overnight in the dark at 25°C. An equal volume of chloroform was added and the mixture was washed with 0.001 N HCl until no further radioactivity could be recovered. The chloroform was then dried in a stream of nitrogen and the lipid was dissolved in methanol–toluene 1:1 and subjected to mild alkaline methanolysis (7). The fatty acid methyl esters were removed by extractions with diethyl ether and the glycerol phosphorylaldehyde was counted on filter paper discs in the toluene-containing scintillation fluid.

Glycerol phosphate esters derived from the lipids by milk alkaline methanolysis were treated with periodate as follows. Each labeled glycerol phosphate ester derived from the lipid by deacylation was combined with non-radioactive  $\alpha\text{-GP}$  to make 1  $\mu\text{mole}$  of phosphate and dissolved in 0.35 ml of water. Then 0.05 ml of 3.0 M acetate buffer pH 4.5, 0.05 ml of 1.0 M formaldehyde, and 0.05 ml of freshly prepared 0.25 M sodium metaperiodate were added. After incubation for 5 min in the dark at 25°C

the reaction was stopped by the addition of 0.05 ml of 10% (v/v) ethylene glycol and the mixture was incubated for an additional 5 min. Water (2 ml) and 0.4 meq of Dowex-1 (200–400 mesh) in the formate form were then added and the mixture was incubated for an additional 5 min in the dark. The suspension was then transferred to a microcolumn containing 0.8 meq of Dowex-1 in a Pasteur pipette, and the column was washed with 3 ml of water. The formaldehyde liberated by periodate oxidation was quantitatively recovered in the water. The formic acid or phosphorylglycolaldehydes were quantitatively retained on the column.

#### *Phospholipase D and the Separation of the Glycerols*

PG isolated by thin-layer chromatography was treated with cabbage phospholipase D (Calbiochem, Los Angeles, Calif.) as described previously (7). 0.02–0.06  $\mu$ mole of lipid phosphate was incubated at 25°C for 4 hr. 60–80% of the lipid was hydrolyzed. The ether was removed in a stream of nitrogen and sufficient amounts of methanol and chloroform were added to give the Bligh and Dyer (15) proportions of chloroform–methanol–water 1:1:0.9. After mixing, the system separated into two phases. The aqueous phase was removed and the chloroform layer was again extracted with an equal volume of 40% aqueous methanol. The aqueous phases were combined. The procedure had quantitatively removed the unacylated glycerol from the organic phase. Part of the unacylated glycerol from PG was counted in the dioxane scintillation fluid and the remainder was used for periodate oxidation.

The chloroform layer of the Bligh and Dyer extraction contained the PA and unhydrolyzed lipid. These lipids were subjected to mild alkaline methanolysis (7). The methyl esters of the fatty acids were recovered in the organic phase, and the glycerol phosphate esters in the aqueous phase were separated on aminocellulose paper with the modified Wawskiewicz solvent (7). The glycerol phosphate esters were located by radioautography and portions of the paper containing them were counted in the toluene-containing scintillation fluid.

The paper discs were recovered from the scintillation fluid and dried, and the esters were quantitatively eluted with two portions of 2 M aqueous ammonium hydroxide. The eluting solvent was removed in a stream of nitrogen and the glycerol phosphate esters were treated with periodate. The radioactivity of a small portion of the glycerol phosphate esters was determined in the dioxane scintillation fluid, in a comparison of the efficiencies of the two scintillation systems.

#### *Hydrolysis of PE*

PE was isolated by thin-layer chromatography and subjected to mild alkaline methanolysis. The fatty acid

methyl esters were recovered in the organic phase and the GPE in the aqueous phase. A portion of the GPE was hydrolyzed in 2 N HCl at 100°C for 1 hr in screw-cap tubes with Teflon liners. The HCl was removed by successive evaporations in a stream of nitrogen and the hydrolysis products were separated by descending chromatography on aminocellulose paper with the modified Wawskiewicz solvent (7). In this system possible reactants gave the following  $R_f$  values: ethanolamine hydrochloride, 0.70; GPE, 0.33;  $\alpha$ -GP, 0.08; Pi, 0.40; and glycerol, 0.56. After hydrolysis of  $^{32}$ P- or  $^{14}$ C-labeled fractions isolated as GPE, radioactivity could be detected only in the  $\alpha$ -GP and ethanolamine hydrochloride fractions. In bacteria grown with glycerol-1,3- $^{14}$ C for 2 doublings, no  $^{14}$ C could be detected in the ethanolamine. Components were eluted and the radioactivity was determined or periodate oxidation performed as described for PG.

#### *Degradation of Cardiolipin*

Cardiolipin is not susceptible to hydrolysis by phospholipase D, so that an alternative method of degradation was developed. The method represents a modification of the Brown and Stewart hydrolysis (16) slightly different from that reported by LeCocq and Ballou (17). Cardiolipin labeled with  $^{32}$ P was deacylated to GPGPG by mild alkaline methanolysis. The GPGPG was separated from the other glycerol phosphate esters by chromatography on aminocellulose paper with a solvent of 3 N formic acid containing 0.4% (v/v) pyridine. The GPGPG was located by radioautography as a band at  $R_f$  value 0.08. A strip of the paper containing the GPGPG band was cut out and soaked in 2 ml of 2 N ammonium hydroxide for a few minutes. The strip was soaked in another 2 ml of ammonia and then in two 0.5 ml portions of water. The washings were pooled and reduced to dryness in a stream of nitrogen.

The GPGPG containing  $^{32}$ P was dissolved in 0.25 ml of water. These were then added: 1  $\mu$ mole of unlabeled GPGPG in 0.1 ml of water, 0.05 ml of a solution containing 150  $\mu$ moles of sodium acetate buffer pH 4.5, 0.05 ml of a solution containing 50  $\mu$ moles of sodium formate, and 0.05 ml of a freshly prepared solution containing 12.5  $\mu$ moles of sodium metaperiodate. The mixture was incubated for 5 min in the dark at 25°C and 0.05 ml of a 10% solution of ethylene glycol (75  $\mu$ moles) was added. The reaction mixture was allowed to stand at room temperature for an additional 5 min in the dark. Then 0.5 ml of a freshly prepared 0.1% (v/v) solution of dimethylhydrazine adjusted to pH 8.4 with acetic acid was added and the reaction mixture was kept at 37°C overnight.

Dimethylhydrazones were extracted from the reaction mixture with 2  $\times$  2 ml chloroform. The aqueous layer

was then reduced to a small volume and the material was applied to paper (Schleicher & Schuell No. 589) for chromatography. The components were separated with a modified Wawszkiewicz solvent (7). In this solvent system the  $R_f$  values for components of the reaction and possible products after ascending chromatography were: PGP, 0.08;  $\alpha$ -GP, 0.23; GPGPG, 0.43; formaldehyde, 0.64; and ethylene glycol, 0.75. After the reaction all the  $^{32}\text{P}$  was found in a spot with an  $R_f$  value of 0.08; traces of formaldehyde and ethylene glycol were present. No other  $^{32}\text{P}$ -containing components or components reacting with periodate and *o*-toluidine (which detects vicinyl glycols) were present.

To confirm that the degradation yielded PGP and that this product contained all the phosphate present in the GPGPG at the start of the reaction, we combined 0.8  $\mu\text{mole}$  of authentic PGP (the gift of R. L. Lester) with GPGPG labeled with  $^{32}\text{P}$  and  $^{14}\text{C}$ . After the degradation reactions the mixture was applied to a column of Dowex-1 (200–400 mesh) and the degradation products were eluted with an ammonium formate–sodium borate gradient (13). The fractions recovered (between 182 and 194 ml) contained 100% of the  $^{32}\text{P}$  and 76% of the  $^{14}\text{C}$  applied to the column. This is the elution volume for authentic PGP (Fig. 2). Formaldehyde and ethylene glycol have retention volumes between 14 and 18 ml, which is where 24% of the  $^{14}\text{C}$  was eluted. Iodate reacts in the colorimetric assay for phosphate and accounts for the broad response between fractions 40 and 60 in Fig. 2.

The  $R_f$  values of other possible products of the reaction mixture are indicated above the elution pattern in Fig. 2. The periodate hydrolysis of GPGPG in the presence of 17 mM dimethylhydrazine at pH 8.4 results in the quantitative generation of PGP.

