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Metabolism of Phospholipid 2-Linked Fatty Acids During the Release of Membrane Fragments from *Haemophilus parainfluenzae* by Ethylenediaminetetraacetic Acid-Tris(hydroxymethyl)-aminomethane

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Membrane fragments containing diacyl phospholipids were released from viable cells of *Haemophilus parainfluenzae* during incubation in ethylenediaminetetraacetic acid (EDTA)- tris(hydroxymethyl)aminomethane (Tris) buffer. The phospholipids located in the part of the membrane that was released during the EDTA-Tris treatment had markedly different proportions of fatty acids than the lipids remaining in the cell residue. Very little metabolism of the 1-linked fatty acid occurred. After a short pulse with ^{14}C, the specific activity of the 1-linked fatty acid was lower in the phospholipids released than in the phospholipids of the residue, indicating an earlier time of synthesis of those lipids released in the membrane fragments. During the EDTA-Tris treatment, the 2-linked fatty acid was metabolized. This metabolism may have involved phospholipase A_2 which stimulates the synthesis of fatty acids and the transfer of acyl groups to the lysophospholipid.

Modifications in the metabolism of the membrane lipids have been shown to accompany changes in the composition of the membrane-bound electron transport system in *Haemophilus parainfluenzae* (13). In attempts to fractionate specific areas of the membrane involved in these modifications, it has been demonstrated that a portion of the membrane can be removed by incubating exponentially growing cells in tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 8.0, containing ethylenediaminetetraacetic acid (EDTA) (9). The membrane fragments that are released contain lipopolysaccharide, cytochromes, 2-demethyl vitamin K_2, and diacyl phospholipids. Further work has shown a markedly different distribution of the diacyl phospholipids released early in the EDTA-Tris treatment compared with the cell residue (10). The membrane fragments have a much larger proportion of cardiolipin (CL) and phosphatidyl glycerol (PG) with a lower proportion of phosphatidyl ethanolamine (PE) and were synthesized earlier than the lipids in the rest of the membrane. In this study the differences in the lipids of the membrane fragments have been shown to extend to the distribution and age of the fatty acids. An active metabolism of the 2-linked fatty acid occurred simultaneously with the release of membrane fragments.

MATERIALS AND METHODS

The culture conditions, media, the EDTA-Tris treatment, and the methods of lipid analysis have been described (9, 10). Cell-free preparations were prepared by sonic vibration (5). The lipids were extracted, separated by thin-layer chromatography, and hydrolyzed by phospholipase *A* from *Ancistrodom piscivorius piscivorius* (14). The lysophospholipids and the free fatty acids derived from the 2-position of the phospholipids were separated by thin-layer chromatography and were quantitatively recovered (14). The lysophospholipids were deacylated by mild alkaline methanolysis, and the glycerol phosphate esters and 1-linked fatty esters were separated (11). The 2-linked fatty acids released by venom phospholipase *A* were methylated, and the fatty acid methyl esters from both the 1- and 2-positions were analyzed by gas-

liquid chromatography (12). The specific activity of the fatty acids was determined after gas chromatographic separation on a portion of the sample in a liquid scintillation spectrometer (12, 14). Fatty acids are designated as the number of carbon atoms: the number of double bonds. Beta hydroxymyristic acid from the lipopolysaccharide (11) is designated as beta hydroxy 14:0. Glycerol phosphate esters derived from the lipids by mild alkaline methanolysis are glycerol phosphorylethanolamine (GPE) from PE and glycerol phosphorylglycerol (GPG) from PG. Lyso-PE indicates lysophosphatidyl ethanolamine.

