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Heterogeneity of Phospholipid Composition in the Bacterial Membrane

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Heterogeneity in the distribution or binding of the membrane phospholipids was demonstrated in the membrane fragments released from *Haemophilus parainfluenzae* by treatment with ethylenediaminetetraacetic acid (EDTA)-tris(hydroxymethyl)-aminomethane (Tris). The membrane fragments released early in the EDTA-Tris treatment contained two- to fivefold higher proportions of cardiolipin and phosphatidylglycerol and less phosphatidylethanolamine as well as phospholipids with threefold lower specific activity of the phospholipid phosphate after a short pulse of ^{32}P than were found in the residue. Heterogeneity was best demonstrated with shorter EDTA-Tris treatments and shorter periods of growth with ^{32}P. EDTA-Tris treatment appeared to progressively strip phospholipids from the cells that were synthesized at progressively later times.

The lipid composition of the limiting membrane of gram-negative bacteria is relatively simple (3). In *Haemophilus parainfluenzae*, 85% of the fatty acids are extractable with solvents and 95% of the extractable fatty acids are found esterified in phosphatidylethanolamine (PE), phosphatidyl glycerol (PG), cardiolipin (CL), phosphatidyl-serine (PS), and phosphatidic acid (PA) (7, 8). The non-extractable fatty acids are associated with the lipopolysaccharide (8). With a relatively simple lipid composition and a large number of membrane-associated activities necessary for metabolism, it might be possible to detect differences in the lipid composition or metabolism that could be correlated with different functions of the membrane.

The lipids are closely associated with the electron transport system in *H. parainfluenzae* (9). Alterations in the composition of the respiratory system coincide with changes in phospholipid metabolism. The slowing of the turnover of the phospholipid phosphate, the change in incorporation of phosphate, the changes in the proportions of PE, PG, and CL, and the increase in the total phospholipid that occur when the electron transport system is modified suggest that lipid metabolism is a necessary part of the modification of the membrane (9). Perhaps these modifications begin at discrete sites in the membrane which could somehow be isolated and studied.

Heterogeneity of membrane activity has been demonstrated in special circumstances. In the *Bacilli*, it has been possible to separate a mesosome fraction from the rest of the membrane which incorporates ^{32}P, acetate-*1*-^{14}C into lipid and ^{59}Fe preferentially (1, 2). The cytochromes and oxidase activities are very low or absent in the mesosome fraction of these cells (4). By using the detergent Sarkosyl, Trembly et al. (5) were able to isolate a membrane fraction from *B. megaterium* containing 90% of the deoxyribonucleic acid (DNA), 75% of the messenger ribonucleic acid (RNA), and 10 to 30% of the membrane. After a short pulse of ^{32}P, the lipids with the highest specific activity are found in the DNA-membrane complex.

In the preceding paper (6), it was shown that portions of the membrane of *H. parainfluenzae* can be released by suspending logarithmically growing cells in ethylenediaminetetraacetic acid (EDTA)-tris(hydroxymethyl)aminomethane (Tris). Between 15 and 20% of the respiratory pigments, the demethyl vitamin K_2, and the phospholipids, and 50% of the lipopolysaccharide are released in 10 min without affecting the viability of the cells.

In the present study, the differences in the phospholipids in the membrane fragments released by the EDTA-Tris treatment and those remaining in the residue indicated that the bacterial membrane had a heterogeneous phospholipid composition.

MATERIALS AND METHODS

The materials, growth of the bacteria, the EDTA-Tris treatment, and the lipid analysis are described in the preceding paper (6). In experiments which involved very short periods of growth with $H_3{}^{32}PO_4$, the cultures

were chilled to 0 C by adding ice, centrifuged at 4 C, and then resuspended in EDTA-Tris buffer at 37 C as before (6). Chilling the cultures at the end of the growth period had no effect on the amount released, the rate of release, or specific activity of the phospholipids released by subsequent EDTA-Tris treatment at 37 C. Phospholipids were separated by thin-layer chromatography, and components of each lipid were separated and analyzed as described (10). Glycerol phosphate esters derived from the diacyl phospholipids by mild alkaline methanolysis are abbreviated as follows: GPE, glycerol phosphorylethanolamine derived from PE; GPG, glycerol phosphorylglycerol derived from PG; GPGPG, di-glycerol phosphorylglycerol derived from cardiolipin; GP, L-α-glycerol phosphate derived from PA; and GPS, glycerol phosphorylserine derived from PS.

