Phospholipid Metabolism During Changes in the Proportions of Membrane-bound Respiratory Pigments in *Haemophilus parainfluenzae*

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After a transition from high to low oxygen tension, there was a twofold to 50-fold increase in the content of membrane-bound respiratory pigments of *Haemophilus parainfluenzae*, and there were concurrent changes in the metabolism of the membrane phospholipids: (i) a twofold decrease in the rate of turnover of the phosphate in all the phospholipids; (ii) a shift from simple one-phase, linear incorporation of phosphate into phospholipids to a complex biphasic incorporation of phosphate into phospholipids; and (iii) an increase in the total phospholipids with a slight increase in the proportion of phosphatidylglycerol (PG) and a slight decrease in the proportion of phosphatidylethanolamine (PE). Changes in the rates of incorporation of phosphate into the phospholipids occurred without a change in the rate of bacterial growth. When the compensatory adjustment of the proportions of the respiratory pigments reached a steady state, the total phospholipid, the rate of incorporation of phosphate into phospholipids, and the proportion of PG fell. At steady-state proportions of cytochromes, the proportion of PE and the rate of turnover of the phosphate in the phospholipids increased. All through an incorporation experiment of 1.5 divisions, the specific activity of the phosphate of PG was twice that of phosphatidic acid (PA). The phosphate of PG turned over 1.2 to 1.5 times more rapidly than the phosphate of PA in cells with high and low cytochrome levels. If the PA was an accurate measure of the precursor for the cytidine-5'-diphosphate-diglyceride, which in turn was the precursor of all the lipids, then the results of these experiments suggested that exchange reactions, in addition to synthesis from PA, were involved in phospholipid metabolism. These reactions were more sensitive to changes in oxygen concentration than was the growth rate.

The electron transport system of *Haemophilus parainfluenzae* can be isolated after cell rupture in the fraction containing the membrane wall complex (21, 22). The 2-demethyl vitamin K_2, the carotenoids, and the fatty acids released after saponification are found exclusively in this fraction (14, 19). The lipids that are extractible from the membrane wall or intact bacteria with chloroform methanol account for 86% of the fatty acids released by saponification, of which 98% are associated with phospholipids. Disorganization of the lipid or respiratory components leads to loss of electron transport function (10, 15). If the lipids are both a part of this membrane and necessary for its electron transport function, the synthesis and turnover of these lipids could be involved in the changes in the proportions of the respiratory components that are adaptations to

differing growth conditions (12, 13, 16). One lipid, 2-demethyl vitamin K_2, is synthesized coordinately with cytochrome b_1 at a molar ratio of 15 to 1 over at least an eightfold range in concentration (14). This lipid is directly involved in electron transport (15).

In *Rhodopseudomonas spheroides*, the total phospholipids increase without much change in the proportions of individual classes of phospholipids during the light-induced formation of the membrane-bound photosynthetic apparatus (9). In *Staphylococcus aureus*, the shift from anaerobic to aerobic growth results in the formation of a functional membrane-bound electron transport system. During the formation of this system, there is a coordinate 1.2-fold increase in mono- and diglucosyldiglyceride, a 1.6-fold increase in vitamin K_2 and cardiolipin, and a two-

fold increase in phosphatidylglycerol (5). This increase in the lipids parallels the changes in respiratory pigments (5). In this period of lipid synthesis, remarkable variation occurs in the magnitude and direction of change in absolute amounts of the 64 fatty acids in these lipids (20).

The lipids of *H. parainfluenzae* have been identified and methods for their assay have been described (17). This study will examine the changes in these lipids during modifications in the electron transport system.

MATERIALS AND METHODS

Growth of the bacteria. The strain of *H. parainfluenzae*, the medium, harvesting procedures, and the determination of purity of the culture were described previously (12). Generally, 12-hour cultures were used as inocula. Cultures in Erlenmeyer flasks were incubated with aeration provided by agitation at high speed in a gyrotory shaker. The doubling time was measured as the time necessary for the absorbancy at 750 nm to double. The doubling time ranged between 40 and 50 min under these growth conditions.