RESULTS

Comparison of 1- and 2-linked fatty acids during EDTA-Tris treatment. When exponentially growing *H. parainfluenzae* cells are treated with EDTA-Tris, membrane fragments containing diacyl phospholipids are lost from the cells (9). In cells grown with $H_3{}^{32}PO_4$, glycerol-*1,3*-^{14}C and serine-*3*-^{14}C prior to the EDTA-Tris treatment, the radioactivities in the glycerol phosphate, ethanolamine, and 1-linked fatty acid of PE (10) as well as the glycerol, glycerol phosphate, and 1-linked fatty acid of PG were all lost from the cell residue at the same rate. During these conditions radioactivity is not lost from the 2-linked fatty acids of PE or PG during the EDTA-Tris treatment. Comparison of the specific activities of the total lipid during the EDTA-Tris treatment showed that the 1-linked fatty acid and the glycerol in the residue remained relatively constant as the 2-linked fatty acid increased slightly during the EDTA-Tris treatment (Fig. 1). The lipid that is lost from the cells is recovered in membrane fragments as diacyl phospholipids (9). In cells grown for 1 hr with ^{14}C prior to the EDTA-Tris treatment, there was little change in the specific activity in the fatty acids or glycerol of the lipid in the membrane fragments (Fig. 2).

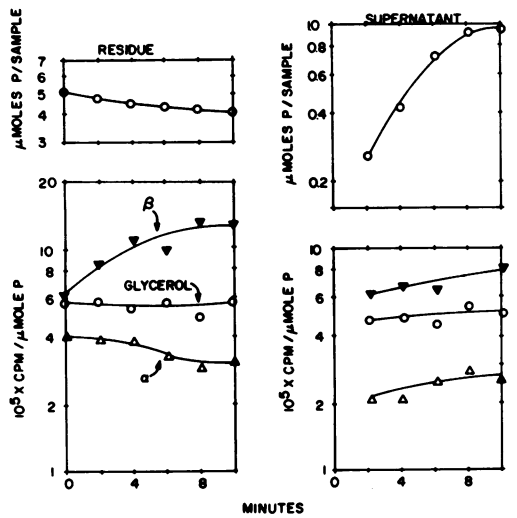

FIG. 1. *Specific activity of ^{14}C in the fatty acids and glycerol of the phospholipids of EDTA-Tris-treated H. parainfluenzae. Cells grown with 100 μc of glycerol-1,3-^{14}C per 1,800 ml for 1 hr prior to the EDTA-Tris treatment were centrifuged and suspended in 0.2 mM EDTA in 0.12 M tris buffer (pH 8.0) at a bacterial density of 0.56 mg (dry weight) per ml at 37 C. At intervals, samples were withdrawn into an equal volume of ice, they were centrifuged at 23,000 × g for 10 min, and the pellet was extracted. The PE and PG were separated by thin-layer chromatography and treated with phospholipase A; the lyso-lipid and fatty acid were separated, and the lyso-lipid was deacylated (14). Phospholipid phosphate in the lipids of the residue and supernatant, analyzed as in reference 10, are illustrated in the upper graphs. In the lower graphs, the specific activity of the β (2-linked) fatty acids (▼), the glycerol (○), and the α (1-linked) fatty acids (△) of the total phospholipids from the residue (left hand column) and the supernatant (right hand column) are illustrated.*

Specific activities of the 1- and 2-linked fatty acids. There was little change in specific activity at the 1-position in the residue in the major fatty acid 16:0 during the EDTA-Tris treatment (Fig. 3). The specific activity of 18:0 increased slightly. The lipids of the membrane fragments showed essentially the same pattern except that the specific activities were about twofold lower. There was insufficient 14:0 in the residue to make an accurate determination. The specific activities of the major 2-linked fatty acids, 16:0 and 16:1, increased slightly in the residue. The specific activities of the 2-linked fatty acid from the lipids of the membrane fragments all showed a rapid two- or threefold increase in specific activity. The specific activity of the 18:0 decreased slightly. The specific activities of the 2-linked fatty acids from the lipids of the membrane fragments all had a two- to threefold lower specific activity than the fatty acids in the residue at the end of the 12-min EDTA-Tris treatment.