#### Phospholipase A Hydrolysis

PE and PG isolated from thin-layer chromatograms were treated with venom from *Ancistrodom piscivorus piscivorus* (Ross Allen's Reptile Institute, Inc., Silver Springs, Fla.) by a procedure slightly modified from that of Hildebrand and Law (18). About 1  $\mu\text{mole}$  of PG or PE was dissolved in 2 ml of ether; 30  $\mu\text{l}$  of 1.5 M  $\text{NH}_4\text{OH}$  and 30  $\mu\text{l}$  of a solution containing 0.1 M  $\text{CaCl}_2$  and 3 mg/ml of lyophilized venom in 0.1 M Tris buffer pH 7.2 were added. The solution was mixed and incubated for 6 hr at room temperature with occasional shaking. The reaction products were separated by thin-layer chromatography in chloroform–methanol–water 12:6:1. In this system  $R_f$  values were: PE, 0.65; lyso-PE, 0.24; PG, 0.35; lyso-PG, 0.17; and fatty acids, 0.79. Lipids were detected after thin-layer chromatography by spraying with Rhodamine 6G and examination of the chromatogram under UV light (13). There were no other products detectable on radioautograms when  $^{32}\text{P}$ -containing PE or PG were used.

To examine the fatty acids from the 1-position, we recovered the lyso-PE or lyso-PG from the silica gel and subjected it to mild alkaline methanolysis. The methyl

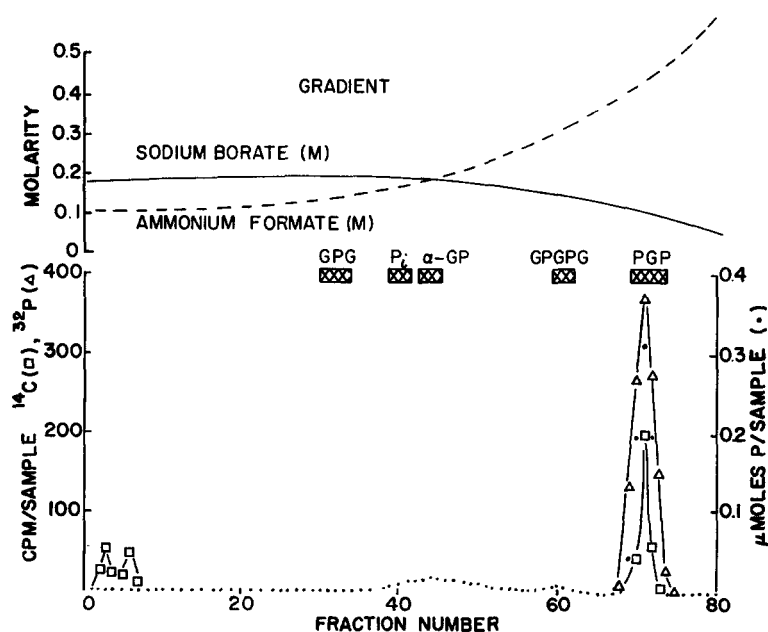


FIG. 2. Column chromatography of GPGPG, isolated from *H. parainfluenzae* grown in the presence of glycerol-1,3- $^{14}\text{C}$  and  $^{32}\text{P}$ , after treatment with periodate and dimethylhydrazine. Upper curve illustrates the gradient used to elute the derivatives from a  $0.6 \times 81$  cm column containing Dowex-1 (formate form). Squares above the chromatogram indicate the elution volumes of reference compounds. ●, total phosphate; □,  $^{14}\text{C}$ ; △,  $^{32}\text{P}$ .

esters were recovered and separated by gas-liquid chromatography. The fatty acids enzymatically hydrolyzed from the 2-position of the phospholipids were recovered from the silica gel, methylated (8), and subjected to gas-liquid chromatography. Recovery was calculated on the basis of the phosphorus recovered in the aqueous phase after mild alkaline methanolysis.

#### Gas-Liquid Chromatography

Fatty acid methyl esters were separated by gas-liquid chromatography on SE-30 (methylpolysiloxo gum) (8). The fatty acid methyl esters were identified from their retention volumes (8). To determine the  $^{14}\text{C}$  in the fatty acid methyl esters, we fitted the column with a 1:1 stream splitter and collected half the sample in  $0.1 \times 30.5$  cm melting point capillaries (8). Each melting point tube containing a fatty acid methyl ester was then rinsed with 5 ml of the toluene-containing scintillation fluid and the radioactivity was determined in the scintillation spectrometer.

#### Measurement of Radioactivity

Samples were assayed for radioactivity in a model 2311 Packard scintillation spectrophotometer. Glycerol phosphate esters were counted on paper discs 1.5-2 cm in diameter in a scintillation fluid of 9.28 mM BBOT in toluene. The efficiency of counting samples on the paper discs was 49.1% for  $^{14}\text{C}$  and 90.7% for  $^{32}\text{P}$ . Volatile, water-soluble components were counted in dioxane containing 1.236 M naphthalene and 6.99 mM BBOT. The efficiency of counting in the dioxane scintillator was 89.7% for  $^{14}\text{C}$  and 100% for  $^{32}\text{P}$ . Esters labeled with both  $^{14}\text{C}$  and  $^{32}\text{P}$  were counted under conditions such that the  $^{32}\text{P}$  channel =  $0.002 \text{ }^{14}\text{C} + 0.817 \text{ }^{32}\text{P}$ , and the  $^{14}\text{C}$  channel =  $0.634 \text{ }^{14}\text{C} + 0.008 \text{ }^{32}\text{P}$  in the toluene scintillator. Under these conditions the efficiency of counting was 29% for  $^{14}\text{C}$  and 81% for  $^{32}\text{P}$ . Radioautograms were made with Kodak no-screen X-ray film (7).

## RESULTS

#### Incorporation of $^{14}\text{C}$ into Phospholipids

Examination of the synthesis and turnover of the phospholipids in detail requires that the fatty acids and the carbon backbone of the lipids be labeled. The data in Table 1 indicate that little  $^{14}\text{C}$  is found in the lipids after growth with  $^{14}\text{C}$ -labeled threonine, choline, ethanolamine, or methionine. Serine and glycerol are satisfactory substrates for labeling the carbon chains of the lipids. Most of the label from serine is found in the fatty acids and rather high levels are required, as the complex medium necessary for the growth of *H. parainfluenzae* contains 1.0 mM serine. Glycerol is readily incorporated into

TABLE 1 INCORPORATION OF  $^{14}\text{C}$  INTO THE LIPIDS OF *H. parainfluenzae*

Source of $^{14}\text{C}$	% in Total Lipid*	% of Lipid $^{14}\text{C}$ in Fatty Acids	% of Lipid $^{14}\text{C}$ in Glycerophosphoryl Derivatives
Threonine- $^{14}\text{C}$	0.043	84	16
Choline (methyl- $^{14}\text{C}$ )	0.054	98	2
Ethanolamine-1,2- $^{14}\text{C}$	0.01	0	100
Methionine (methyl- $^{14}\text{C}$ )	0.002	41	59
Glucose-1- $^{14}\text{C}$	1.3	83	17
Serine-3- $^{14}\text{C}$	6.4	98	2
Glycerol-1,3- $^{14}\text{C}$	4.1	63	37

*H. parainfluenzae* was grown in the presence of  $10 \mu\text{C}$  of  $^{14}\text{C}$ -labeled compound ( $50 \mu\text{C}$  for methionine) in 1.5 liters of medium for 18 hr at  $37^\circ\text{C}$ . The bacteria were harvested and 26-30  $\mu\text{moles}$  of lipid phosphate was isolated. The lipids were deacylated and the methyl esters of the fatty acids were separated from the glycerol phosphate derivatives.

\* Radioactivity in total lipid extract as a percentage of radioactivity in the medium before inoculation.

the glycerol portions of the phospholipids. *H. parainfluenzae* incorporates 0.4% of the  $\text{H}_3\text{ }^{32}\text{PO}_4$  in the medium per doubling at a specific activity in the medium of 20,000 cpm/ $\mu\text{mole}$  of phosphate.

#### Incorporation of Glycerol into Phospholipids

Since glycerol was a satisfactory substrate for the labeling of the glycerol of the phospholipids, it was important to know if the glycerol was incorporated directly into the lipid or indirectly via some other metabolic product. If the phospholipids are deacylated and the glycerol phosphate esters that result are treated with periodate, the specificity of the labeling can be determined, for if the position of the  $^{14}\text{C}$  of the glycerol in the lipid is the same as that of the glycerol added to the medium, a metabolic precursor of the lipid glycerol that randomizes the label is ruled out.

A method that uses a microcolumn of Dowex-1 to retain the formate and the glycolaldehyde phosphate esters (but not the formaldehyde) that are produced after periodate oxidation was developed by R. L. Lester. The method proved to be quantitative, as neither GPE nor its hydrolysis products phosphoethanolamine glycolaldehyde or formaldehyde was retained by the column, and the recovery of radioactivity was 100%. None of the radioactivity from glycerol-1,3- $^{14}\text{C}$  and all of the radioactivity of glycerol-2- $^{14}\text{C}$  was retained on the column after periodate treatment. These experiments are illustrated in the last two lines of Table 2.