For measurement of the turnover of the phospholipids, about 150 ml of bacterial culture was incubated in the presence of 1.5 mc of $H_3{}^{32}PO_4$ (supplied in plastic bottles by Tracerlab, Richmond, Calif.). After a period from 10 min to 1 hr, the culture was centrifuged at room temperature and the radioactive medium was decanted. The cells were suspended in 10 ml of 0.05 M phosphate buffer (pH 7.6) at 34 to 37 C, centrifuged, suspended in 30 ml of medium not containing ^{32}P at 37 C, and inoculated into 1.7 liters of warm medium in a low-form Erlenmeyer flask. In these operations, there was essentially no change in the growth rate of the bacteria before and after the pulse of ^{32}P. For the measurement of phospholipid turnover in bacteria actively forming cytochromes, the cultures were grown to a density of 0.3 mg (dry weight) of cells per ml with agitation, and the agitation was stopped. After 1 or 2 hr of growth without agitation, portions of the culture were removed and incubated with ^{32}P. During the period of growth in the nonradioactive medium, 200-ml samples were removed aseptically and added to an equal volume of ice. The ice effectively stopped phospholipid metabolism. A culture added to this volume of ice incorporated only 108 counts/min of ^{32}P into the lipids compared with an incorporation of 6,300 counts/min of ^{32}P into lipids at 37 C. The culture and ice were centrifuged at 4 C and resuspended in 0.05 M phosphate buffer at *p*H 7.6, and the lipids were extracted. For incorporation studies, the bacteria were incubated in medium containing between 800,000 and 2,000,000 counts/min per μmole of phosphate. Samples were withdrawn and treated as in the turnover experiments. In experiments where the cytochrome levels were to be determined, the sample was divided into two portions; one portion was incubated with ^{32}P, and the other was centrifuged for assay of the cytochromes.

Extraction. The most effective method of lipid extraction in terms of total lipid phosphate removed

is the modification of the procedure of Bligh and Dyer (2) described previously (18). A sample of bacteria divided into four equal portions yielded 21.1, 22.0, 19.6, and 21.7 μmoles of lipid phosphate per 0.409 g (dry weight) of cells.

Deacylation. The phospholipids were deacylated by mild alkaline methanolysis, which yields water-soluble glycerol phosphate esters and lipid-soluble methyl esters of the fatty acids (17). The methanolysis mixture was neutralized by the carboxylic acid resin Biorex 70 (Bio-Rad Laboratories, Richmond, Calif.) as described (17). From this procedure, there is no detectable cyclic glycerol phosphate from overhydrolysis. The reaction is complete in 2-hr at 0 C, and the recovery of the glycerol phosphate esters and fatty acid methyl esters is quantitative (17). The following abbreviations are used to represent glycerol phosphate esters after deacylation of the phospholipids: GPC, glycerolphosphoryl choline derived from phosphatidylcholine; GPE, glycerolphosphoryl ethanolamine derived from phosphatidylethanolamine; MM-GPE, glycerolphosphoryl monomethylethanolamine derived from phosphatidyl monomethylethanolamine; DM-GPE, glycerolphosphoryl dimethylethanolamine derived from phosphatidyl demethylethanolamine; GPG, glycerolphosphoryl glycerol derived from phosphatidylglycerol; αGP, L-α-glycerol phosphate derived from phosphatidic acid; GPS, glycerolphosphoryl serine derived from phosphatidylserine; and GPGPG, diglycerolphosphoryl glycerol derived from cardiolipin.

Separation of glycerol phosphate esters. R. L. Lester has developed a highly reproducible method for assay of glycerol phosphate esters involving elution from a Dowex-1 column with a gradient of ammonium formate and sodium borate. He also developed a two-dimensional chromatographic method for separation of these esters on aminocellulose paper. These methods as applied to *Haemophilus* phospholipids have been described previously (17). The data in Table 1 indicates that the column and paper chromatographic methods are reproducible and give essentially the same proportions of glycerol phosphate esters. The paper chromatographic separation of the glycerol phosphate esters and the thin-layer chromatographic separation of the intact phospholipids give the same distribution of phosphate (17). A radioautogram of ^{32}P-labeled lipids is illustrated in Fig. 1.

Assay of the glycerol phosphate esters. Glycerol phosphate esters containing ^{32}P were located by radioautography as described (17). Paper discs corresponding to the dark areas of the radioautogram were cut out, and their radioactivity was determined as described (17). The discs were then removed, cut into tiny fragments, and digested with 3.9 M perchloric acid at 200 C; the phosphate in the digest was determined as described (19). Comparison of the specific activity of the separated glycerol phosphate esters with that of the mixture of deacylated phospholipids indicates there are no substantial quenching problems in this assay (Table 2).

