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Release of Membrane Components from Viable *Haemophilus parainfluenzae* by Ethylenediaminetetraacetic Acid-Tris(hydroxymethyl)-aminomethane

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Logarithmically growing *Haemophilus parainfluenzae* lost 15 to 20% of the phospholipids, demethyl vitamin K_2, cytochrome *b*, and cytochrome *c*, and 50% of the lipopolysaccharide when incubated in ethylenediaminetetraacetic acid (EDTA)-tris-(hydroxymethyl)aminomethane (Tris) for 10 min. This loss of membrane components occurred without loss in viability, and the lost components were recovered as membrane fragments in the surrounding buffer. The phospholipids recovered in the membrane fragments had a slightly lower specific activity than the phospholipids in the residue. Lysis of a portion of the cells could not account for the release of membrane components, as the cells lost neither glucose-6-phosphate dehydrogenase activity nor deoxyribonucleic acid. The treated cells were osmotically stable and contained the same proportions of the individual phospholipids as pretreatment cells. Prolongation of the EDTA-Tris treatment did not induce further loss of phospholipid or demethyl vitamin K_2, but caused a decrease in viability. If the cells were returned to the growth medium after 10 min, the cells immediately resumed growth at the pretreatment rate. During growth in the recovery period, the phospholipids increased logarithmically in the pretreatment proportions, although there was a marked decrease in the turnover and a shift from the use of extracellular lipid precursors to the use of intracellular pools of precursors.

The treatment of suspensions of gram-negative bacteria in tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0) with ethylenediaminetetraacetic acid (EDTA) renders the bacteria susceptible to lysozyme (4, 23), makes the cells permeable to charged molecules without affecting some permease systems (13, 15, 16, 30), makes resistant organisms sensitive to antibiotics (26, 31), and causes the release of lipopolysaccharide-phospholipid complexes from the cell walls (14, 17). These changes occur with very little loss of viability, and the cells return to the pretreatment growth rate immediately after they are diluted into media containing Mg^{2+}.

The essential action of the EDTA-Tris treatment may be a result of the chelation of Mg^{2+}. Mg^{2+} may cross-link the units in the cell wall complex (1). The EDTA-Tris treatment results in the loss of one-third to two-thirds of the intracellular Mg^{2+} in logarithmically growing cells (18). *Pseudomonas aeruginosa* is lysed in EDTA (1, 8, 9), but, if the organism is grown with Mg^{2+} limitation, the walls are resistant to lysis by EDTA (2). The detergent activity of EDTA is no longer considered to be a factor in the disruptive process (9).

Phospholipids are the principal lipids in gram-negative bacteria. Loss of phospholipids may be responsible for the lysozyme sensitivity after EDTA treatment (4). The lipopolysaccharide (LPS) that is lost from the cells in the EDTA-Tris treatment is associated with phospholipid (17). Isolated LPS forms micelles with phospholipids (25, 32), and the formation of these micelles is an absolute requirement for the activity of the uridine diphosphate (UDP)-glucose and UDP-galactose LPS transferase activities (24). These enzymes synthesize part of the core of the LPS and exhibit a phospholipid requirement that is specific for a negatively charged phospholipid containing unsaturated or cyclopropane fatty acids (24). A lysine-requiring auxotroph of *Escherichia coli*, when grown with limiting lysine, secretes LPS-phospholipid spherules which have

an appearance similar to the complexes formed from isolated LPS and phosphatidylethanolamine (12). This mutant appears to lose the outer layer of its membrane-wall tricomplex (12).

Since the metabolism of phospholipids in the membrane of *Haemophilus parainfluenzae* has been studied extensively (38, 39, 41, 42), the EDTA-Tris treatment that induces permeability changes in the membranes of other gram-negative bacteria might provide insight into the involvement of the membrane lipids in permeability. The present study documents that the removal of up to 20% of the phospholipids, demethyl vitamin K_2 isoprenologues, cytochromes *b* and *c*, and 50% of the lipopolysaccharide in a 10-min EDTA-Tris treatment does not affect the viability or the ability to initiate growth immediately on return to growth medium in these organisms. The loss of membrane components has profound effects on the phospholipid metabolism, but there is no release from the tight controls over the total amount or proportions of the phospholipids in the membrane. In the accompanying paper (29), the EDTA-Tris treatment was used to document heterogeneity of phospholipid composition in the membrane.

MATERIALS AND METHODS

Materials. The best grade of commercially available reagents was used without further treatment unless specifically indicated. Cutscum (Fisher Scientific Co., St. Louis, Mo.) is isooctylphenoxypolyoxyethylene ethanol, a neutral detergent. Labeled compounds were purchased as described previously (42).

Growth of H. parainfluenzae. *H. parainfluenzae* strain "Boss No. 7" was the gift of G. Leidy. The medium contained 2% proteose peptone, 0.5% yeast extract (Difco), 102 mM NaCl, 9 mM KNO_3, 50 mM sodium-gluconate, 0.12 mM $Na_2S_2O_4$, and 20 mM Tris at *p*H 7.6. The medium was boiled, filtered, and autoclaved at 120 C for 25 min in 2.5-liter low-form Erlenmeyer flasks containing 1.7 liters of medium. After cooling, filter-sterilized nicotinamide adenine dinucleotide (NAD) was added to a final concentration of 1.5 μM. Cells were preserved in 15% (v/v) glycerol in growth medium at −60 C. The flasks were inoculated with 5 ml of a 10-hr culture of the organisms and shaken as described (37). Since this organism requires NAD, contamination of the culture was checked by incubating the cells in the absence of NAD. Dry weight was determined from the absorbance at 750 nm (35) except during treatment and recovery periods, when washed pellets from 50-ml portions were dried to constant weight at 40 C in a vacuum oven (36).