In lipids extracted from bacteria grown with glycerol-1,3- $^{14}\text{C}$  the  $\alpha$ -GP derived from PA by deacylation, the  $\alpha$ -GP derived from PG after treatment with phospholipase D and deacylation, and the  $\alpha$ -GP derived from

TABLE 2 PERIODATE TREATMENT OF GLYCEROL PHOSPHATE ESTERS FROM THE PHOSPHOLIPIDS OF *H. parainfluenzae* GROWN WITH GLYCEROL-1,3-<sup>14</sup>C

Lipid	Periodate Treatment		Recovered Formaldehyde	% Recovered	% Expected*
	Deacylated Product	Un-treated			
		cpm/sample			
PA	α-GP	822	391	48	50
PS	GPS	85	34	42	50
PG	GPG	8286	3824	47	50
PG	α-GP	537	283	53	50
PE	GPE	2091	2084	100	100
PE	α-GP	2167	1108	52	50
CL	GPGPG	678	174	26	33
Glycerol-1,3- <sup>14</sup> C	—	7642	7549	98	100
Glycerol-2- <sup>14</sup> C	—	9176	12	0.1	0

Lipids isolated from *H. parainfluenzae* grown with glycerol-1,3-<sup>14</sup>C for 1.4 doublings were separated by thin-layer chromatography and deacylated, and the glycerol phosphoryl derivatives were treated with periodate. The periodate reactions were stopped by addition of ethylene glycol and the mixtures were placed on microcolumns of Dowex-1. The formaldehyde produced in the periodate reaction was eluted from the columns with water and the phosphoryl glycolaldehyde derivatives (except for glycolaldehyde phosphoryl ethanolamine) remained bound to the column. Deacylated phospholipids and formaldehyde were counted on paper discs in the toluene scintillation fluid.

\* On the basis of no randomization of radioactivity during metabolism.

PE by acid hydrolysis of GPE each yield 50% of the radioactivity as formaldehyde after periodate oxidation (Table 2). GPG and GPS yield about 50% of the radioactivity as formaldehyde after periodate oxidation. These experiments strongly suggest that glycerol is incorporated directly into the phospholipids without randomization of the label. The GPGPG that was derived from CL yielded only one-quarter of the radioactivity as formaldehyde after periodate oxidation. If randomization of the label does not occur, the middle glycerol probably contains more <sup>14</sup>C than each outside glycerol.

#### Turnover of <sup>14</sup>C and <sup>32</sup>P

The decrease in radioactivity remaining in the phospholipids after a short period of growth in <sup>14</sup>C and <sup>32</sup>P gives a measure of the metabolism of the lipids during bacterial growth. In the experiment illustrated in Fig. 3, *H. parainfluenzae* was allowed to grow for 1 hr in the presence of 500 μc of H<sub>3</sub><sup>32</sup>PO<sub>4</sub> and 50 μc of glycerol-2-<sup>14</sup>C per 100 ml. The culture was then centrifuged at 37°C, suspended in warm, nonradioactive medium, and reincubated at 37°C. Samples of 100 ml were removed, the lipids were extracted and deacylated, and the glycerol phosphate esters were separated by paper chromatography. The esters were located by radioautography, and the radioactivity of the spots corresponding to the dark

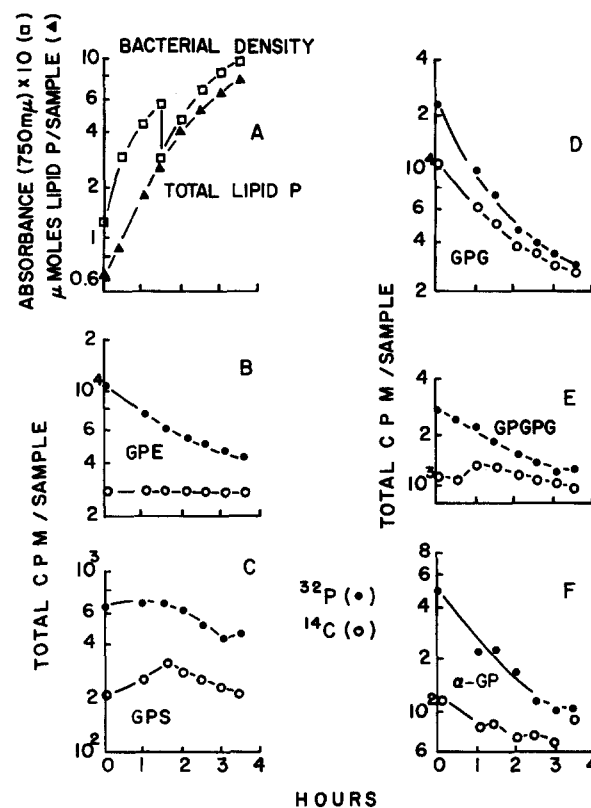


FIG. 3. Turnover of <sup>14</sup>C and <sup>32</sup>P in the lipids of *H. parainfluenzae*. The culture was grown in <sup>32</sup>P and <sup>14</sup>C for 1 hr, harvested, washed, and suspended in nonradioactive medium. The lipids were then extracted, deacylated, and separated by chromatography and their radioactivity was determined. Curves in A indicate the bacterial density and total lipid phosphate during the period of growth in nonradioactive medium. In curves shown in B to F, the total <sup>32</sup>P (●) and <sup>14</sup>C (○) in each of the glycerol phosphate esters is given.

spots on the film was determined in the scintillation spectrophotometer.

During the early period of the turnover experiments the bacterial density was kept below an absorbance of 0.6 by dilution. If the bacterial density is kept below 0.3 mg dry weight per ml and the flask is vigorously aerated, rapid cytochrome synthesis does not occur. Rapid cytochrome synthesis has been shown to affect phospholipid phosphate metabolism (3). The last four points on each curve in Fig. 3 represent the turnover during rapid cytochrome synthesis. The striking feature of the experiment is that the turnover of <sup>32</sup>P is faster than the turnover of the <sup>14</sup>C in the glycerol. Times for the initial loss of half the radioactivity for <sup>32</sup>P and <sup>14</sup>C for the glycerol phosphate esters were: GPE, 127 and 2400 min; GPS, 150 and 230 min; GPG, 50 and 80 min; GPGPG, 150 and 230 min; and α-GP, 85 and 210 min. The time necessary to double the total phospholipid was 40 min. In the period during which the last four samples were taken, rapid cytochrome synthesis was occurring, and there was

a reduction in the rate of turnover of both  $^{32}\text{P}$  and  $^{14}\text{C}$ . In view of the discrepancy of the turnover of  $^{14}\text{C}$  and  $^{32}\text{P}$ , a detailed analysis of the metabolism of PG, PE, and CL was initiated.

#### Metabolism of PG

The lipids of *H. parainfluenzae* were labeled with glycerol-1,3- $^{14}\text{C}$  for 1 hr and the PG was isolated from the lipid extract by thin-layer chromatography. The PG was treated with ethanolic periodic acid as described in Materials and Methods and the formaldehyde from the 1-position of the unacylated glycerol was recovered by repeated extractions of the reaction mixture with 0.001 N HCl. A second portion of the PG was deacylated by mild alkaline methanolysis and the  $^{14}\text{C}$  in the GPG was determined. The  $\text{C}_5$  aldehyde that is a product of the periodate oxidation of PG was also deacylated and its  $^{14}\text{C}$  determined. Using phosphate as a measure of the amount of material, we compared the amount of  $^{14}\text{C}$  in the  $\text{C}_5$  aldehyde to that in the GPG. The  $^{14}\text{C}$  missing from the aldehyde was 37.6% of the total  $^{14}\text{C}$  in the GPG and represents the proportion of the  $^{14}\text{C}$  in C-1 of the unacylated glycerol. If both glycerols in the PG were equally labeled and there was no randomization, 25% of the radioactivity should be in C-1 of the unacylated glycerol. The fact that it contains more than 25% of the radioactivity suggests that there is a difference in the metabolism of the unacylated and diacyl glycerols of PG. This implication has been established by the experiments reported below.

#### Turnover of PG

PG was labeled by growth of the bacteria with 400  $\mu\text{C}$  of  $^{32}\text{P}$  and 75  $\mu\text{C}$  of glycerol-1,3- $^{14}\text{C}$  per 150 ml for 1 hr. The culture was then centrifuged and resuspended in warm medium that contained no radioactivity. During the growth in the nonradioactive medium, 250-ml samples were withdrawn, the lipids were extracted, and the PG was isolated by thin-layer chromatography. The PG was incubated with phospholipase D and the unacylated glycerol was recovered in two washes with water. The organic phase, containing PG and PA, was subjected to mild alkaline methanolysis and the GPG and  $\alpha\text{-GP}$  were separated by chromatography on aminocellulose paper with the modified Wawszkiewicz solvent (7). The glycerol phosphate esters were located by radioautography, the spots were cut out, and the  $^{32}\text{P}$  and  $^{14}\text{C}$  were determined in the scintillation spectrometer. Under these conditions the PG was about 50% hydrolyzed by the phospholipase D. The total fatty acids were recovered from the mild alkaline methanolysis.