Cytochromes. Cytochromes were estimated from the difference spectra measured by comparing a suspension of bacteria with the respiratory pigments re-

duced in the presence of 10 mM formate to a part of the same suspension in which the respiratory pigments are oxidized by shaking in air. The measurements were performed in a Cary 14-CM spectrophotometer as described (12, 14). Cytochrome a_2 was measured as the absorbancy increment between the maximum at 630 nm and a line connecting points at 610 and 660 nm; cytochrome c_1 was measured as the absorbancy increment between the maximum at 553 nm and a line connecting points at 540 and 580 nm; cytochrome b_1 was measured as the absorbancy increment between its maximum at 560 nm and the line used for cytochrome c_1. The overlaps of cytochromes b_1 and c_1 were corrected for each other by use of the expression derived previously (21). Cytochrome oxidase o was measured as the absorbancy increment between the maximum at 416 nm and the minimum between 430 and 440 nm in difference spectra obtained with bacteria with pigments reduced and saturated with carbon monoxide versus bacteria with pigments reduced.

Oxygen uptake. Oxygen utilization was measured with the Clark oxygen electrode as described (13).

RESULTS

Modification of the electron transport system. Rates of turnover and incorporation of phosphate

TABLE 1. *Comparison of paper and column chromatography of deacylated phospholipids[a]*

Lipid	Paper	Column
	%	%
GPE	77.4 ± 0.1	76.0 ± 2.0
GPG	18.4 ± 0.2	19.4 ± 1.0
aGP	0.7 ± 0.1	0.8 ± 0.2
GPS	0.5 ± 0.2	0.4 ± 0.2
GPGPG	3.0 ± 0.1	3.4 ± 0.2

[a] Values are expressed as per cent of total glycol phosphate esters. Lipids were extracted from bacteria grown with aeration in the presence of ^{32}P and deacylated by mild alkaline methanolysis. The methanolysis was neutralized with Biorex-70 resin and the fatty acid methyl esters were removed with ethyl ether. Recovery of the phosphate in the aqueous phase after mild alkaline methanolysis was 101%. Less than 0.5% of the ^{32}P was found in the ethyl ether. Four samples containing 2.10 μmoles of phosphate were spotted on aminocellulose paper and chromatographed in two dimensions (Fig. 1). The deacylated lipids were detected by radioautography; the spots were cut out and their radioactivity was determined, after which the papers were digested and analyzed for phosphate. Two samples of deacylated lipids containing 4.54 μmoles of phosphate were made 0.02 M with NaB$_2$O$_7$, loaded on a Dowex column, and eluted with the ammonium formate-sodium borate gradient described (18). One-sixth of each fraction was then pooled, dried in vials, and counted in the scintillation spectrometer.
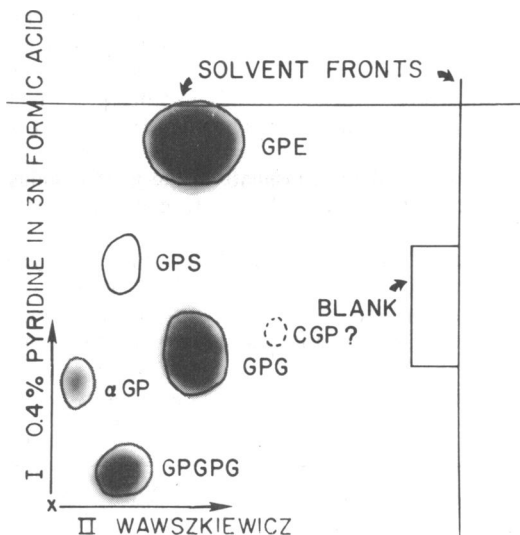

FIG. 1. *Radioautogram after paper chromatography of deacylated ^{32}P-labeled phospholipids from H. parainfluenzae. Whatman AE-81 amino cellulose paper was treated by chromatography with 3 M formic acid, dried and used for two-dimensional chromatography. The solvent for the first dimension was 3 M formic acid containing 0.4% (v/v) pyridine; the solvent for the second dimension was 1.15 M ammonium acetate with 11.8 mM ethylenediaminetetraacetic acid made to pH 5.0 with acetic acid and diluted 3 to 7 with 95% ethanolic 0.26 M ammonium (Wawszkiewicz solvent; 17). The paper was dried and placed on Kodak-no-screen X-ray film for 12 hr; the radioautogram was developed. CGP indicates cyclic glycerol phosphate.*