There was little change in the specific activity of the 1-linked fatty acids during the EDTA-Tris treatment in the cells grown with ^{14}C for 60 min prior to EDTA-Tris treatment, and the specific activity of these fatty acids was 1.5 times higher in the residue than in the membrane fragments (Fig. 1). In cells grown with ^{14}C for 10 min prior to the EDTA-Tris treatment, there was a much greater difference in specific activities between residue and supernatant in both the 1- and 2-linked fatty acids (Fig. 2). The change in specific activity of the 2-linked fatty acid in the membrane fragments during the EDTA-Tris treatment was accentuated in the cells grown with ^{14}C for the shorter time. Shorter exposure to ^{32}P prior to EDTA-Tris treatment also accentuated the

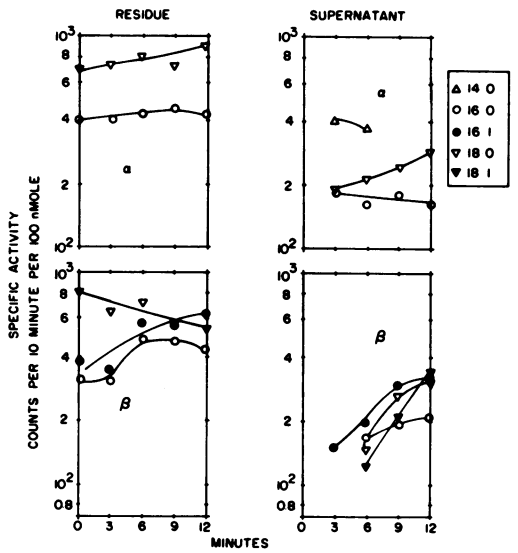

FIG. 2. *Specific activity of individual 1- and 2-linked fatty acids of EDTA-Tris treated H. parainfluenzae. Cells were grown with 200 μc of sodium acetate-1-^{14}C for 10 min in 2,000 ml, chilled by addition of ice to 7 C, centrifuged, and suspended in 500 ml of EDTA-Tris as in Fig. 1. At intervals, samples were withdrawn and centrifuged; the pellet and supernatant were extracted as in Fig. 1. Methyl esters of the fatty acids were prepared and separated on ethylene glycol succinate columns by gas chromatography (12). A portion of the eluate corresponding to each ester was recovered and counted in a scintillation spectrometer. Between 500 and 70 nmoles of each fatty acid was recovered containing between 5,000 and 700 counts per 10 min of ^{14}C above background (200 counts per 10 min). Each sample was counted 12 times and under conditions where the efficiency of counting was 53%. The specific activity of the 1-linked fatty acids (labeled as α) are illustrated in the upper curves, the specific activity of the 2-linked fatty acids (labeled as β) are shown in the lower curves. The lipids from the supernatant of the EDTA-Tris treatment are shown in the right-hand column; the lipids from the residue are in the left-hand column.*

specific activity differences between cell residue and membrane fragments (10).

During the EDTA-Tris treatment, *H. parainfluenzae* loses about 50% of the lipopolysaccharide (9). The lipid A of the lipopolysaccharide contains beta-hydroxy 14:0 (9). In cells grown with acetate 2-^{14}C for 5 min prior to the EDTA-Tris treatment, the specific activity of the beta-hydroxy 14:0 was 360 to 380 counts per min per nanomole after a 10-min treatment in both the supernatant fraction and the cell residue.

Metabolism of the 2-linked fatty acid during the EDTA-Tris treatment. The specific activities of the 16:0 and 16:1 increased and the 18:0 decreased in the cell residue during the EDTA-Tris treatment (Fig. 2). In the EDTA-Tris treatment the specific activity of the 1-linked 16:0 remained constant. The reactions most likely to account for the changes in fatty acid specific activities during the EDTA-Tris treatment involve phospholipase A_2 hydrolysis with replacement of the 2-linked fatty acid. The new 2-linked fatty acid could be synthesized from ^{14}C-labeled precursors and incorporated into the lipid via acyltransferase activity.

Phospholipase A. Preliminary experiments indicate that homogenates of *H. parainfluenzae* hydrolyze PE to lyso-PE which can be recovered (Y. Ono, *unpublished experiments*). Phospholipase A_1 and A_2 activities can be predicted from the data on fatty acid turnover in *H. parainfluenzae* during exponential growth (14). The proportions of the fatty acids in the three classes of PE, two classes of PG, phosphatidylserine, and CL differ markedly from the proportions of fatty acids in phosphatidic acid, the predicted precursor of the diacylated phospholipids (11). This strongly suggests the activity of phospholipase *A*.