The results of the turnover experiments are illustrated in Fig. 4. The doubling time of the bacteria was 45 min. Half of the radioactivity was lost from the total fatty

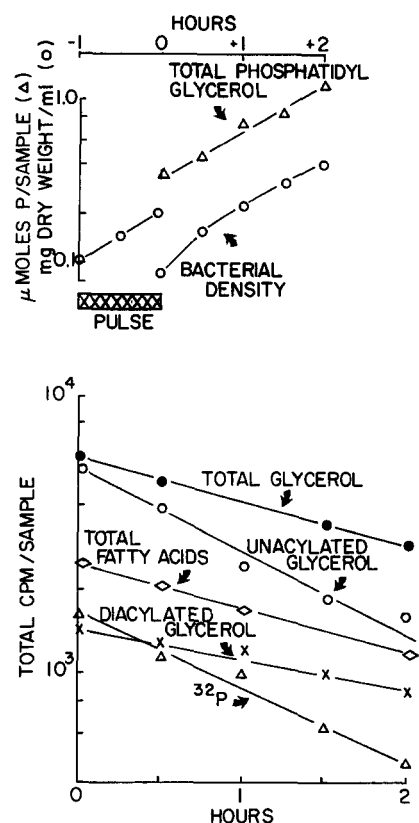


FIG. 4. Turnover of  $^{14}\text{C}$  and  $^{32}\text{P}$  in the PG of *H. parainfluenzae*. The culture was grown in the presence of the isotopes during the period marked "pulse" in the upper portion of the figure. The culture was centrifuged, washed, inoculated into nonradioactive medium in 10–15 min (not illustrated in the figure), and then allowed to grow. The total radioactivity in the unacylated glycerol, phosphate, diacylated glycerol, and total fatty acids during the period of growth in nonradioactive medium is shown in the lower portion.

acids in 102 min (2.27 doublings), from the diacyl glycerol in 162 min (3.6 doublings), from the unacylated glycerol in 58 min (1.3 doublings), and from the phosphate in 60 min (1.32 doublings). The unacylated glycerol and phosphate lose radioactivity at the same rate, which is 2.7 times as rapid as the loss of radioactivity from the diacyl glycerol.

*H. parainfluenzae* was grown in the presence of 1  $\mu\text{C}$  of  $\text{H}_3^{32}\text{PO}_4$  and 120  $\mu\text{C}$  of glycerol-1,3- $^{14}\text{C}$  per 1300 ml and the kinetics of incorporation of isotopes into the diacyl glycerol, unacylated glycerol, phosphate, and total fatty acids were measured. Radioactivity in the unacylated glycerol increased four times as fast (Fig. 5) as in the diacyl glycerol or fatty acid, if the precursor pools for each had the same specific activity. The phosphate incorporation fortuitously parallels the incorporation into the unacylated glycerol. In these experiments the specific activity of the medium was 20,300 cpm of  $^{32}\text{P}$  and 2760 cpm of  $^{14}\text{C}$  per  $\mu\text{mole}$  of phosphate. By the end of the experiment the specific activity of the  $^{32}\text{P}$  had dropped 13% and the  $^{14}\text{C}$  dropped 38%.

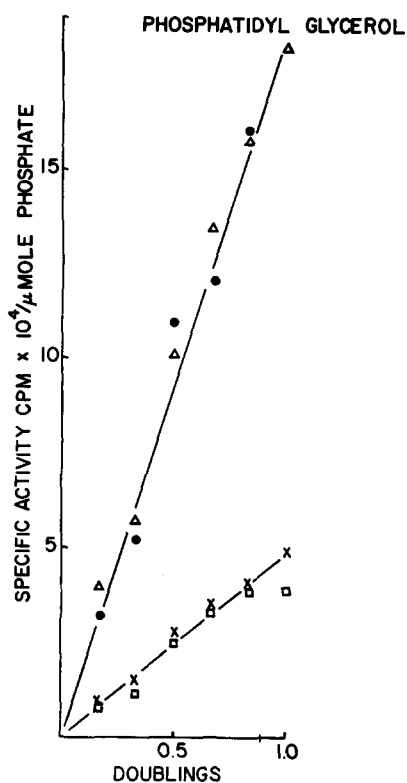


FIG. 5. Incorporation of  $^{32}\text{P}$  and  $^{14}\text{C}$  into PG in *H. parainfluenzae* grown in the presence of 1 mc of  $\text{H}_3^{32}\text{PO}_4$  and 120  $\mu\text{C}$  of glycerol-1,3- $^{14}\text{C}$ .  $\Delta$ ,  $^{32}\text{P}$ ;  $\bullet$ , unacylated glycerol;  $\square$ , total fatty acids;  $\times$ , diacylated glycerol.

The  $\alpha$ -GP derived from the diacyl glycerol and the glycerol released from PG by phospholipase D were treated with periodate; samples from an incorporation experiment like that illustrated in Fig. 5 were used. The formaldehyde was then separated on Dowex-1 from the formate and from the phosphoryl glycolaldehyde produced by periodate oxidation (Fig. 6). There is no randomization of glycerol-1,3- $^{14}\text{C}$ , as 50% of the radioactivity was recovered from the  $\alpha$ -GP derived from the diacyl glycerol and 100% of the radioactivity was recovered from the unacylated glycerol as formaldehyde during growth in glycerol-1,3- $^{14}\text{C}$  for 1 doubling.

#### Metabolism of PE

If the ethanolamine of the PE is to be labeled efficiently the bacteria must be grown in serine- $^{14}\text{C}$ . An experiment in which *H. parainfluenzae* was incubated in 50 mM phosphate buffer (pH 7.6) containing 2 mM sodium gluconate, 0.15  $\mu\text{M}$  NAD, and  $^{14}\text{C}$ -labeled serine is illustrated in Table 3. Under these conditions the bacteria, at a density of 0.58 mg dry weight per ml, incorporated 18% of the serine-3- $^{14}\text{C}$  and 0.01% of the serine-1- $^{14}\text{C}$  into the lipids in 2 hr in the presence of 10  $\mu\text{C}$  of  $^{14}\text{C}$  per 50 ml. The fatty acid accounts for 35% of the  $^{14}\text{C}$  from carboxyl-labeled serine and 93.5% of the  $^{14}\text{C}$  from

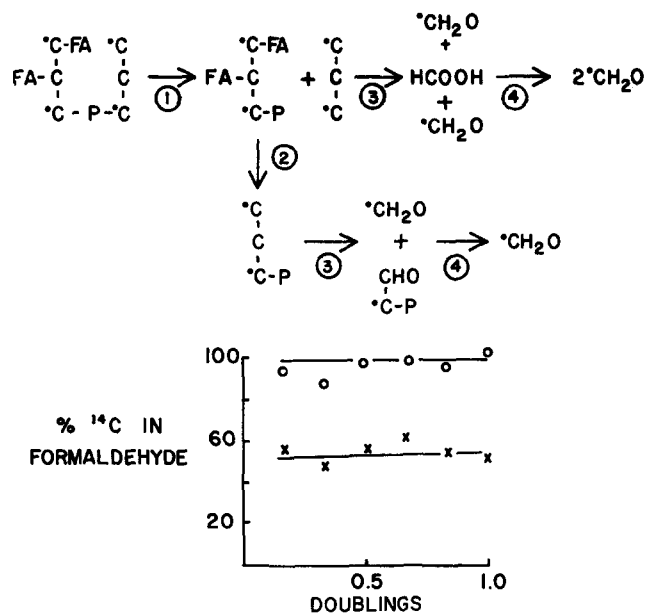


FIG. 6. Determination of position of  $^{14}\text{C}$  in the glycerol of PG of *H. parainfluenzae* grown with glycerol-1,3- $^{14}\text{C}$ . The operations involved in this experiment, illustrated in the upper part of the figure, are: 1, treatment with phospholipase D; 2, deacylation of the phosphatidic acid after separation from the unacylated glycerol by extraction with  $\text{CHCl}_3$ ; 3, periodate treatment; 4, column chromatography on Dowex-1 with recovery of the formaldehyde from the glycerol. The  $\bullet$  indicate the position of the  $^{14}\text{C}$ . The curves in the bottom part of the figure show the percentage of the total  $^{14}\text{C}$  obtained after step 3 that was present in formaldehyde:  $\circ$ , for the top sequence of reactions corresponding to unacylated glycerol;  $\times$ , for the sequence of reactions corresponding to diacylated glycerol.