into the phospholipids of the protoplast membrane of *H. parainfluenzae* are compared under conditions during which changes in the composition of the membrane-bound electron transport system take place. Previous work has shown that the bacterium modifies the respiratory system in response to lowering oxygen tension in the growth medium by forming a much more extensive cytochrome system (12, 16). This more extensive system is capable of a much more rapid rate of electron transport at low oxygen tensions than the system formed during growth at high oxygen concentrations (13). A high oxygen tension is obtained by rapidly agitating a culture with a bacterial density less than 0.3 mg (dry weight) of cells per ml (16). If agitation of the growth vessel is stopped and the bacterial density is greater than 0.2 mg/ml, there is essentially no oxygen detectable 5 mm below the surface of the medium after a few minutes of metabolism (16). When the agitation of the culture is stopped, there is a rapid synthesis of cytochromes. There is *at least* a twofold to 50-fold increase in the

relative concentration of the cytochromes be-
tween the steady-state aerobic and the nonagi-
tated culture (Table 3). The proliferation of the
cytochromes continues for about 5 hr, after which
a steady-state high cytochrome complement is
maintained.

Turnover of the phosphate in the phospholipids.
The turnover of the phosphate in the phospho-
lipids was compared during the maintenance of
low cytochrome levels and during active cyto-
chrome formation when the cytochrome levels
were approximately 60% of the nonaerated level.
If the bacteria are allowed to grow in the presence

TABLE 2. *Specific activity of the separated and
total deacylated phospholipids
of H. parainfluenzae[a]*

Lipid	Counts/min	Phosphate	Specific activity[b]
		μmoles	
GPE.............	11,060	0.693	
GPG.............	971	0.127	
GPGPG.........	342	0.036	
aGP.............	61	0.008	
GPS.............	95	0.005	
Total, separated..	12,529	0.869	14,471
Total, lipid......	12,648	0.850	14,880

[a] Deacylated phospholipids from bacteria
grown with ^{32}P were spotted on four aminocellu-
lose papers. Two of the papers were subjected to
chromatography as in Fig. 1; the spots were
located by radioautography and cut out. Their
radioactivity was determined, after which the
paper was digested and analyzed for phosphate.
The two remaining papers were allowed to equili-
brate with the two solvents; then 2-cm circles were
cut out around the origin, and the paper discs
were treated as the papers actually subjected to
chromatography.

[b] Expressed as counts/min per μmole of phos-
phate.

of ^{32}P for 25% of their doubling time and then
transferred to medium not containing ^{32}P, the
turnover of the phospholipid phosphate can be
examined (Fig. 2). After 2 hr in the nonaerated
medium, the bacteria have the same 42-min
doubling time as bacteria grown in aerated
cultures. The striking result of these experiments
is that there is a marked decrease in the turnover
of the phosphate of the lipids during rapid cyto-
chrome synthesis. This is best seen in GPG, for
which the time necessary to lose 50% of its ^{32}P
decreases from 47 min in the agitated culture to
118 min after an hour of incubation without
agitation and to 150 min after 2 hr of incubation
without agitation. A second feature of this experi-
ment is the time necessary to saturate the phos-
phate pool that forms the cardiolipin phosphate.
This pool is saturated in less than 10 min with
aerobically growing cells, but it requires 70 to 90
min during active cytochrome biosynthesis. If the
bacteria are allowed to grow in ^{32}P for 1.3 gen-
erations, the phosphate pools are all saturated
and the turnover of cardiolipin can be examined
(Fig. 3). Under these conditions, the time, in
generations, necessary for 50% of the ^{32}P to be lost
in the aerobic cells is 3.0, 3.9, and 1.7 generations
for GPE, GPG, and GPGPG, respectively. After
2 hr of growth without agitation, the time neces-
sary for 50% of the ^{32}P to be lost from the GPE,
GPG, and GPGPG is 6.8, 2.1 and 3.5 generations,
respectively. There is a 1.6-fold to twofold *de-
crease* in turnover during rapid cytochrome for-
mation, even though the doubling time of the
bacteria remains the same.

If cells of *H. parainfluenzae* which contain the
high levels of cytochrome are suddenly shifted to
growth conditions where low cytochrome levels
are normally formed, there is a 1.5-fold increase in
the turnover of GPG, a 1.1-fold increase in the
turnover of GPGPG, but a 1.2-fold decrease in
the turnover of GPE (Fig. 4).