RESULTS

Phospholipid composition. The proportions of the phospholipids in the membrane fragments released early in EDTA-Tris treatment were quite different from the proportions in the residue (Fig. 1). The membrane fragments released into the incubation mixture during the first part of the treatment contained a 5-times higher proportion of CL and a 1.4-times higher proportion of PG than were present in the cell residue. After 3 min of treatment, the fragments released contained 5% of the total phospholipid, 15% of the CL, 6% of the PG, and 4% of the PE present in the pretreatment cells. After 9 min of treatment, the proportions of PE, PG, and CL were equal in membrane fragments and residue. Proportions of PS and PA were similar in supernatant fluid and residue through the treatment.

Phospholipid specific activity. In cells grown for short periods with $H_3{}^{32}PO_4$ prior to treatment, the specific activity of the phospholipids released by EDTA-Tris treatment was up to three times lower than that of the phospholipids remaining in the residue (Fig. 2). With a longer EDTA-Tris treatment, the difference in the specific activities of the phospholipid phosphate between the membrane fragments and the residue became less (Fig. 2, upper portion). The longer the period of growth with ^{32}P, the less the difference in specific activities between the membrane fragments and residue after a 10-min EDTA-Tris treatment (Fig. 2, lower portion). When a culture was grown with ^{32}P and ^{14}C for 25 min and then treated with EDTA-Tris, the specific activity of the lipids remaining in the pellet was consistently 1.1 to 1.4 times greater than the specific activity of the lipids released into the medium (Table 1).

Marked differences in the specific activities of each phospholipid between membrane fragments and residue were demonstrated (Fig. 3).