3-labeled serine. Carboxyl-labeled serine and serine labeled in the 3-position were incorporated into GPE equally efficiently, but there was 100 times more  $^{14}\text{C}$  incorporated into GPE from serine-3- $^{14}\text{C}$  than from serine-1- $^{14}\text{C}$ . A larger proportion of the 3-labeled serine appears in the GPG and GPGPG as well.

The turnover of PE was measured in *H. parainfluenzae* grown in the presence of 20  $\mu\text{C}$  of L-serine-3- $^{14}\text{C}$  for 2 hr; 25  $\mu\text{C}$  of glycerol-1,3- $^{14}\text{C}$  and 360  $\mu\text{C}$  of  $\text{H}_3^{32}\text{PO}_4$  per 100

TABLE 3 INCORPORATION OF SERINE- $^{14}\text{C}$  INTO THE LIPIDS OF *H. parainfluenzae*

Lipid	Serine-1- $^{14}\text{C}$	Serine-3- $^{14}\text{C}$
	<i>cpm/μmole phosphate</i>	
GPE	273	237,000
GPS	12,500	10,900
GPGPG	1,000	6,440
GPG	2,500	29,700

*H. parainfluenzae* was incubated with  $^{14}\text{C}$ -labeled serine, the lipids were isolated and deacylated by mild alkaline methanolysis, and the glycerophosphoryl derivatives were separated by two-dimensional paper chromatography. The separated, deacylated phospholipids were detected by radioautography, the spots were cut out, and their radioactivity was determined. The paper discs were then digested and the phosphate was determined.



ml of medium were added during the final hour of incorporation. The culture was centrifuged and resuspended in nonradioactive medium as described for the experiments with PG. At 30-min intervals 50-ml samples were withdrawn onto an equal volume of ice and centrifuged, and the lipids were extracted. The PE was isolated by thin-layer chromatography and recovered from the silica gel. The PE was then deacylated and the GPE hydrolyzed in HCl as described in Materials and Methods. The resulting  $\alpha$ -GP and ethanolamine hydrochloride were separated on aminocellulose paper and located by radioautography; the paper corresponding to the spots on the radioautogram were cut out and counted by liquid scintillation. The acid hydrolysis was quantitative and no radioactive products other than  $\alpha$ -GP or ethanolamine hydrochloride could be detected after chromatography.

The data from this experiment are illustrated in Fig. 7. The bacteria and total PE doubled in 42 min. Half the

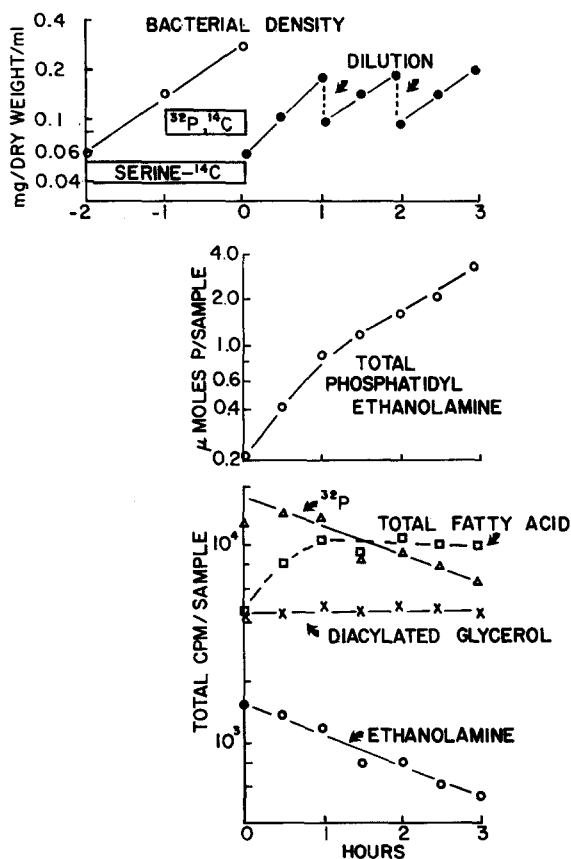


FIG. 7. Turnover of  $^{32}\text{P}$  and  $^{14}\text{C}$  in PE of *H. parainfluenzae*. The curves in the upper part of the Figure indicate the bacterial density during pulse labeling and the subsequent period of growth in nonradioactive medium. The culture was diluted to restrain the proliferation of the electron transport system. The middle curve indicates the total PE per sample during the period examined. The curves in the lower part illustrate the changing radioactivity of the total fatty acids, ethanolamine, glycerol, and phosphate during the turnover.

radioactivity in the ethanolamine disappeared in 114 min (2.7 doublings), and from the phosphate in 120 min (2.8 doublings). The glycerol did not turn over in 4.3 doublings.

The incorporation of radioactivity into PE was measured in the presence of  $600\ \mu\text{C}$  of  $\text{H}_3^{32}\text{PO}_4$ ,  $100\ \mu\text{C}$  of serine- $3\text{-}^{14}\text{C}$ , and  $100\ \mu\text{C}$  of glycerol- $1,3\text{-}^{14}\text{C}$  per 500 ml of medium. Samples were withdrawn onto ice every 10 min and treated as described above. The results of this experiment are plotted in Fig. 8. The fatty acids, ethanolamine, and phosphate incorporate radioactivity more rapidly than the glycerol.

The  $\alpha$ -GP derived from PE was treated with periodate in an experiment to determine if randomization in the position of  $^{14}\text{C}$  in the glycerol- $1,3\text{-}^{14}\text{C}$  occurred during the incorporation period. There was no apparent randomization of the  $^{14}\text{C}$ , as half the radioactivity was found in C-1 of the glycerol derived from PE (Fig. 9).

#### Turnover of Cardiolipin

Periodate-dimethylhydrazine hydrolyzes GPGPG with the quantitative formation of PGP, as described in Materials and Methods. The PGP can be isolated by paper chromatography and the  $^{14}\text{C}$  of the GPGPG and PGP can be used to calculate the  $^{14}\text{C}$  in the end glycerols. In the experiment illustrated in Fig. 10, *H. parain-*

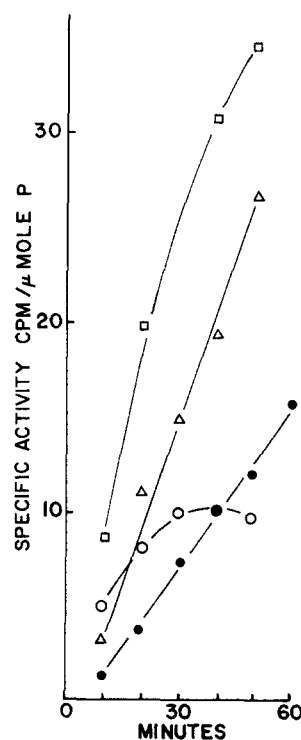


FIG. 8. Incorporation of  $^{32}\text{P}$  and  $^{14}\text{C}$  into PE of *H. parainfluenzae* in the presence of  $600\ \mu\text{C}$  of  $\text{H}_3^{32}\text{PO}_4$ ,  $100\ \mu\text{C}$  of serine- $3\text{-}^{14}\text{C}$ , and  $100\ \mu\text{C}$  of glycerol- $1,3\text{-}^{14}\text{C}$ .  $\square$ , total fatty acids;  $\Delta$ ,  $^{32}\text{P}$ ;  $\circ$ , ethanolamine;  $\bullet$ , glycerol.

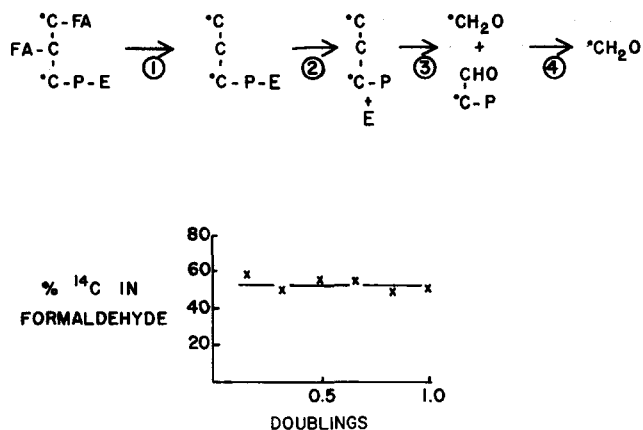


FIG. 9. Determination of position of  $^{14}\text{C}$  in the glycerol backbone of PE from *H. parainfluenzae* grown with glycerol-1,3- $^{14}\text{C}$ . The operations involved in this experiment are: 1, deacylation by mild alkaline methanolysis; 2, acid hydrolysis and separation of E (ethanolamine) and  $\alpha$ -GP by chromatography; 3, periodate treatment; 4, column chromatography on Dowex-1 with recovery of the formaldehyde of the glycerol. The  $^{\circ}\text{C}$  indicates the position of the  $^{14}\text{C}$ .