TABLE 3. *Cytochrome components and oxygen utilization of H. parainfluenzae grown
with vigorous and limited aeration[a]*

Growth condition	Cytochromes					Oxygen utilization
	a_2	a_1	b_1	c_1	o	
Aerated...........	0.0001	0.0005	0.0036	0.002	0.0056	12.40
Nonaerated.......	0.0065	0.0031	0.065	0.149	0.600	114.00

[a] Cytochromes are expressed as the absorbance increment per 10 mg (dry weight). Oxygen utilization
is expressed as nanomoles of oxygen utilized per sec per 10 mg (dry wt) in the presence of 10 mM formate.
Actual measurements of the cytochromes were performed with bacteria at densities of 15.6 mg (dry wt)
per ml of cells (aerated) and 17.0 mg/ml (nonaerated). Bacteria were grown under the same conditions
described in Fig. 3, except that the $H_3{}^{32}PO_4$ was omitted. The measurements were performed at the
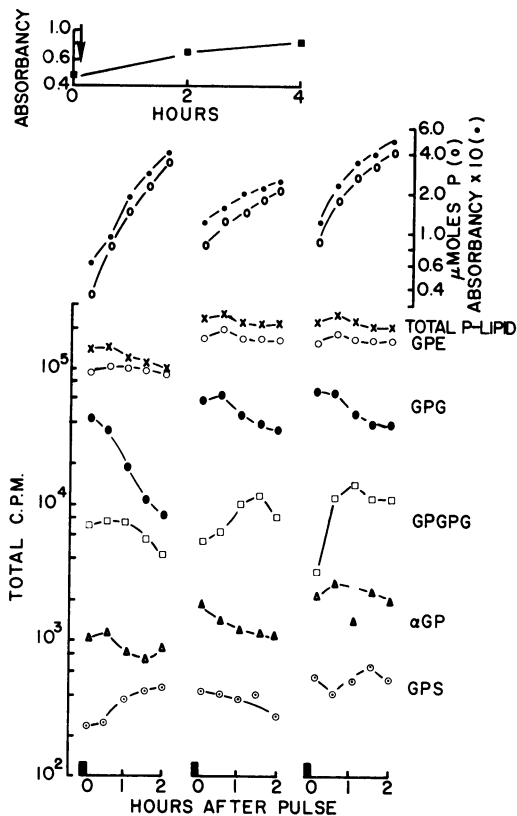zero-time point of the turnover.

FIG. 2. *Turnover of* ^{32}P *in the phospholipids of H. parainfluenzae during aerobic growth and while adapting to growth with poor aeration. Cultures were grown to a density of 0.27 mg (dry weight) per ml with aeration; a portion was removed, incubated in the presence of 1.5 mc of* $H_3{}^{32}PO_4$ *in 150 ml for 10 min, centrifuged, washed in warm 0.05 M phosphate buffer (pH 7.6), and then diluted into 1.7 liters of nonradioactive medium. During growth in the medium without radioactivity, samples of 200 ml were taken. The lipids were extracted, deacylated, and separated; the total radioactivity in each sample was calculated. The original culture from which the first 150-ml portion had been removed had been incubated without agitation during the period of the first turnover experiment; in 1 hr, it had grown to a density of 0.43 mg (dry weight) per ml. At this point, a second portion was removed, incubated with* ^{32}P, *and analyzed as before. A third sample was removed when the bacterial density was 0.48 mg (dry weight) per ml and treated similarly. The upper curve indicates the growth of the bacteria that were used for the incubations with* ^{32}P. *The arrow indicates the time at which the agitation was stopped. The middle curves indicate the bacterial density and the total lipid phosphate per sample during the growth after removal of* ^{32}P. *Left hand column indicates bacteria withdrawn from the culture just before aeration was stopped. These bacteria were aerated during the pulse and the turnover. The middle column indicates bacteria withdrawn after growth for 1 hr without*

Kinetics of incorporation of phosphate into the phospholipids. Kinetics of incroporation of ^{32}P into the lipids of bacteria maintaining low cytochrome levels and bacteria that are rapidly forming cytochromes are illustrated by the data in Fig. 5. In the presence of 800,000 counts/min of ^{32}P per μmole of phosphate, there is a linear incorporation in aerobically growing cells that begins in 1 to 2 min in GPE, GPG, and GPS. There is an apparent 8-min lag in the incorporation of ^{32}P into αGP, and there is a 5-min lag in the incorporation into GPGPG. When the bacteria are rapidly forming cytochromes, the lag is shorter and there is a more rapid initial ^{32}P incorporation. This is seen most strikingly with the GPGPG.