EDTA-Tris treatment. Logarithimically growing cells were treated with EDTA-Tris by the methods described by Leive (15). Cells were grown with agitation to a density of 0.22 mg (dry weight) per ml, centrifuged at 23,000 × *g* for 10 min, and resuspended in 0.12 M Tris buffer (*p*H 8.0) containing 15 μM NAD at a bacterial density of 0.86 mg (dry weight) per ml. NAD rapidly leaks out of this organism. EDTA was then added to a final concentration of 0.2 mM. Centrifugation and resuspension were performed at 37 C. To terminate the reactions, sufficient $MgCl_2$ was added to make the final concentration 10 mM.

Lipid analysis. Samples of 50 to 200 ml were withdrawn onto an equal volume of ice and centrifuged at 23,000 × *g* for 10 min at 4 C. The supernatant fluid and resuspended pellet were then extracted. The methods of lipid extraction, chromatographic separation, recovery, methanolysis and enzymatic hydrolysis, analysis for phosphate, amino nitrogen, and carbohydrate, and the determination of radioactivity were described in detail (38, 40–42). The following abbreviations will be used for the glycerol phosphate esters derived from the lipids by mild alkaline methanolysis: glycerol phosphorylethanolamine (GPE) derived from phosphatidylethanolamine (PE), glycerol phosphorylglycerol (GPG) derived from phosphatidylglycerol (PG), di-glycerol phosphorylglycerol (GPGPG) derived from cardiolipin (CL), L-α-glycerol phosphate (GP) derived from phosphatidic acid (PA), and glycerol phosphorylserine (GPS) derived from phosphatidylserine (PS).

Fatty acids were separated by gas chromatography (39) and are indicated as the number of carbon atoms and the number of double bonds. β-Hydroxy myristic acid is indicated as 14:0, OH.

Characterization of the LPS. LPS was isolated from *H. parainfluenzae* by phenol extraction at 68 C (33). The LPS was then dialyzed, treated with ribonuclease, and dialyzed again (17). Lipids were then extracted (11). The LPS was collected on glass wool and hydrolyzed in 0.05 N HCl for 30 min at 95 C, and the lipid A was extracted with chloroform (3). The lipid A was hydrolyzed in 6 N HCl for 6 hr at 95 C, and the fatty acids were extracted and then methylated for analysis by gas chromatography (39). The aqueous phase was dried several times with nitrogen to remove the HCl, and the carbohydrate was chromatographed on paper with isopropanol-acetic acid-water (3:1:1, v/v). The carbohydrate was assayed with anthrone and ninhydrin (40). After the lipid A was removed, the LPS was divided into several portions. One portion was hydrolyzed in 4 N H_2SO_4 for 2 hr at 95 C, and the sugars were tentatively identified after paper chromatography in *n*-butyl alcohol-pyridine-water (9:5:4, v/v). A second portion of the LPS was hydrolyzed in 0.2 N H_2SO_4 for 15 min at 95 C, and colitose and 3-deoxyoctulosonate (KDO) were separated on a Dowex column (6). The deoxy sugars were assayed with thiobarbituric acid (5). The analyses of sugars were performed by E. C. Heath, Department of Physiological Chemistry, Johns Hopkins University, Baltimore, Md. A third portion of the LPS was hydrolyzed in 1 N HCl for 6 hr at 95 C; the HCl was removed in a stream of nitrogen, and the hydrolysate was chromatographed on Dowex-50 as described by Grollman and Osborn (10). Each fraction was analyzed for total phosphate, inorganic phosphate, and amino nitrogen.

Analysis of the electron transport system. The oxygen utilization of the respiratory system was

measured polarographically (36), and the cytochromes were measured by difference spectroscopy as described in previous reports (34, 37). Cytochrome *b* and *c* absorption increments were corrected for overlap by using an expression which gives values for the cytochromes that are proportional to the protoheme and heme *c* content of the cells (28). The absorption increment at 560 nm also includes up to 20% cytochrome oxidase *o* (28, 34). The quinone, 2-demethyl vitamin K_2 (DMK_2), was extracted and assayed spectrophotometrically (35).

Nucleic acids. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were assayed after precipitation with cold 10% (w/v) trichloroacetic acid and hydrolysis in 5% trichloroacetic acid at 90 C for 15 min (27). DNA was assayed colorimetrically with diphenylamine (7) and total pentose was assayed with orcinol (20) with deoxyribose as standard.

Enzyme activities. Dehydrogenases that were found in the supernatant fluid after centrifugation of cell-free preparations were assayed with pyridine nucleotide spectrophotometrically (36).