Acyltransferase activity. The acyltransferase activity must be active since no lysophospholipids could be detected in cells labeled with ^{32}P (9, 11, 14). Exponentially growing *H. parainfluenzae*

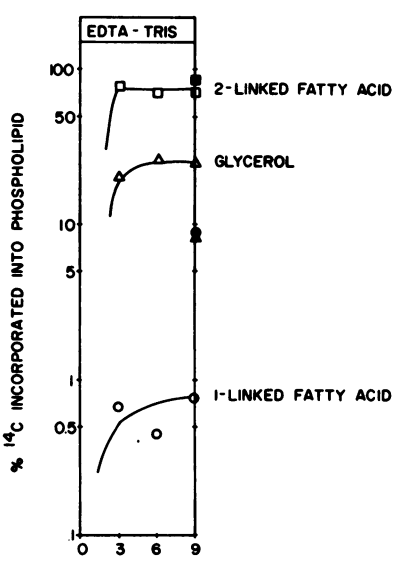

FIG. 3. *Distribution of ^{14}C in the phospholipids synthesized during the EDTA-Tris treatment. Cells were incubated in 0.12 M Tris (pH 8.0), and either acetate-1-^{14}C [3 μc per 15 ml (solid symbols)] or glycerol-1,3-^{14}C [10 μc per 50 ml (open symbols)] was added with the EDTA. Cells were recovered after centrifugation and treated as in Fig. 1.*

containing 1.8 μmole of phospholipid were treated with EDTA-Tris in the presence of 2.36 μmoles of lyso-^{32}PE (specific activity 500,000 counts per min of ^{32}P per μmole) at 37 C. The lyso-PE was dissolved in 0.47 M Na_2CO_3 and added to the EDTA-Tris buffer to a final Na_2CO_3 concentration of 4.7 mM. After incubation for 10 min, $MgCl_2$ (20 mM final concentration) and ice were added, and the culture was centrifuged. The lipids were extracted from the cell residue and separated by thin-layer chromatography. A total of 59% of the exogenous lyso-^{32}PE was isolated from the cells of which 12% was PE and 82% was lyso-PE. In a similar experiment, cells were incubated with 2.64 μmoles of *H. parainfluenzae* fatty acids (75,000 counts per min of ^{14}C per μmole dissolved in Na_2CO_3). After 10 min, 19% of the fatty acids were found in the cells of which 3% were in lyso-PE, 9% were in PE, and 89% were free fatty acids. Lyso-PE and PE containing ^{14}C from the added fatty acids represented 0.47 and 1.6% of the total phospholipid of the cells.

Fatty acid recovery. If phospholipase A_2 and acyltransferase were responsible for the specific activity changes of the 2-linked fatty acids illustrated in Fig. 1 and 2, then the free fatty acids might be recovered. Free fatty acids were not detected in the lipid mixture during the EDTA-Tris treatment. Growth of *H. parainfluenzae* for 20 divisions in acetate-*1*-^{14}*C* resulted in the incorporation of 17% of the radioactivity in the growth medium into the fatty acids of the phospholipids. Less than 0.05% of the ^{14}C from acetate is incorporated into the glycerol portion of the lipids (Y. Ono, *unpublished data*). *H. parainfluenzae* was grown in sodium acetate-*1*-^{14}*C* for 2 hr and then incubated in EDTA-Tris for 12 min. At 2-min intervals, samples were removed and centrifuged; lipids were extracted from the pellet and the supernatant fluid. The chloroform was removed in a stream of nitrogen, and the lipids were dissolved in petroleum ether. Partitioning against 4% (w/v) Na_2CO_3 removed no ^{14}C, indicating that no free fatty acids were formed in either the supernatant or the cells.