With the effect of low oxygen tension on turnover and incorporation known, an examination of (i) the rates of incorporation of ^{32}P into the phospholipids, (ii) the proportions of the various phospholipids, and (iii) the total phospholipid per gram (dry weight) was made where the growth rate and the cytochrome concentrations were measured simultaneously (Fig. 6–8). In the experiment illustrated in Fig. 6, these parameters of phospholipid metabolism were determined under conditions of rapid cytochrome synthesis. This was achieved by growing the culture to a density of 0.19 mg (dry weight) of cells per ml with vigorous agitation and then stopping the agitation. After the agitation was stopped, samples were removed as the bacteria adapted to the low oxygen tension. In the experiment illustrated in Fig. 7, the parameters were determined during the terminal stages of the adaptation. This was achieved by growing the culture to 0.38 mg (dry weight) of cells per ml and incubating the culture for 8 hr without shaking. In the experiment illustrated in Fig. 8, the parameters were measured during a decrease in the cytochrome content. Bacteria with cytochrome levels typical of cells growing with low aeration were diluted to a density of 0.19 mg (dry weight) of cells per ml, and the culture was agitated vigorously. After the agitation was started, repeated dilutions kept the bacterial density below 0.27 mg (dry weight) of cells per ml during this experiment. The cytochrome

aeration, and the right hand column indicates bacteria withdrawn after 2 hr of growth without agitation. The cultures taken 1 and 2 hr after agitation was stopped were not shaken during the pulse or the turnover. The lower curves indicate the total ^{32}P *in each lipid of the samples. The black bars indicate the duration of the pulse with* ^{32}P. *In the time scale, there is a 20-min period for centrifuging and washing the cultures between the black bars and the turnover that is not shown in the figure.*

content of bacteria during these growth conditions is illustrated in part A of Fig. 6–8. In Fig. 8, the solid line gives the decrease in the content of cytochrome expected if there were no synthesis or breakdown during the adaptation. This rate of decrease represents the rate of dilution of the cytochrome during growth. Responses of cytochromes with more rapid rates of decrease (steeper slopes) indicate cytochrome breakdown; responses of cytochromes with slower rates of
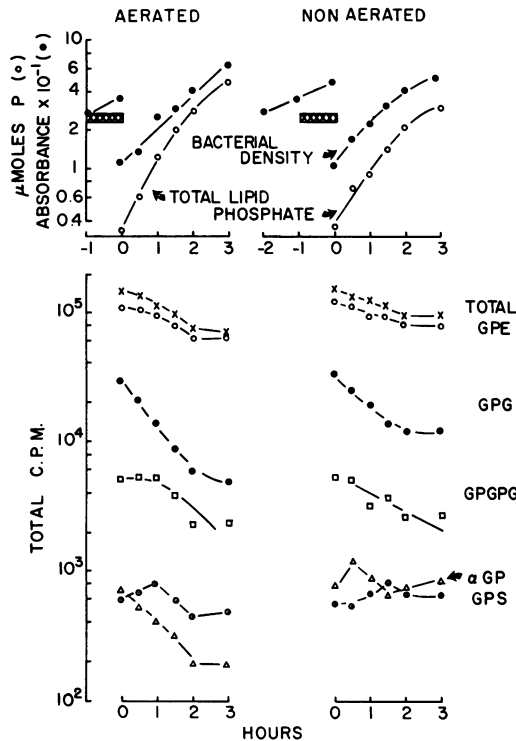

FIG. 3. Turnover of ^{32}P in the phospholipids of H. parainfluenzae during the shift from low to high content of cytochromes. The upper curve indicates the changes in bacterial density measured as absorbance at 750 nm and the total lipid phosphate in the 200-ml samples during the manipulation like those described in Fig. 2. The cross-hatched areas indicate the period of exposure of each culture to $H_3{}^{32}PO_4$. The specific activity of the medium was 875,000 counts/min per µmole of phosphate for the aerated culture and 1,188,000 counts/min per µmole for the nonaerated culture. Before zero-time in each experiment, both cultures were centrifuged, washed, and resuspended in nonradioactive, warm media, which represented 20 min not illustrated on the time scale. There was no lag in growth caused by these manipulations. The aerobic cultures were incubated with agitation on a gyrotory shaker at 300 cycles/min in low-form Erlenmeyer flasks containing 1.7 liters of medium. The nonaerated cells were incubated under the same conditions, except that the flasks were not agitated.
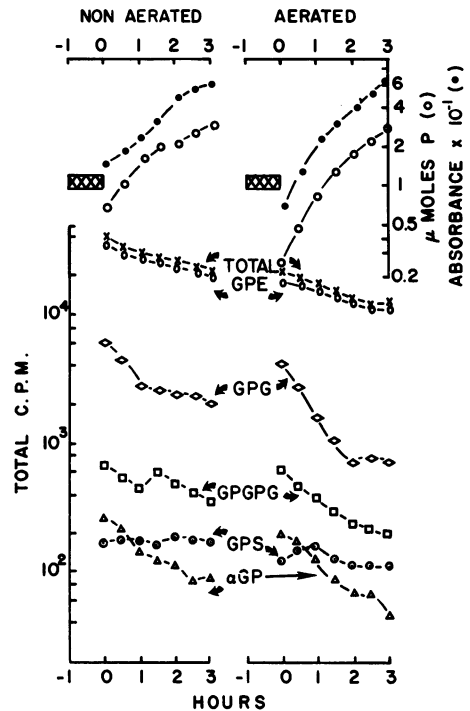