RESULTS

When *H. parainfluenzae* in the log phase of growth was centrifuged and resuspended in EDTA-Tris at 37 C, there was a loss of bacterial density and of phospholipid from the bacteria. There was no change in absorbance when the EDTA was deleted. When the treatment in EDTA-Tris was terminated with 10 mM $MgCl_2$ within 10 min and the cells were diluted 10-fold into warm medium, there was an immediate resumption of growth at the pretreatment rate (Fig. 1). At the end of the EDTA-Tris treatment, the phospholipid content of the cells decreased 20% (1.5 to 1.2 μmoles of lipid phosphate per 150-ml sample). The relative concentrations of PE, PG, and PS remained constant during the loss of lipid. There was a rapid preferential loss of CL followed by an accumulation during the treatment period. During the recovery period all the phospholipids but PS were formed in their pretreatment proportions (Fig. 1). The concentrations of the various phospholipid constituents present in the cells are shown in Fig. 2.

Conditions for the loss of lipid. In *E. coli* both Tris and EDTA must be present for maximal loss of phospholipids (16). When *H. parainfluenzae* was grown for 4 hr with 50 μc of glycerol-*1-3-*^{14}C and 500 μc of $H_3{}^{32}PO_4$ in 200 ml of medium, 18% of the ^{32}P and ^{14}C from the glycerol and fatty acids was lost in 15 min from the cells in 0.12 M Tris (*p*H 8.0) containing 0.2 mM EDTA. No radioactivity was lost in 30 min from these cells when suspended in 0.12 M Tris or phosphate buffer at *p*H 8.0. Cells treated with EDTA in phosphate buffer lost no lipid phosphate in 10 min. Cells suspended in Tris buffer containing 0.5% (v/v) Cutscum detergent lost 42% of the phospholipid in 20 min, but did not recover when transferred to the growth medium. *H. parainfluenzae* grows normally in 0.1% Cutscum.

Prolongation of the EDTA-Tris treatment does not result in greater loss of phospholipid but causes a decrease in viability (Fig. 3).

Recovery of the phospholipid. Cells were grown in the growth medium with labeled glycerol and phosphate for 25 min before the EDTA-Tris treatment. During a 12-min treatment, samples were removed at 3-min intervals and centrifuged; the pellet and supernatant fraction were extracted. The sum of the lipid analyses from the supernatant fraction plus the pellet equalled between 99 and 102% of the lipid phosphate, ^{32}P, and ^{14}C in the fatty acids, and glycerol in the zero-time sample.

LPS release. The LPS of *H. parainfluenzae* was isolated by phenol extraction. This treatment removed 95% of the 14:0, OH acid from the cells. The lipid A of the LPS contained all of the 14:0, OH. A single carbohydrate which co-chromatographed with glucosamine was found in the lipid A hydrolysate. Carbohydrate was detected by ninhydrin and periodate reactivity. The molar ratios of amino nitrogen measured with ninhydrin, to carbohydrate measured with anthrone, to 14:0, OH measured by gas chromatography was 1:1:1 in the lipid A. No colitose and a trace of KDO were detected in the LPS. After strong acid hydrolysis, sugars with the chromatographic mobility of glucose, galactose, mannose, ribose, and rhamnose were detected in the LPS. Analysis for *O*-phosphorylethanolamine or other phosphate esters in the LPS indicated that only inorganic phosphate was released by hydrolysis. No amino nitrogen-containing phosphate esters were detected in the hydrolysate.

In a 12-min EDTA-Tris treatment, the bacteria lost 50% of the total 14:0, OH fatty acid present in the cells. In the same period, the carbohydrate in the medium increased 3.2-fold, suggesting LPS fragments were released from the cells. These LPS fragments were much smaller than those released from *E. coli* (17). Only 18% of the 16:1 fatty acid was lost from the cells in this period (Fig. 4). The rate of 14:0, OH acid loss from the lipopolysaccharide was 2.5 times the rate of loss of 16:1 from the phospholipids. Over 99% of the 16:1 is in the phospholipids, 85% of which is at the 2-position (39, 42).

Effect on the electron transport system. The phospholipids, DMK_2, and electron transport system are a part of the same membrane complex (38), and changes in the composition of the electron transport system are reflected in the amount of DMK_2 (35) and in the metabolism of the phospholipids (41). In a 15-min EDTA-Tris treatment, the cells lost 20% of the phospholipid,