*fluenzae* was incubated in the presence of 100  $\mu\text{C}$  of glycerol-1,3- $^{14}\text{C}$  and 700  $\mu\text{C}$  of  $^{32}\text{P}$  in 250 ml of medium for 1 hr. The culture was centrifuged and resuspended in nonradioactive medium. The doubling time during both the "pulse" and the "chase" was 40 min. To maintain aerobic growth without modification of the electron transport system we diluted the culture with warm medium after the third sample had been taken. During the growth in nonradioactive medium, 250-ml samples were withdrawn onto ice and the lipids were extracted as in the other experiments. The lipids were deacylated and the GPGPG recovered from paper chromatograms was treated with periodate-dimethylhydrazine. The radioactivity of the PGP and GPGPG was determined.

The times for half the radioactive phosphate and  $^{14}\text{C}$  in the middle glycerol to disappear were 162 and 165 min, or about 4 bacterial doublings. The  $^{14}\text{C}$  in the end glycerols increased during the 1st hr, then decreased by half in 360 min or 9 bacterial doublings. In 3 doublings the  $^{14}\text{C}$  in the middle glycerol decreased from 50% to 25% of the total  $^{14}\text{C}$  in the GPGPG. Clearly, the middle glycerol and phosphates turn over at least three times more rapidly than the end glycerols (or what would be the diacyl glycerols in the cardiolipin) during aerobic growth of *H. parainfluenzae*.

#### Incorporation of Glycerol and Phosphate

*H. parainfluenzae* in the logarithmic growth phase was placed in a medium containing 700  $\mu\text{C}$  of  $\text{H}_3^{32}\text{PO}_4$  and 100  $\mu\text{C}$  of glycerol-1,3- $^{14}\text{C}$  per 2000 ml and allowed to grow. Growth was initiated without a lag period with a doubling time of 42 min and continued at that rate for 2 hr. Samples were withdrawn during the first 45 min

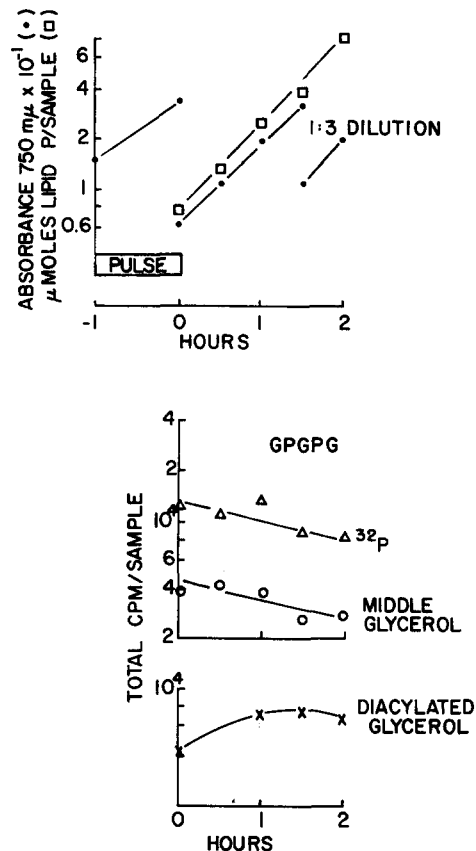


FIG. 10. Turnover of CL in *H. parainfluenzae*. The upper curves illustrate the bacterial density during the pulse labeling and period of growth in nonradioactive medium. The radioactivity in the middle glycerol diphosphate and in the outside (diacylated) glycerol moieties during the growth in nonradioactive medium is illustrated in the lower curves.

of growth and the specific activity of the glycerol phosphate esters was determined (Fig. 11). During the period of growth the specific activity of the medium fell from 265,000 to 228,000 cpm of  $^{32}\text{P}$  per  $\mu\text{mole}$  of P and from 18,000 to 16,000 cpm of  $^{14}\text{C}$  per  $\mu\text{mole}$  of P. Both glycerol and phosphate were incorporated into the glycerol phosphate ester portions of the phospholipids at rates proportional to the concentration of each lipid in the membrane. Both were incorporated into PS at rates significantly lower than for the other lipids.

#### Incorporation of $\alpha$ -GP

*Sn*-Glycerol-2- $^{14}\text{C}$  3-phosphate- $^{32}\text{P}$  was prepared from ATP labeled with  $^{32}\text{P}$  in the terminal phosphate with glycerol kinase (EC 2.7.1.30) by Dr. Marion Steiner. In a growth cycle, 2.04% of the  $^{32}\text{P}$  and 8.4% of the  $^{14}\text{C}$  of the doubly-labeled glycerol phosphate in the medium was incorporated into the lipid. Under these growth conditions 0.8% of the  $^{32}\text{P}$  and 4.9% of the  $^{14}\text{C}$  was incorporated from  $\text{H}_3^{32}\text{PO}_4$  and glycerol-2- $^{14}\text{C}$  in the medium. About 62% of the  $^{14}\text{C}$  in the lipid was found in

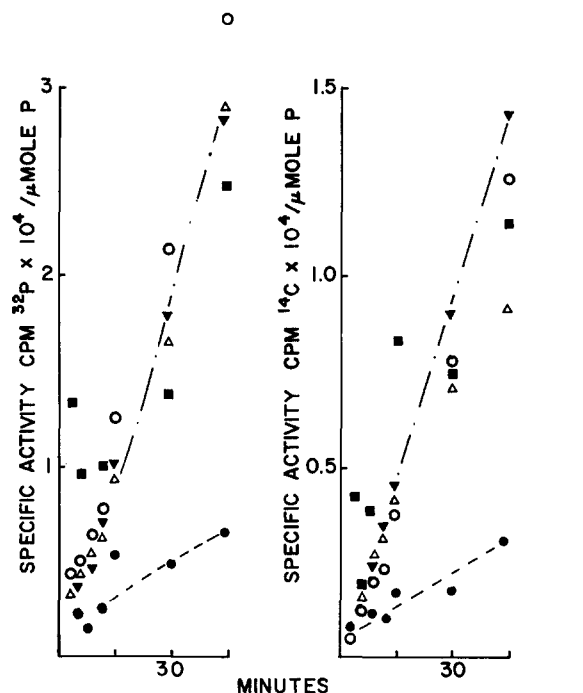


FIG. 11. Incorporation of  $^{14}\text{C}$  and  $^{32}\text{P}$  into the glycerol phosphate esters derived from the lipids of *H. parainfluenzae*. Left, incorporation of  $^{32}\text{P}$ ; right, incorporation of glycerol-1,3- $^{14}\text{C}$ . O, GPE;  $\Delta$ , GPG;  $\nabla$ , GPGPG;  $\bullet$ , GPS;  $\blacksquare$ ,  $\alpha$ -GP.

the fatty acids, whether glycerol or  $\alpha$ -GP was the source of  $^{14}\text{C}$ . Both the phosphate and glycerol were incorporated into phospholipids twice as efficiently from  $\alpha$ -GP as from glycerol and phosphate.

When cells were grown in the presence of radioactive  $\alpha$ -GP with a  $^{32}\text{P}/^{14}\text{C}$  ratio of 1:5.2 for 15 min (an increase from 0.1 to 0.14 mg dry weight per ml), the ratio of  $^{32}\text{P}/^{14}\text{C}$  was 1:6.0 in GPE, 1:13.2 for GPG, 1:11.7 for GPGPG, 1:6.4 for  $\alpha$ -GP, and 1:6.0 for GPS. If  $\alpha$ -GP were incorporated into GPG and GPGPG as in GPE without any dilution of the  $\alpha$ -GP, the expected ratio would be 1:12 for GPG and 1:9 for GPGPG.