FIG. 4. Turnover of ^{32}P in phospholipids of H. parainfluenzae during the shift from high to low content of cytochromes. The data are plotted as in Fig. 3, except that the bacteria were grown without aeration and then shifted to conditions of high aeration for 2 hr before being compared to bacteria grown without aeration in the same type of experiment.

breakdown (flatter slopes) represent cytochrome synthesis during this period.

Rate of phospholipid biosynthesis. In part B of Fig. 6–8, the rate of phospholipid synthesis during changes in the composition of the membrane-bound electron transport system is illustrated. The rate of synthesis was calculated from the specific activity of the ^{32}P in the medium and the ^{32}P recovered in each lipid after analysis. At the times indicated in the figures, 100-ml samples of the culture were removed and incubated with ^{32}P for 10 min; they were then poured into an equal volume of ice, centrifuged, and washed. The lipids were extracted, deacylated, separated, and assayed, as described in Materials and Methods. A 10-min incorporation was chosen because it corresponds to the rapid phase of incorporation demonstrated in Fig. 5. In 10 min, the turnover of ^{32}P in the lipids involves at most a 10% underestimation of the rate of synthesis of GPG during aerobic growth. (The most rapid turnover occurs in this lipid and under this growth condition.) The difference in the rate of

synthesis of the phospholipid between the first and second points in Fig. 6 and 7, part B, shows the effect of aeration. The samples used for the first point were aerated by shaking during the incorporation, and the remaining samples were not agitated during incubation. In Fig. 8, part B, the incubation mixture was not shaken for the first sample but was agitated for the remainder. Agitation of the culture during the incubation period causes a marked increase in phospholipid

biosynthesis. Comparison of the rate of phospholipid biosynthesis to the rate of growth, measured as the increase in dry weight per minute, indicates that phospholipid biosynthesis is much more sensitive to the concentration of terminal electron acceptor for the electron transport system than is the overall rate of growth. The initial phases of cytochrome biosynthesis are reflected in an increase in the rate of synthesis of GPE, GPG, and GPGPG. Even though there is a marked de-
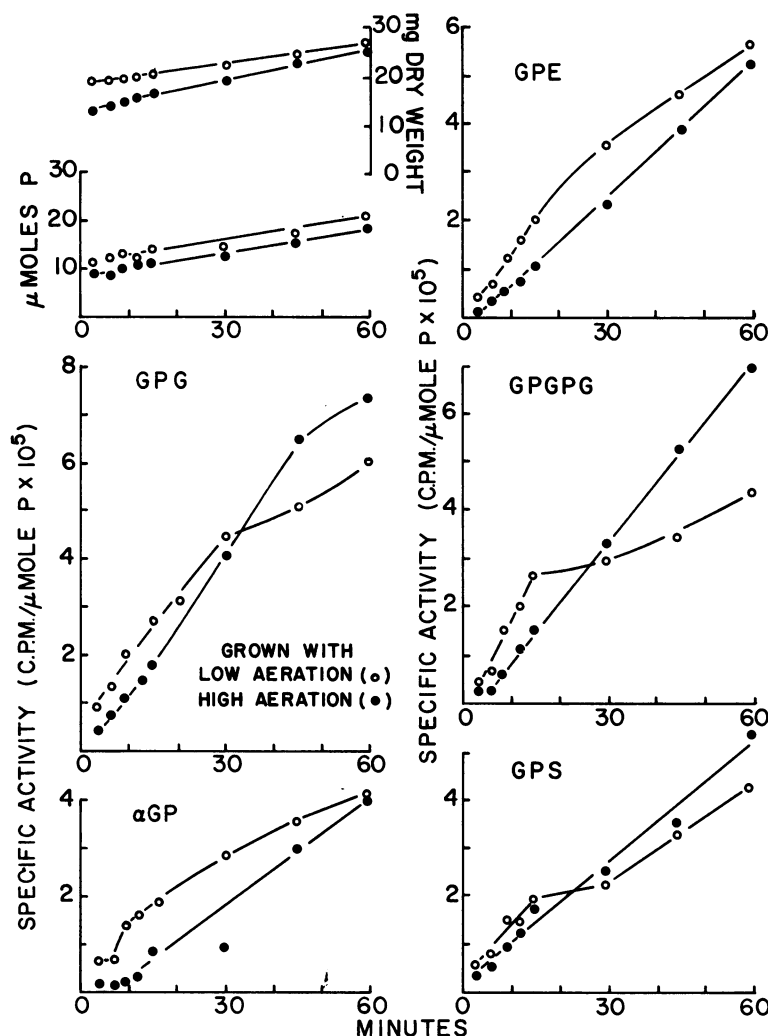