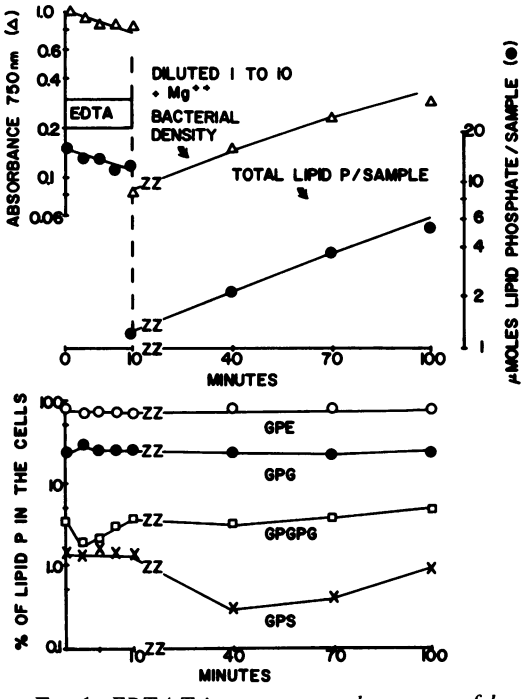

FIG. 1. *EDTA-Tris treatment and recovery of log-phase Haemophilus parainfluenzae. Growing cells were centrifuged and suspended in 0.2 mM EDTA in 0.12 M Tris buffer (pH 8.0) at a bacterial density of 0.56 mg (dry weight) per ml at 37 C. After 10 min, the cells were diluted into 10 volumes of warm growth medium containing 10 mM $MgCl_2$. Samples of 40 ml were withdrawn into an equal volume of ice and centrifuged at 23,000 × g for 10 min, and the pellet was extracted. Samples of 150 ml were used after the EDTA-Tris treatment. The lipids were recovered, deacylated by mild alkaline methanolysis and separated chromatographically; the phosphate content was determined. Bacterial density was measured as absorbance at 750 nm. GPE, GPG, GPGPG, and GPS indicate the glycerol phosphate esters derived from PE, PG, CL, and PS by mild alkaline methanolysis.*

16% of the DMK_2, and 17% of the dry weight. The DMK_2 and phospholipid were lost in about 10 min (Fig. 5). During the EDTA-Tris treatment there was a loss of about 20% of the cytochrome *b* (plus cytochrome oxidase *o*) and the total cytochrome *c* (Fig. 6). This paralleled the loss of DMK_2. The amount of formate-reducible cytochrome *c* decreased by 36% in 9 min. The fact that enzymatic reducibility was lost faster than chemically reducible cytochrome *c* indicates damage to the electron transport system. Apparently cytochrome oxidase a_2 was not lost, although the small amount present makes small decreases difficult to detect. There was no loss of oxidative activity measured as the rate of oxygen utilization in the presence of formate. The endogenous respiration of *H. parainfluenzae* can be stopped by washing the cells in phosphate buffer (34). Endogenous respiration of unwashed cells was lost in 3 min during the EDTA-Tris treatment.

Loss of nucleic acid. Nucleic acids and polysaccharides from the cells and supernatant fraction were precipitated with 10% trichloroacetic acid at 4 C for 3 hr, and the precipitate was hydrolyzed. During the 10-min EDTA-Tris treatment, the bacterial dry weight decreased 9.4% and the total pentose decreased by 20%. The total orcinol-positive components accounted for 34% of the dry weight at the start of the experiment and includes the ribose detected in the lipopolysaccharide. The deoxyribose accounted for 0.45% of the bacterial dry weight. Essentially no DNA was lost from the bacteria during the EDTA-Tris treatment (less than 10 μg of the 405 μg of deoxyribose in the first sample), and none could be detected in the supernatant fraction.

Effect on enzyme activities. Some enzymatic activities were found in the supernatant portion after high-speed centrifugation of bacteria disrupted with sonic vibration (36). During a 10-min EDTA-Tris treatment, 6-phosphogluconate dehydrogenase activity and glyceraldehyde-3-phosphate dehydrogenase activity leaked out and were detected in the supernatant portion after centrifu-

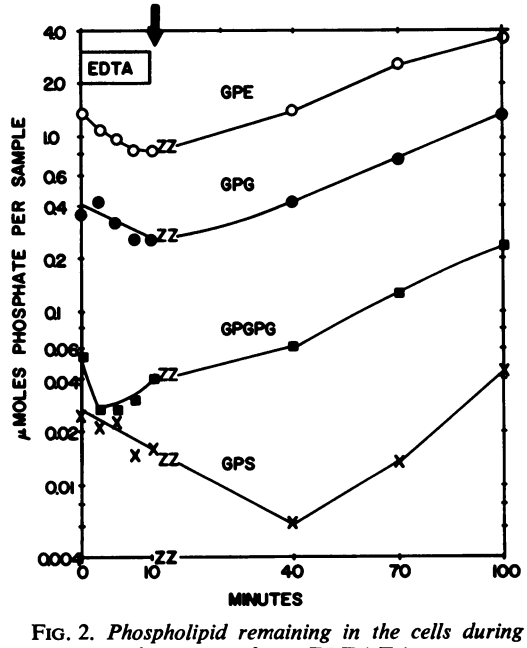

FIG. 2. *Phospholipid remaining in the cells during treatment and recovery from EDTA-Tris treatment. The experiment was performed as in Fig. 1.*