#### Turnover of the Fatty Acids

The phospholipase A of *A. piscivorus piscivorus* specifically hydrolyzes the 2-fatty acid from diacyl phospholipids (18). We examined the metabolism of the fatty acids of the lipids of *H. parainfluenzae* during growth using this specific enzymatic hydrolysis to differentiate between the 1- and 2-positions of the diacyl glycerol in PE and PG. PG and PE were isolated from the lipids by one-dimensional thin-layer chromatography and hydrolyzed with snake venom phospholipase A. The hydrolysis products were separated by thin-layer chromatography. The 2-fatty acids were recovered from the silica gel, methylated, and analyzed by gas-liquid chromatography. The lyso-PG and lyso-PE were recovered from the

TABLE 4 DISTRIBUTION OF FATTY ACIDS BETWEEN THE 1- AND 2-POSITIONS IN PG AND PE FROM *H. parainfluenzae*

Fatty Acid	Phosphatidyl Glycerol			Phosphatidyl Ethanolamine		
	1	2	Total	1	2	Total
	% of total fatty acid in the lipid					
14:1		0.4	0.4	0.4	6.7	7.1
14:0	1.5		1.5	3.7	0.1	3.8
16:1	1.7	31.9	33.6	2.5	16.0	18.5
16:0	31.1	2.1	33.2	28.5	0.4	29.0
17:1	1.0	1.0	2.1	0.7	1.3	2.0
17:0	1.4	0.6	2.0	1.3	1.5	2.8
17:0cyc	0.8	0.6	1.3	0.9	1.0	1.9
18:1	1.5	1.3	2.7	0.7	2.4	3.1
18:0	4.0	2.3	6.3	3.5	3.4	7.0
19:1		1.1	1.1		3.2	3.2
19:0	3.3	2.2	5.5	5.0	6.5	11.6
21:1		0.9	0.9	0.8	0.8	1.6
22:0	1.6	3.0	4.6	0.6	2.0	2.6
23:0		0.9	0.9		3.1	3.1

Fatty acids are designated by the number of carbon atoms: number of bonds; cyc = cyclopropane and br = branched. Fatty acids were identified by their retention volumes on SE-30 and their percentages were calculated as previously described (8). The fatty acids 12:1, 12:0, 13:1, 13:0, 15:1, 15:0, 17:0 br, 18:0 cyc, 19:0 cyc, 20:0, and 21:1 together represented 2.4% and 1.9% in PG and 1.1% and 1.3% in PE of the total at the 1- and 2-positions respectively.

silica gel, the 1-fatty acids were obtained as methyl esters after mild alkaline methanolysis, and the methyl esters were subjected to gas-liquid chromatography.

The distribution of fatty acids between the 1- and 2-positions in PE and PG is given in Table 4. The 2-position contains 90–92% of the monoenoic fatty acids and 74–76% of the total fatty acids with chain lengths greater than  $\text{C}_{20}$  in both PE and PG. Of the total fatty acids with chain length less than  $\text{C}_{20}$ , 85.6% in PG and 78% in PE occupied the 1-position. 58% of the total cyclopropane fatty acids occupy the 2-position in PG and 78% in PE. Although the total fatty acid composition differs between PG and PE, the distributions between 1- and 2-positions are remarkably similar.

In experiments designed to measure the turnover of the fatty acids of PG and PE, *H. parainfluenzae* was grown in the presence of 100  $\mu\text{C}$  of glycerol-2- $^{14}\text{C}$  per 150 ml for 1 hr, the culture was centrifuged and diluted into nonradioactive medium, and allowed to grow for an additional 2 hr. During the growth periods the bacterial density was kept below 0.18 mg dry weight per ml by dilution; the doubling time was 40 min. During the period of growth in nonradioactive medium samples were withdrawn, the PG and PE were separated by thin-layer chromatography, and the fatty acid compositions at the 1- and 2-positions were determined as before.

The turnover of the fatty acids of PE is illustrated in Fig. 12. The glycerol of PE does not turn over in 3 hr (4.5 doublings). According to the data in Fig. 3 half the

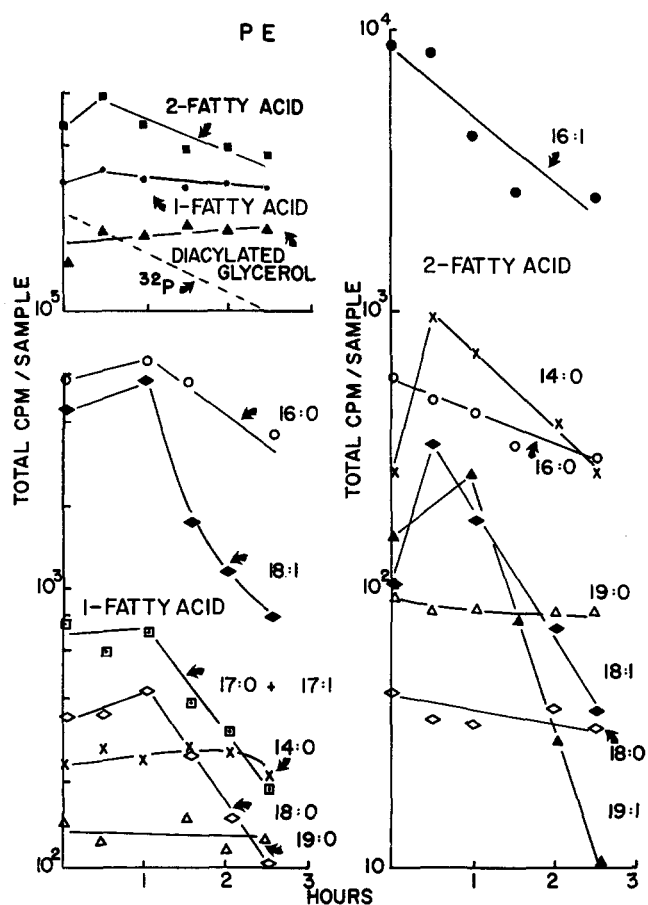


FIG. 12. Turnover of the 1- and 2-fatty acids of PE of *H. parainfluenzae*. PE was treated with phospholipase A and the fatty acids were analyzed by gas-liquid chromatography. The turnover of  $^{32}\text{P}$  in the upper left-hand corner is taken from data in Fig. 3. The turnover of the 1-fatty acids is illustrated on the left and that of the 2-fatty acids, on the right.

radioactivity in the phosphate and ethanolamine would be lost in 127 min (3.2 doublings). The fatty acids in the 1-position lose half their radioactivity in 260 min (6.6 doublings), whereas half the radioactivity of the 2-fatty acids is lost in 156 min (3.9 doublings). 16:1, 18:1, and 19:1 in the 2-position all turn over faster than the saturated acids in the same position. In the 1-position 14:0 and 19:0 do not turn over at all, while  $\text{C}_{17}$  and  $\text{C}_{18}$  acids lose half of their radioactivity in less than a doubling, after a lag period of about 2.5 doublings.

The turnover of the fatty acids of PG is illustrated in Fig. 13. The unacylated glycerol and phosphate lose half of their radioactivity in 1.2 doublings and the acylated glycerol loses half of its radioactivity in 3.0 doublings (dashed lines in Fig. 12, based on Fig. 3); total 1-fatty acids do the same in 3.5, and 2-fatty acids in 1.45 doublings. As for PE, the individual fatty acids show marked differences: 14:1, 16:0, 16:1, 17:0, 17:1, and 18:0 from the 2-position and 16:0, 17:0, and 18:1 from the 2-position lose the radioactivity faster than the aver-

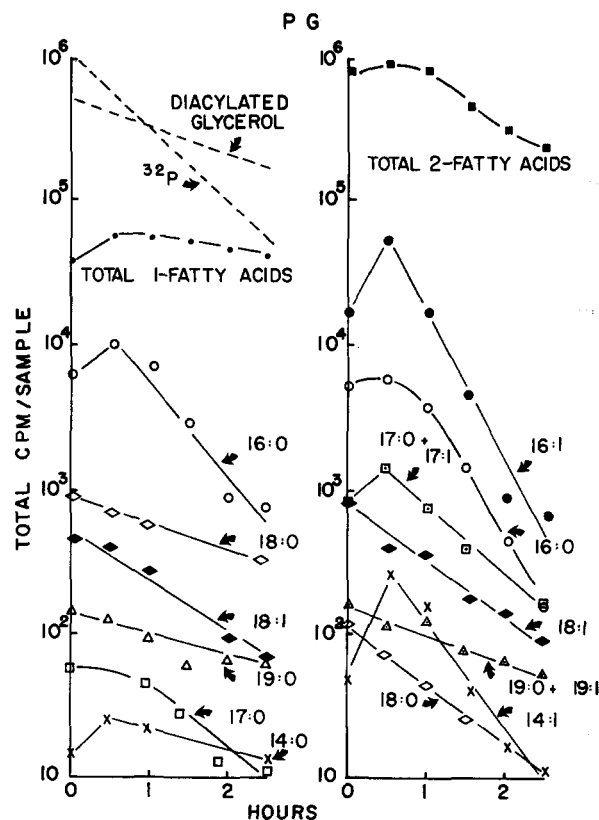


FIG. 13. Turnover of the 1- and 2-fatty acids of PG from *H. parainfluenzae*. The data are plotted as in Fig. 12. The turnover rates of the diacylated glycerol and  $^{32}\text{P}$  were taken from Fig. 3. For convenience in illustration the data for 14:0 and 17:0 in the 1-fatty acids and 14:1, 18:0, and 19:0 in the 2-position are plotted at one-tenth the actual values.

age. As in PE, 14:0 and 19:0 from the 1-position and the  $\text{C}_{19}$  acids from the 2-position turn over very slowly. In the 2-position the turnover of 16:0, 16:1, and 18:0 is relatively rapid in PG and relatively slow in PE.