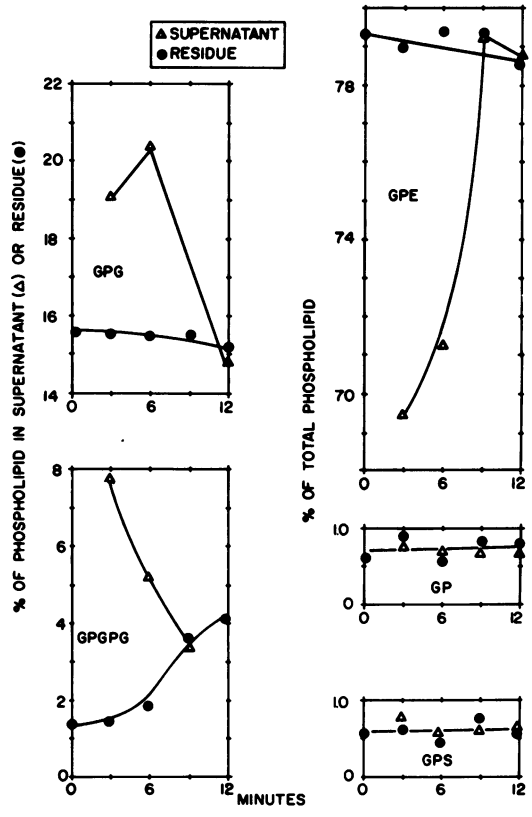

FIG. 1. *Proportions of the phospholipids in the membrane fragments and treated cell residue after EDTA-Tris treatment. Logarithmically growing Haemophilus parainfluenzae were incubated with 3.4 mc of $H_3{}^{32}PO_4$ per 1,800 ml at a density of 0.22 mg (dry weight) per ml for 5 min in a beaker, and the contents were stirred with a magnetic stirrer. The time between the addition of ^{32}P and the point at which the temperature of the incubation mixture was 0 C after the addition of ice was 5 min. Cooling occurred in 20 sec. After cooling, the culture was centrifuged at 4 C, suspended to a density of 0.9 mg (dry weight) per ml in 375 ml of 0.12 M Tris buffer (pH 8.0) containing 0.2 mM EDTA and 15 μM nicotinamide adenine dinucleotide at 37 C. Samples of 75 ml were removed periodically into $MgCl_2$ (final concentration 10 mM) and ice and then centrifuged at 23,000 × g for 10 min at 4 C. The treated cells (●) and the supernatant membrane fragments (△) were separated, the lipids were extracted and deacylated by mild alkaline methanolysis, and the glycerol phosphate esters were separated chromatographically. After localization by radioautography, the amount of each ester was determined* (7).

In the membrane fragments released in the first 3 min of EDTA-Tris treatment, the specific activities of PE, PG, and CL were 2.3, 3.1, and 7.5 times lower than the specific activities of the

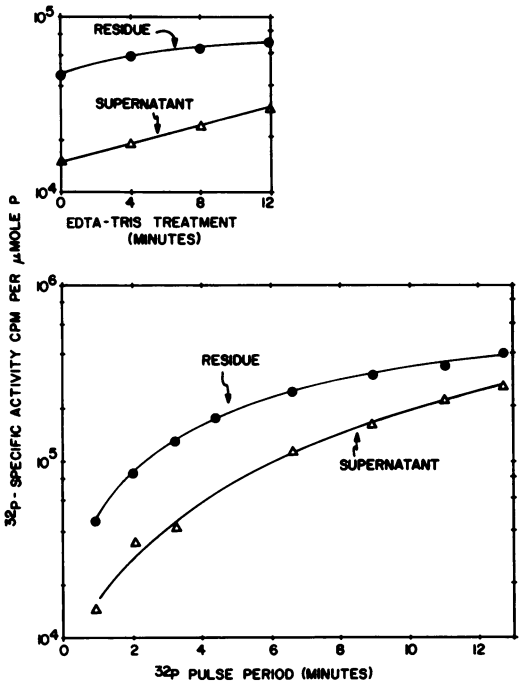

FIG. 2. *Specific activity of the phosphate of the phospholipids in the supernatant fluid and residue of H. parainfluenzae treated with EDTA-Tris. In the upper figure, cells were incubated with 1 mc of $H_3{}^{32}PO_4$ per 400 ml for 2 min. The cells were then suspended in EDTA-Tris and samples were withdrawn as in Fig. 1. In the lower figure, cells were incubated at 37 C with 2 mc $H_3{}^{32}PO_4$ in 1,300 ml of medium. Samples of 150 ml were withdrawn into ice at the times specified; they were then centrifuged, suspended in 40 ml of EDTA-Tris, and incubated for 10 min. Specific activities of the supernatant membrane fragments (△) and residue (●) were determined as in Fig. 1.*

phospholipid phosphate of these lipids remaining in the residue. These differences decreased as the length of the EDTA-Tris treatment was prolonged. The specific activities of the PS and PA were slightly higher in the membrane fragments than in the residue.

Phospholipid metabolism during the EDTA-Tris treatment. Phosphate was not significantly incorporated into the phospholipids during the EDTA-Tris treatment. Incubating cells in 200 ml of EDTA-Tris containing 100 μc of $H_3{}^{32}PO_4$ lead to the incorporation of less than 0.2% (69,000 counts/min) of the total ^{32}P into the phospholipids in the first 3 min. No further incorporation occurred in an additional 9 min of incubation.