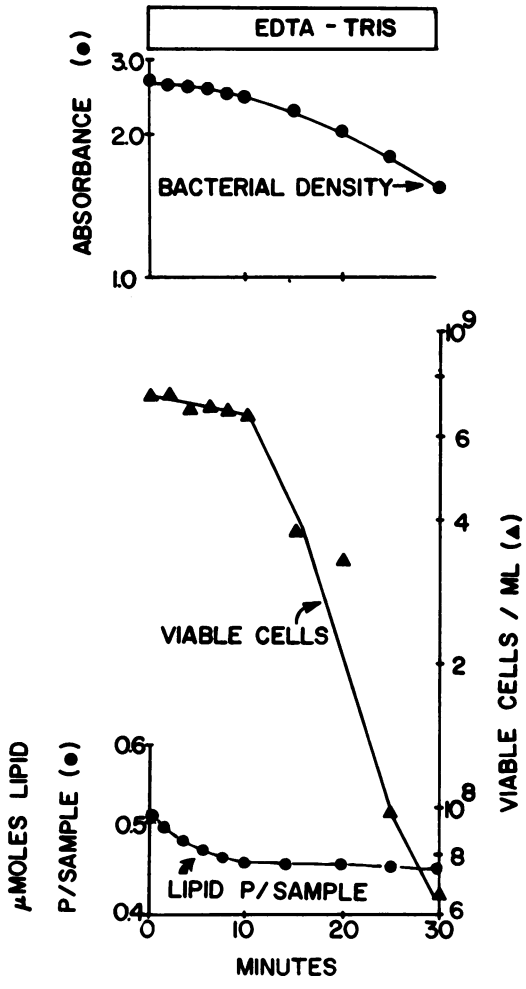

FIG. 3. *Effect of EDTA-Tris treatment on the viability and rate of phospholipid loss in Haemophilus parainfluenzae. The experiment was performed as in Fig. 1. Samples of 6 ml were removed, and 5 ml was used for phospholipid extraction. Viable counts were made from the samples at the indicated time intervals by diluting in growth medium plus 10 mM $MgCl_2$. Triplicate 1-ml samples of the 10^{-6} dilution were used for the inoculation of pour plates of the growth medium plus 1.5% agar.*

gation (Table 1). Glucose-6-phosphate dehydrogenase activity apparently did not leak out of the cells during the EDTA-Tris treatment.

Osmotic fragility. Osmotically sensitive spheroplasts of *H. parainfluenzae* can be prepared by growth in the presence of lactose and penicillin (43). The cells were not osmotically sensitive after the EDTA-Tris treatment. No ghosts were detectable by phase-contrast microscopy. The cells appeared smaller but retained the coccobacillary form typical of log-phase cells. The cells after the EDTA-Tris treatment could be centrifuged and resuspended in distilled water without a change in bacterial density measured as the the absorbance at 750 nm.

Nature of the material lost from the cells. Cells were recovered by centrifugation at 23,000 × *g* for 10 min after a 10-min EDTA-Tris treatment in which 10% of the dry weight was lost. The supernatant portion was then centrifuged at 90,000 × *g* for 2 hr. Portions of the supernatant fluid were removed sequentially and the lipids were extracted. The top 4 ml contained 3.5%, the central 22.0 ml contained 18.6%, the bottom 4 ml contained 48.1%, and the pellet contained 29.8% of the phospholipid. The pellet contained protein, carbohydrate, and DMK_2, in addition to the

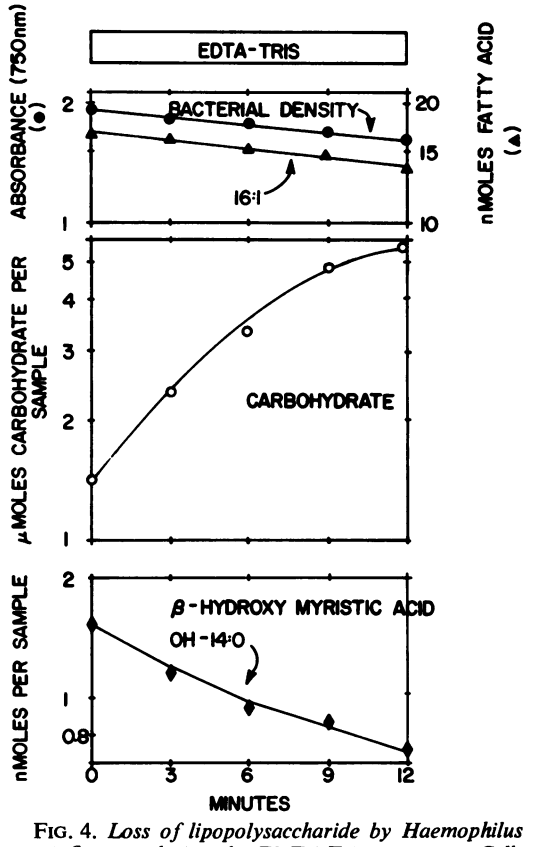

FIG. 4. *Loss of lipopolysaccharide by Haemophilus parainfluenzae during the EDTA-Tris treatment. Cells were treated with EDTA-Tris as in Fig. 1, and the cells and supernatant fluid were separated by centrifugation. The carbohydrate in the supernatant fluid was measured with anthrone, with glucose used as a standard. The cells were saponified, and the fatty acids were recovered, methylated, and analyzed by gas chromatography.*

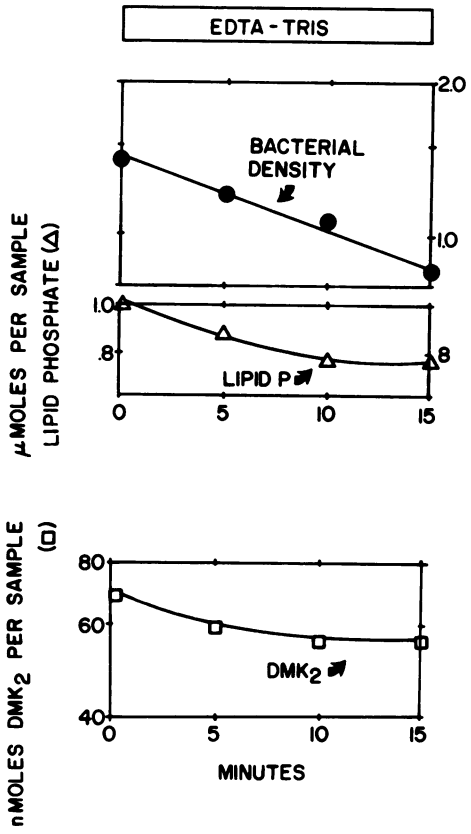

FIG. 5. *Loss of DMK_2 during the EDTA-Tris treatment of Haemophilus parainfluenzae. DMK_2 was extracted and assayed as described (35).*

phospholipid. When the original supernatant fraction was evaporated to dryness in a flash evaporator, a white proteinaceous precipitate was recovered after resuspension. This precipitate contained all the heme (measured as the reduced pyridine hemochrome) that was lost from the cells.