Free fatty acids are not found in the lipids of *H. parainfluenzae* during normal growth (11, 12, 14). The fact that no ^{14}C fatty acids were recovered from the lipid extracts during the EDTA-Tris treatment indicated that catabolism to nonlipid products might have occurred. There was no loss of ^{14}C from labeled fatty acids of fatty acid methyl esters added to the cells during the EDTA-Tris treatment. The fatty acids were dissolved in 4.7 mM Na_2CO_3 and the methyl esters were dissolved in 0.01% (v/v) Cutscum (Fisher Chemical Co.), an isoactyl phenoxy-polyoxyethylene ethanol, in the presence of the cells.

Incorporation of ^{14}C into lipids during the EDTA-Tris treatment. Another requirement for the phospholipase A_2-acyltransferase cycle is the synthesis of fatty acids from ^{14}C-labeled precursors to be added to the 2-position of the lipids. Addition of either sodium acetate-*1*-^{14}*C* or glycerol-*1,3*-^{14}*C* to the cells during the EDTA-Tris treatment resulted in incorporation of ^{14}C into cellular lipid. Less than 0.01% of the acetate-*1*-^{14}*C* added to the preparation was recovered in the lipids compared with 2.9% of the glycerol-*1,3*-^{14}*C*. All of the incorporation of ^{14}C occurred in the first 3 min of the EDTA-Tris treatment. The lipids from each experiment were treated with venom phospholipase A_2, and the distribution of ^{14}C between glycerol and the 1- and 2-linked fatty acids was determined (Fig. 3). About 74% of the ^{14}C from the glycerol-*1,3*-^{14}*C* and 85% of the ^{14}C from acetate-*1*-^{14}*C* were incor-

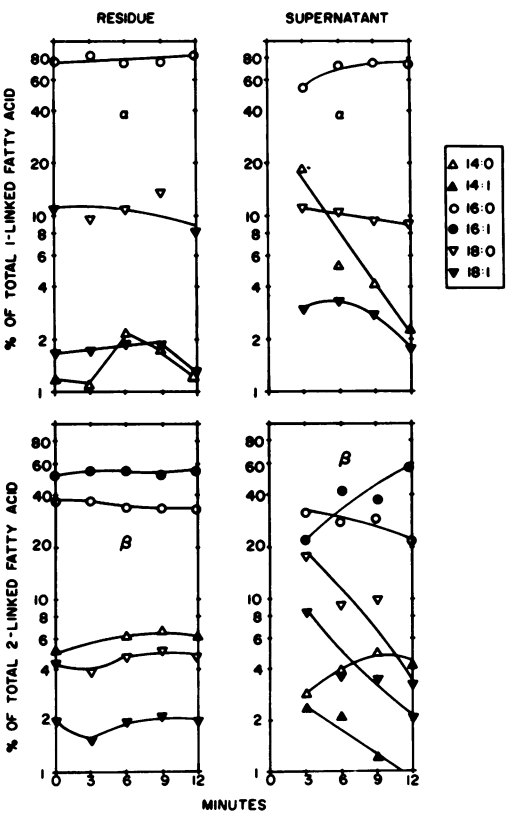

FIG. 4. *Proportions of the fatty acids in the phospholipids of the cell residue and supernatant membrane fragments during the EDTA-Tris treatment. Fatty acids were analyzed as in Fig. 2.*

porated into the 2-linked fatty acids. There was no redistribution of ^{14}C after the first 3 min of the EDTA-Tris treatment. Acetate was also less efficiently incorporated into the 2-demethyl vitamin K_2 (0.0003% of added ^{14}C per generation) than glycerol (0.12% of added ^{14}C per generation).

Proportions of the fatty acids in the cell residue and membrane fragments. There was a marked difference in the fatty acid composition of the lipids in the membrane fragments released by EDTA-Tris and those remaining in the residue (Fig. 4). This difference was most striking earlier in the EDTA-Tris treatment. At the 1-position, the supernatant lipids contained higher proportions of 14:0 and less 16:0 than the residue. At the 2-position, the supernatant lipids contained more 14:1, 18:0, and 18:1 but less 16:1 than the residue. When the EDTA-Tris treatment was continued the fatty acid composition of the membrane fragments approached that of the cell residue.