Phospholipid catabolism during the EDTA-Tris treatment was examined by growing the cells with $H_3{}^{32}PO_4$, serine-*3*-^{14}C, and glycerol-*1,3*-^{14}C for three generations prior to treatment. The loss of radioactivity from the parts of the two major lipids in the residue was then followed during the EDTA-Tris treatment (Fig. 4). There was an exponential loss of radioactivity from the ethanolamine, glycerol, phosphate, and 1-linked fatty acid of PE, and the two glycerols and phosphate of PG. This loss paralleled the loss of lipid phosphate from the two lipids. In the course of the EDTA-Tris treatment, the specific activity of the glycerol and phosphate of GPE (7.2×10^4 counts/min of ^{32}P and 3.3×10^4 (counts/min ^{14}C per μmole of phosphate) and of both glycerols and phosphate of GPG (1.1×10^5 counts/min of ^{32}P and 3.6×10^4 counts/min of ^{14}C per μmole of phosphate) remained constant. Apparently hydrolysis and replacement from ^{14}C-labeled precursors was active, as the 2-linked fatty acid of PE lost radioactivity at a much slower rate than the rest of the molecule (Fig. 4). This was also true of the PG, as the average of the two fatty acids lost radioactivity more slowly than the rest of the lipid (Fig. 4).

Synthesis of CL during the EDTA-Tris treatment. During a 12-min EDTA-Tris treatment, 0.13 μmole (3.3%) of the PE and 0.17 μmole

TABLE 1. *Specific radioactivity in the diacyl phospholipids released from Haemophilus parainfluenzae by EDTA-Tris after a pulse of ^{14}C and ^{32}P*

Time of incubation[a] (min)	Pellet			Supernatant		
	^{32}P	^{14}C glycerol	^{14}C fatty acid	^{32}P	^{14}C glycerol	^{14}C fatty acid
	10^{-3} × CPM per μmole lipid P					
0	160	21	9.0			
3	140	20	8.5			
6	125	22	8.6	90.1	15	5.4
9	140	22	8.3	126	15	7.4
12	138	23	8.0	118	16	7.0
15	150	21	9.0	108	20	6.7

[a] Log-phase *H. parainfluenzae* was grown with 1 mc of $H_3{}^{32}PO_4$ and 100 μc of glycerol-*1-3*-^{14}C per 250 ml for 25 min, centrifuged, and resuspended in warm 0.12 M Tris (*p*H 8.0). A 25-ml zero-time sample was taken, the mixture was made to 0.2 mM with EDTA, and the rest of the samples were taken at 3-min intervals. The samples were withdrawn onto an equal volume of ice and centrifuged at 23,000 × *g* for 20 min; the supernatant fraction and the pellet were extracted. The total lipid was then deacylated by mild alkaline methanolysis, and the ^{32}P and ^{14}C in the glycerol phosphate esters and the ^{14}C in the fatty acid methyl esters were determined. Between 0.14 and 0.23 μmole of lipid phosphate was recovered in the 25-ml samples of the supernatant fluid in the 6- through 15-min samples.

(19.3%) of the PG were lost from the combined cell residue and membrane fragments (Fig. 5). In this period, 0.14 μmole of CL was synthesized, representing an increase of 88% over that originally present in the pretreatment cells. The increase in CL can account for 87% of the loss of PG. The residue lost 0.8 μmole (16%) of phospholipid, and 0.7 μmole of phospholipid was recovered in the membrane fragments for an overall recovery of 97% of the phospholipid originally present at the start of treatment. The specific activity of the CL in the residue increased 35% during the treatment, whereas the PE increased only 7% and the PG did not increase at all (Fig. 3). The increase in specific activity in the lipids in the released membrane fragments was 42% for PE, 36% for PG, and 75% for CL.

DISCUSSION

Striking heterogeneity of the phospholipid composition in the membrane of *H. parainfluenzae* has been demonstrated. Membrane fragments released from viable cells by EDTA-Tris contained phospholipids with much higher proportions of CL and PG and a lower proportion of PE (Fig. 1), as well as phospholipids with a lower phosphate specific activity (Fig. 2, 3) as compared with the phospholipids retained in the residue. The most striking differences in both composition and specific activities of the phospholipids in the membrane fragments and residue were detected very early in the EDTA-Tris treatment (Fig. 1–3). This indicated that the membrane