Recovery from the EDTA-Tris treatment. During the EDTA-Tris treatment, the cells lost total mass measured as dry weight faster than they lost phospholipid. After 10 min, the total phospholipid increased from 70 μmoles per g (dry weight) to 90 μmoles per g (dry weight). In the recovery period the cells doubled the dry weight in 42 min and the phospholipid in 75 min. This slow rate of phospholipid synthesis (in relation to the synthesis of other components of the cells) continued for about 1 hr until the phospholipid content was again 70 μmoles of lipid P per g, dry weight (Fig. 7).

The fate of ^{14}C and ^{32}P introduced into the cells before the EDTA-Tris treatment is illustrated in Fig. 8. During the treatment, lipid was lost as has been described above. During the recovery period there was a slow loss of ^{32}P from the lipids. The turnover rate of ^{32}P in PE was 2-fold slower, the rate in PG was 1.7-fold slower, and the rate in CL was 1.2-fold faster than during normal aerobic growth. These rates were calculated in terms of the bacterial doubling times (45 min during the recovery and 43 min during the pretreatment growth period). ^{14}C was not lost from the lipids but accumulated in the fatty acids and the glycerol of PE and PG.

During the period when there was logarithmic increase in the total phospholipid (Fig. 9), the incorporation of ^{32}P added to the cells was not logarithmic. ^{32}P was actively incorporated only near the end of the recovery period for all of the lipids but PE. In the recovery period, ^{14}C glycerol was rapidly incorporated into the glycerol

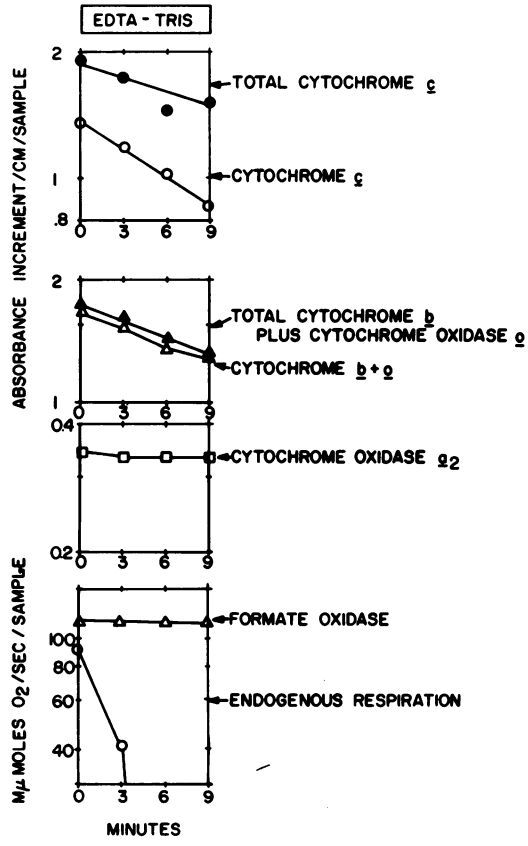

FIG. 6. *Changes in the membrane-bound electron transport system of Haemophilus parainfluenzae during the EDTA-Tris treatment. Cytochromes were determined by difference spectroscopy (34, 37) and the oxygen utilization was determined by polarography (36).*

TABLE 1. *Enzyme activities lost during EDTA-Tris treatment of Haemophilus parainfluenzae*

Enzyme	Specific activity[a]	
	Pellet[b]	Supernatant fraction
6-Phosphogluconate dehydrogenase[c]	.045	.042
Glucose-6-phosphate dehydrogenase[c]	.021	<.001
Glyceraldehyde-3-phosphate dehydrogenase[d]	.055	.016

[a] Expressed as micromoles per minute per milliliter.

[b] Log-phase *H. parainfluenzae* cells were treated with EDTA for 10 min as in Fig. 1. After centrifugation, the pellet and supernatant fraction were separated. The pellet was treated with sonic vibration, and the mixture was centrifuged. The supernatant portion of the sonically disrupted pellet was assayed. The supernatant portion of the EDTA-Tris treatment was assayed directly. A 1-ml amount of reaction mixture contained the extract from 1.05 mg (dry weight) of cells.

[c] Measured as reduced nicotinamide adenine dinucleotide phosphate appearance at 25 C.

[d] Measured as reduced nicotinamide adenine dinucleotide appearance at 25 C.