DISCUSSION

During the EDTA-Tris treatment, membrane fragments containing diacyl phospholipids are released from the cells (9, 10). When the lipids of these cells were labeled with ^{32}P and ^{14}C just prior to the EDTA-Tris treatment, the radioactivity of the 1-linked fatty acid, the glycerols, the phosphate, and the ethanolamine in the lipids remaining in the residue all decreased. Radioactivity in the 2-linked fatty acids of PE and PG did not decrease during the EDTA-Tris treatment (Fig. 1–2). These data can be accounted for if a cycle involving cleavage of the 2-linked fatty acid by phospholipase A_2, catabolism of the fatty acid released, synthesis of new fatty acids from ^{14}C containing precursors, and acyltransferase activities as illustrated in Fig. 5 were active.

Phospholipase *A* has been demonstrated in cell-free homogenates. The activity of both phospholipase A_1 and A_2 are necessary to account for the turnover of the 1- and 2-linked fatty acids in *H. parainfluenzae* during exponential growth (14). Phospholipase *A* activity has been detected in a number of bacteria (1–4, 7, 8). No free fatty acids or fatty acid containing neutral lipids can be detected in *H. parainfluenzae* during the EDTA-Tris treatment. Consequently, a catabolic reaction involving the 2-linked fatty acid is postulated (reaction 2, Fig. 5). ^{14}C-labeled fatty acids or methyl esters added outside the cells during the EDTA-Tris treatment were not degraded. Possibly these fatty acids did not reach the proper compartment of the cells. Reaction 2

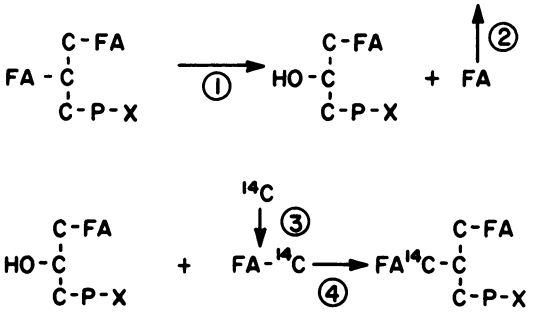

FIG. 5. *Postulated cycle of phospholipase A and acyltransferase activity.*

has not been established as yet. Reaction 3, the synthesis of fatty acids from ^{14}C-labeled precursors during the EDTA-Tris treatment, can be demonstrated (Fig. 3). The incorporation from glycerol-*1,3*-^{14}C occurred within the first 3 min and then stopped. Perhaps the permeation mechanism for exogenous substrates is damaged by the EDTA-Tris treatment such that incorporation stops. The endogenous respiration of *H. parainfluenzae* is lost within 3 min during the EDTA-Tris treatment (9). Reaction 4, the acyltransferase activity, can be readily demonstrated. Lyso-PE was acylated by endogenous fatty acids and even a small portion of added fatty acids was incorporated into phospholipids during the EDTA-Tris treatment. Lyso-phospholipid acyltransferase activity has been demonstrated in *Escherichia coli* (2, 6). Since the composition of the fatty acids of each of the phospholipids differed widely and was markedly different from the fatty acid composition of the supposed precursor phosphatidic acid, the widespread activity of phospholipase *A* and acyltransferase activity during exponential growth has been postulated previously (11, 14).

The diacyl phospholipids released from the cells early in the EDTA-Tris treatment have much higher proportions of CL and PG and were synthesized much earlier in time than the rest of the membrane (10). In this study the phospholipids released early in the EDTA-Tris treatment had a markedly different fatty acid composition than the rest of the phospholipids (Fig. 4). The specific activities of the 1-linked fatty acids [which do not undergo metabolism during the EDTA-Tris treatment (Fig. 1–2)] were lower in the membrane fragment lipids, suggesting their incorporation occurred earlier in time than in the rest of the lipids. As with the specific activity of the phosphate and the proportions of the lipids, the fatty acid composition of the membrane fragments approached the composition of the

rest of the cell membrane as the incubation in EDTA-Tris continued.

ACKNOWLEDGMENT

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