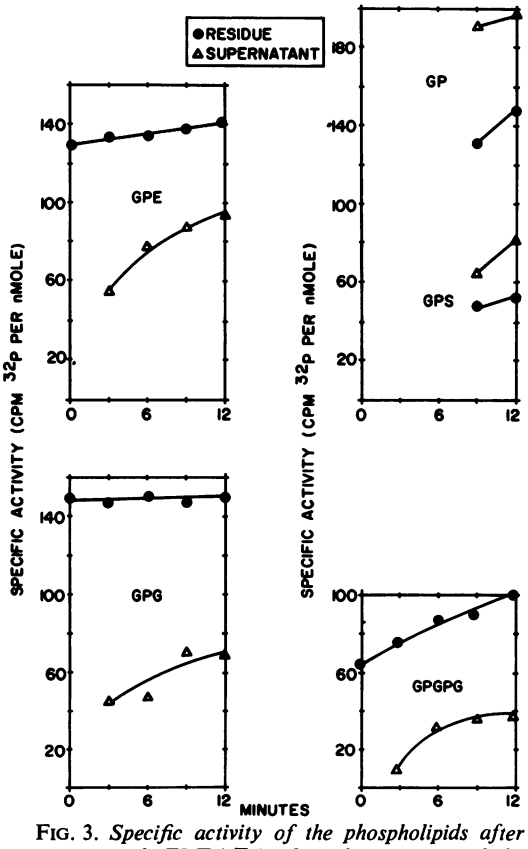

FIG. 3. *Specific activity of the phospholipids after treatment with EDTA-Tris. Specific activities of the lipids were determined from the experiment described in Fig. 1.*

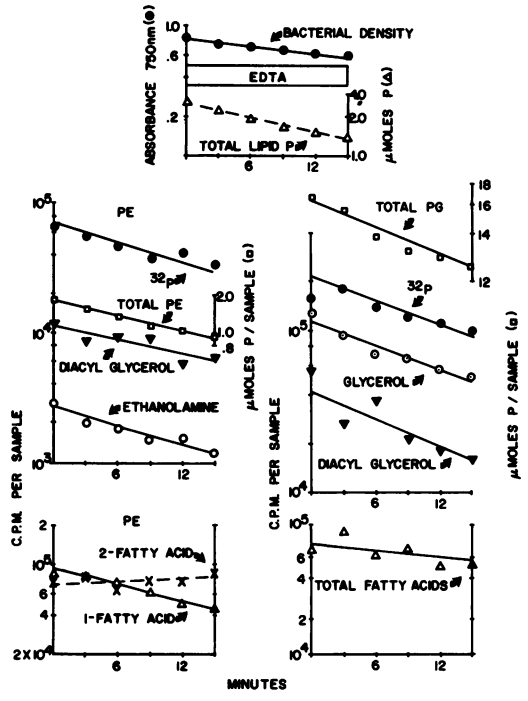

FIG. 4. *Turnover of PE and PG remaining in the residue from H. parainfluenzae during the EDTA-Tris treatment. Bacteria were grown with serine-3-^{14}C, glycerol-1,3-^{14}C, and $H_3{}^{32}PO_4$ for 2 hr and then were centrifuged and suspended in EDTA-Tris for 10 min. After 10 min, the cells were diluted into 10 volumes of warm growth medium containing 10 mM $MgCl_2$. Samples of 40 ml (150 ml after dilution) were withdrawn onto an equal volume of ice and centrifuged; the pellet was extracted, and the PE and PG were separated by thin-layer chromatography. Part of the PE was treated with phospholipase A, the 2-fatty acid and lyso-lipid were separated, and the 1-fatty acid was released after saponification and then recovered. The PE was deacylated, the GPE was hydrolyzed in acid, and the glycerol and O-phosphorylethanolamine were recovered. The PG was hydrolyzed with phospholipase D and the unacylated glycerol was recovered. The PA was deacylated, forming GP and fatty acid methyl esters, and the radioactivity was determined. The methodology has been described in detail (10).*