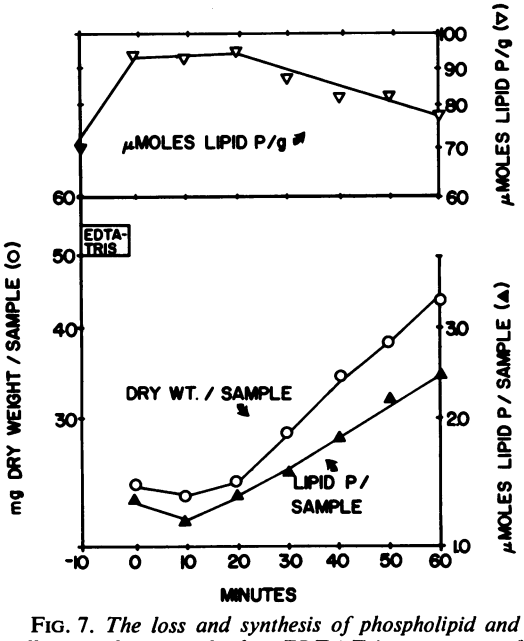

FIG. 7. *The loss and synthesis of phospholipid and cell mass during and after EDTA-Tris treatment of Haemophilus parainfluenzae. The dry weight was determined directly (36).*

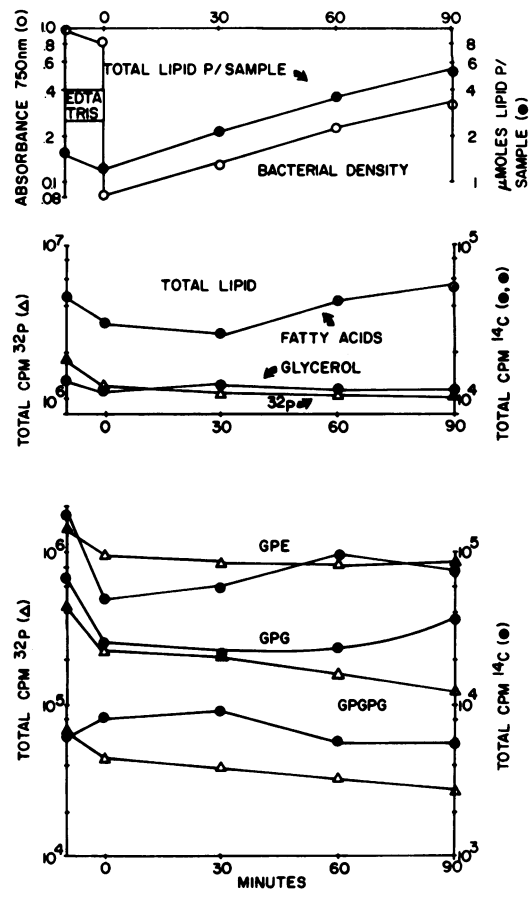

FIG. 8. *Turnover of ^{14}C and ^{32}P in the lipids of Haemophilus parainfluenzae during EDTA-Tris treatment and the recovery period. Cells were grown with 500 μc of $H_3{}^{32}PO_4$ and 50 μC of glycerol-1-3-^{14}C per 500 ml for 4 hr and then treated with EDTA-Tris as in Fig. 1.*

phosphate esters and fatty acids of the lipids without a lag period (Fig. 10).

DISCUSSION

Treatment of logarithimically growing *H. parainfluenzae* with EDTA-Tris resulted in the loss of phospholipids, LPS, components of the electron transport system, RNA, and some enzymatic activities into the surrounding medium (Fig. 1–6). The material that was lost could be recovered in the medium. The phospholipids, DMK_2, and the heme that were lost were found in small membrane fragments. If the duration of the treatment was less than 10 min despite loss of cell components, the cells were viable, and osmotically stable; when transferred to warm medium containing $MgCl_2$, they immediately began growing at the same rate as before the

treatment (Fig. 1). Prolonging the EDTA-Tris treatment decreased the viability although continued loss of lipid did not occur (Fig. 3).

The response of *H. parainfluenzae* to the EDTA-Tris treatment was like the response of *E. coli*. It was difficult to assess the effects of EDTA-Tris on the permeability, as *H. parainfluenzae* is normally permeable to many molecules (34, 36). The rapid loss of endogenous respiratory activity (Fig. 6) suggested that permeability changes in the membrane were as rapid as in *E. coli* (16). Glucose-6-phosphate dehydrogenase activity was not lost from the cells during the treatment in both organisms (Table 1, reference 19).