## DISCUSSION

This study indicates that the metabolism of the phospholipids of the bacterial membrane is complex. The findings in an earlier study (3), that the phosphate of PG turns over more rapidly than the phosphate of PE, have been confirmed and extended. The polar portions of the phospholipid molecules turn over two to four times as rapidly as the diacyl glycerols of these molecules. The ethanolamine and phosphate of PE (Fig. 7), the unacylated glycerol and phosphate of PG (Fig. 4), and the unacylated glycerol and phosphates of CL (Fig. 10) have identical turnover rates. These data suggest that a phospholipase C hydrolyzes these lipids during growth of the organism. Phospholipase C has been demonstrated in homogenates of *Escherichia coli* (19). The different rates of hydrolysis of PE, PG, and CL as reflected in the

turnover rates could be due to different degrees of accessibility of the phospholipids to a single enzyme or could indicate that several enzymes are involved.

The proportions and total amount of phospholipids in the membrane of *H. parainfluenzae* remain constant during log phase growth in highly aerated culture media (3). The specific activities of the glycerol and phosphate increase at similar rates for GPG, GPGPG, GPE, and  $\alpha$ -GP (Fig. 11). This indicates that the rate of incorporation is proportional to the concentration of the lipids in the membrane and is not affected by turnover rate. This latter is the rate at which a radioactive component is replaced by an unlabeled component, and the value obtained is a minimum, since any recycling of radioactive material depresses the apparent turnover rate. It is unlikely, however, that the more rapid turnover of unacylated glycerol and phosphate relative to diacylated glycerol in PG or CL represents recycling of most of the unacylated glycerol and phosphate into diacyl glycerols, for the following reasons.

(a) All of the  $\alpha$ -GP returned to the pool from the turnover of the unacylated glycerol and phosphate of PG could account for only 40% of the  $\alpha$ -GP needed to maintain a constant level of diacyl glycerol in PG during log phase growth. The unacylated glycerol and phosphate from PG turnover could account for only 8% of the  $\alpha$ -GP necessary to maintain a constant level of PE.

(b) About 60% of the  $^{14}\text{C}$  of the glycerol and  $\alpha$ -GP utilized for lipid synthesis is found in the fatty acids (Table 1).  $\alpha$ -GP labeled in the glycerol and phosphate is incorporated into the lipids twice as efficiently as glycerol and phosphate. The ratio of  $^{14}\text{C}/^{32}\text{P}$  in the glycerol phosphate esters derived from all the lipids indicates that  $\alpha$ -GP is incorporated without hydrolysis. If the  $\alpha$ -GP released from the turnover of PG were incorporated via the same mechanism, at least 60% of the  $^{14}\text{C}$  would be utilized for the synthesis of fatty acids. The data of Fig. 11, which show very similar rates of incorporation of glycerol and phosphate for the lipids, suggest very strongly that any  $\alpha$ -GP released in the turnover that is recycled must be incorporated as  $\alpha$ -GP.

(c) Periodate oxidation of the PG or the GPGPG (Table 2) indicates that the unacylated glycerol incorporates more glycerol-1,3- $^{14}\text{C}$  than the diacyl glycerol does. There is no randomization of the position of  $^{14}\text{C}$  in the glycerols of the lipids when cells are grown in the presence of glycerol-1,3- $^{14}\text{C}$  (Table 2, Figs. 6 and 9).

(d) The incorporation of  $^{14}\text{C}$ , judged from the rate of increase of the specific activity of the unacylated glycerol compared to the diacylated glycerol in PG (Fig. 5), and of the ethanolamine compared to the diacylated glycerol in PE (Fig. 8), is consistent with the concept that the polar portions of these lipids turn over more rapidly than the diacyl glycerol.

The metabolism of PE is remarkable in that there is essentially no turnover of the glycerol (Figs. 3 and 7). Also, the phosphoethanolamine or serine-3- $^{14}\text{C}$  lost during the turnover of PE makes an insignificant contribution of  $^{14}\text{C}$  to the glycerol moiety, as there is no randomization of the label in 1 doubling (Fig. 9). Serine does contribute a small proportion of the  $^{14}\text{C}$  to the glycerol moieties of PG and CL after a long period of growth (Table 3). The major portion of the serine-3- $^{14}\text{C}$  is incorporated into lipids as fatty acids. The findings that serine-1- $^{14}\text{C}$  and serine-3- $^{14}\text{C}$  contribute  $^{14}\text{C}$  equally to GPS but virtually only serine-3- $^{14}\text{C}$  contributes to PE suggest that the PE is formed by decarboxylation of PS. This pathway of PE synthesis has been established in *E. coli* (20). The ethanolamine and phosphate of PE turn over at half the rate of the unacylated glycerol and phosphate of PG. In this instance the metabolism of *H. parainfluenzae* differs from *E. coli*. There is either very slight or no turnover of the phosphate of PE during growth of *E. coli* (10, 12).

Cardiolipin has a very active phosphate turnover in *Mycobacteria* (9) and a slow turnover in *E. coli* (11, 12). In *H. parainfluenzae* the phosphate and middle glycerol turn over more slowly than the phosphate and ethanolamine of PE (Fig. 10). The fact that the middle glycerol and phosphates turn over equally rapidly suggests that an enzyme exists that catalyzes the hydrolysis of CL to PGP.

In PA, the phosphate turns over rapidly but the glycerol is relatively stable (Fig. 3). The fact that the glycerol does not turn over rapidly may indicate that the synthesis of all the lipids from PA via cytidine 5'-diphosphate diglyceride as outlined by Chang and Kennedy (20) is not a total explanation of phospholipid metabolism in *H. parainfluenzae*. Studies of the fatty acid patterns of isolated lipids in *H. parainfluenzae* (7) and *Staphylococcus aureus* (21) have shown that the PA has a fatty acid composition definitely different from those of the major lipids of the bacteria. Perhaps exchange reactions between fatty acids and the polar portions of the lipids are significant features of bacterial phospholipid metabolism.

The distribution of fatty acids between the 1- and 2-positions of PE and PG in *H. parainfluenzae* is the same as in other bacteria (18). The incorporation and turnover of fatty acids from glycerol-1,3- $^{14}\text{C}$  into the fatty acids proceed with simple kinetics. As in rat liver phosphatidyl choline (22), the fatty acids esterified to the 2-position have the most active turnover. The fatty acids from PG turn over much more rapidly than those from PE. The individual acids have markedly different turnover rates, which indicates possible enzymatic specificities for fatty acid chain lengths and unsaturation. The rapid turnover of the fatty acids esterified at the 2-position suggests that

a phospholipase A is involved. The presence of phospholipase A has been suggested in homogenates of *E. coli* (19). Enzymes that rapidly hydrolyze certain fatty acids (18:1, C<sub>17</sub> acids) from the 1-position must also be present in *H. parainfluenzae*. Since there are no lysophospholipids in *H. parainfluenzae* (7), there must be an active acylase (especially for the diacyl glycerols of PE, PA, and CL), as the diacyl glycerol of these lipids is so stable. Acylases with the appropriate specificities have been detected in *E. coli* (23).

The question proposed at the outset of this work still remains unanswered. The turnover of the membrane phospholipids is not likely to be directly involved in the active transport of ions or small molecules into the cell: the turnover rate of the most active lipid (PG)—1 doubling for half the lipid phosphate and unacylated glycerol—is simply too slow for active transport. One fact is known, however; when the membrane-bound electron transport system is actively changing in composition, there is a marked decrease in the turnover of the phospholipid phosphate (3). Induction of formation of the membrane-bound electron transport system in *S. aureus* results in marked changes in the metabolism of the phospholipids (2). Perhaps some part of the lipid takes part in the formation of new bacterial membrane.

Another possibility could be that the relatively slow turnover is due to the presence of phospholipase C in the space between the membrane and the wall. This would result in a high concentration of diglyceride at the outside of the membrane. If there were diglyceride kinases or transferases at the inside of the membrane that could add phosphoethanolamine to the diglyceride, a gradient of decreasing concentration of PE and an increasing concentration of diglyceride between the inside and outside of the membrane could exist. This gradient could possibly be responsible for movement of the lipids between the inside and outside of the membrane and serve as a vehicle for transporting macromolecules from the cytoplasm to the cell wall.

These considerations are further complicated by the possibility that the membrane is a mosaic of domains with different lipid compositions and different functions. Our study of the whole bacteria gives the average

metabolism of each lipid. It is hoped that fractionation of the membrane and characterization of the enzymes involved in the phospholipid metabolism can shed light on the function of these lipids.

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