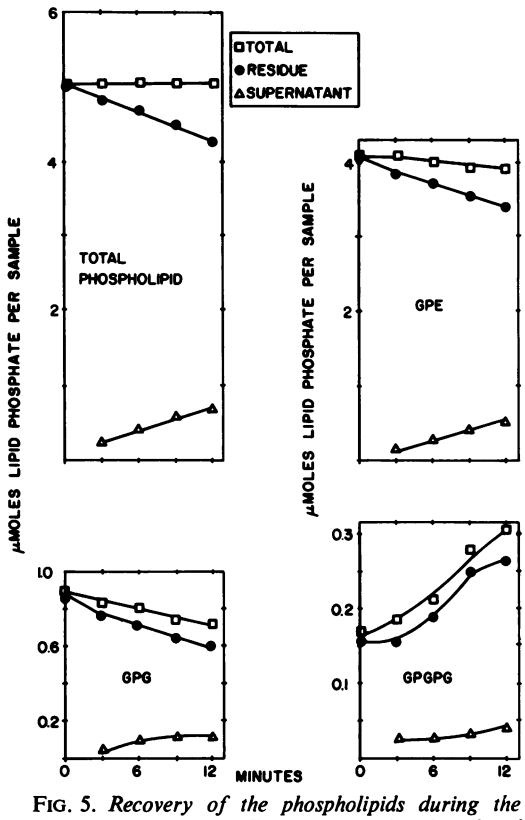

FIG. 5. *Recovery of the phospholipids during the EDTA-Tris treatment, cells were treated and analyzed as in Fig. 1. The total lipid phosphate (□) represents the sum of the lipid phosphate of the treated cells (●) and the supernatant membrane fragments (△). In this treatment the cells lost 30% of the dry weight.*

fragments released initially showed greater differences from the bulk of the membrane than did the fragments which were released later. Greater differences in specific activities between membrane fragments and residue were detected after shorter periods of growth with ^{32}P before the EDTA-Tris treatment (Fig. 2). The lower specific activities of the PE, PG, and CL released by the EDTA-Tris treatment indicated that these lipids were synthesized earlier than were these same lipids in the residue. The somewhat higher specific activities of PA and PS in the membrane fragments may reflect difficulties in the analysis due to the small amounts present.

During the EDTA-Tris treatment, the specific activity of the PG in the residue remained constant, but the specific activity of the PG in the membrane fragments increased (Fig. 3). The specific activity of PE in the membrane fragments increased more rapidly than in the residue during the EDTA-Tris treatment (Fig. 3). This suggested that the EDTA-Tris treatment progressively strips off lipids synthesized more and more recently as the treatment was continued. The lipid composition of the membrane fragments more and more closely approached that of the bulk membrane as the EDTA-Tris treatment was prolonged (Fig. 1-3).

CL synthesis occurred during the EDTA-Tris treatment (Fig. 5). The total CL increased 88%, which accounted for nearly all of the PG that was lost. The specific activity of the CL phosphates increased in both membrane fragments and residue, indicating synthesis from precursors of higher specific activity.

There was no evidence that the relatively rapid turnover of the polar portions of the phospholipids, without changes in lipid proportions that is characteristic of logarithmically growing cells (10), occurred during the EDTA-Tris treatment (Fig. 4). Rapid turnover of the 2-linked fatty acid of the PE and PG is also characteristic of logarithmically growing *H. parainfluenzae* (10).

It is possible that the EDTA-Tris treatment causes membrane release from a portion of the population that has a markedly different lipid composition and in some stage of the division cell cycle that makes it particularly susceptible. In 10 min, 50% of the lipopolysaccharide is lost (6), so that at least 50% of the population is involved after 10 min. In the first 3 min of the EDTA-Tris treatment, 15% of the total CL is lost from the cells, so that at least 15% of the cells whose lipid composition must be exclusively CL are involved. There has been no evidence that the proportions of PE, PG, and CL vary either during the growth after EDTA-Tris treatment (6) or throughout the whole growth cycle from earliest logarithmic phase to late stationary phase (Tucker and White, *unpublished data*). Consequently, the heterogeneity documented in this study, both in the time of synthesis and the proportions of the phospholipids, at least partially reflects the mosaic nature of lipid distribution in the membrane. It remains for future studies to correlate these phospholipid differences with modifications of the membrane-associated electron transport system or with other membrane activities.

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