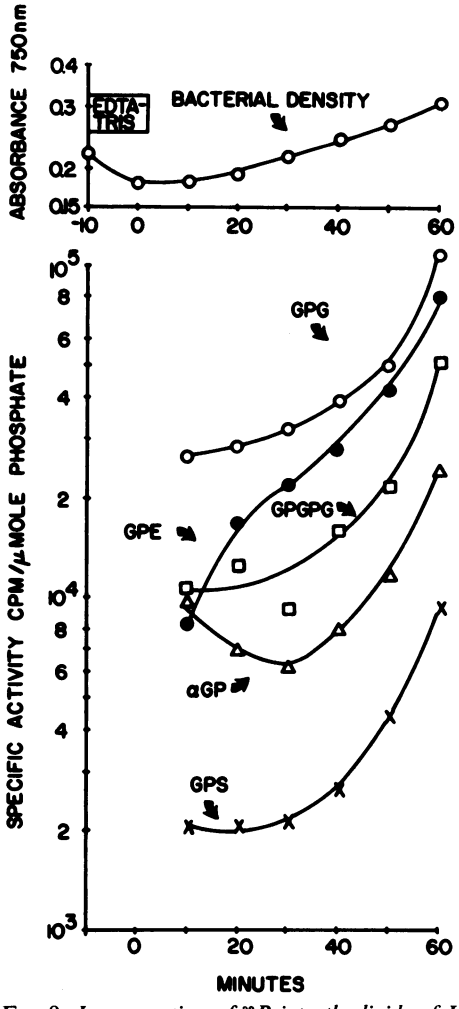

FIG. 9. *Incorporation of ^{32}P into the lipids of Haemophilus parainfluenzae during the recovery from EDTA-Tris treatment. Cells were treated with EDTA-Tris for 10 min, as in Fig. 1, and lost 17% of the phospholipid. They were diluted into 1,500 ml of warm medium containing 20 mM $MgCl_2$ in the presence of ^{32}P at a specific activity of 1.13 × 10^6 counts per min per μmole of P. Samples were drawn into an equal volume of ice and centrifuged; the lipids were extracted, deacylated by mild alkaline methanolysis, and separated chromatographically; the radioactivity of the glycerol phosphate esters was determined.*

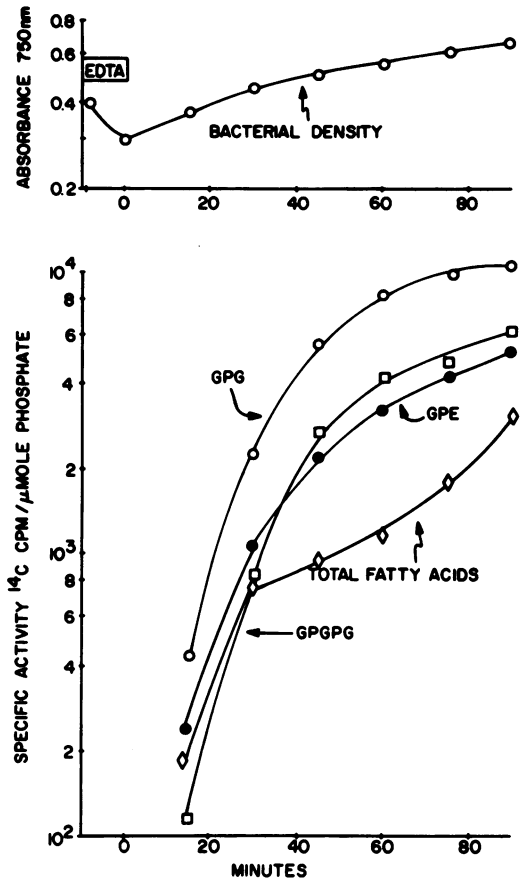

FIG. 10. *Incorporation of ^{14}C into the lipids of Haemophilus parainfluenzae during the recovery period from EDTA-Tris treatment. Cells were treated with EDTA-Tris for 8 min, as in Fig. 1, and lost 22% of their phospholipid. They were then diluted into 1,000 ml of warm medium containing 100 μc of glycerol-1,3-^{14}C, and were analyzed as in Fig. 9.*

Gram-negative organisms supposedly have a three-layered wall-membrane complex with an outer layer, a mucopeptide layer, and an inner layer (22). Spheroplast membranes can be fractionated into two fractions: one supposedly from the outer membrane containing the bulk of the carbohydrates and another with little carbohydrate, 70% of the phospholipid, and the respiratory pigments (21). In *H. parainfluenzae* the EDTA-Tris treatment damaged both membranes,

since LPS as well as respiratory pigments were lost. The loss of membrane fragments does not represent the lysis of 20% of the cells, as no lysis was detectable with phase-contrast microscopy, no DNA or glucose-6-phosphate dehydrogenase activity was lost, and the cells were viable and osmotically stable. The phospholipid remaining in the cells had a slightly higher specific activity than the lipid recovered from the medium after the EDTA-Tris treatment (Table 1), but the lipids were lost at the same rates except for CL (Fig. 2). The EDTA-Tris treatment makes the cells much more susceptible to nonionic detergents. Incubation of treated cells in 0.5% Cutscum in Tris buffer resulted in the loss of 88% of the phospholipids in 20 min. These cells were not osmotically stable.

The damage to the membrane by the EDTA-Tris treatment had profound effects on the metabolism of the phospholipids during the recovery period. Although the cells began to grow immediately at the pretreatment growth rate, the turnover of the phospholipid phosphate of PE and PG was slowed (Fig. 8). Slowing of the phospholipid turnover occurs also when the membrane-bound electron transport system is modified (41). In the recovery period the cells utilized intracellular phosphate preferentially over $H_3{}^{32}PO$ in the medium as rapid incorporation occurred only late in the recovery period (Fig. 9). Glycerol-*1,3*-^{14}C added to the growth medium was rapidly incorporated into the glycerol and fatty acids of the phospholipids in the recovery period. The glycerol pool appeared to be saturated in 30 min (Fig. 10). ^{14}C also came from the nonlipid precursors during the recovery period, as cells grown with labeled acetate or glycerol before the EDTA-Tris treatment accumulate ^{14}C in the lipids during the recovery period (Fig. 8).

Several of the control mechanisms normally operative remained effective. Since cell mass was lost faster than phospholipid during the treatment, the 20% increase in relative phospholipid content was corrected by a slower rate of phospholipid synthesis during the recovery period (Fig. 7). The proportions of the lipids remained the same throughout the recovery period (Fig. 1 and 2), indicating that the control of end products of phospholipid metabolism was operative.

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