FIG. 5. *Comparison of the kinetics of $H_3{}^{32}PO_4$ incorporation into phospholipids of H. parainfluenzae during growth with high aeration (low cytochromes) and low aeration (high cytochromes). Bacteria from cultures in exponential growth were either grown with high aeration in the ${}^{32}P$-containing medium or allowed to grow without aeration, as described in the text. The specific activity of the medium was 762,000 counts/min per µmole for the aerated (●) and 827,000 counts/min per µmole for the nonaerated (○). The samples (100 ml) were withdrawn into an equal volume of ice, centrifuged, and extracted. The lipids were deacylated and separated, and their specific activity was determined. The cytochrome content of the aerated and nonaerated cultures at the start of the incorporation was essentially as given in Table 3.*
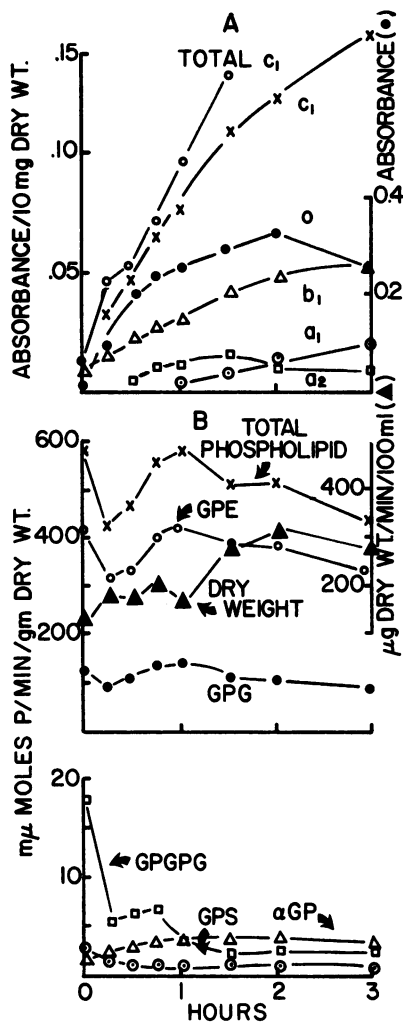
FIG. 6. *Changes in phospholipid phosphate incorporation and proportions during the rapid formation of cytochromes. In this experiment, bacteria were grown with aeration to a density of 0.19 mg (dry weight) per ml and then allowed to grow without aeration. This causes the induction of the formation of the cytochromes illustrated in part A. Samples (100 ml) were withdrawn for cytochrome assay, and other 100-ml samples were placed in 1-liter flasks containing $H_3{}^{32}PO_4$ in medium of specific activity of 1.2 to 1.6 × 10⁶ counts/min per μmole of phosphate and incubated without aeration for 10 min. At this time, 100 ml of ice was added; the culture was centrifuged and the lipids were extracted, deacylated, and separated by two-dimensional chromatography. The specific activity of each lipid was determined. In A, the absorbancy increments for the cytochromes are illustrated after measurement, as described in Table 3. Both total cytochrome c_1 and enzymatically reducible cytochrome c_1 are given. The right hand axis refers to cytochrome oxidase o (●). In B, the rate of ${}^{32}P$ incorporation per minute per gram (dry weight) is*
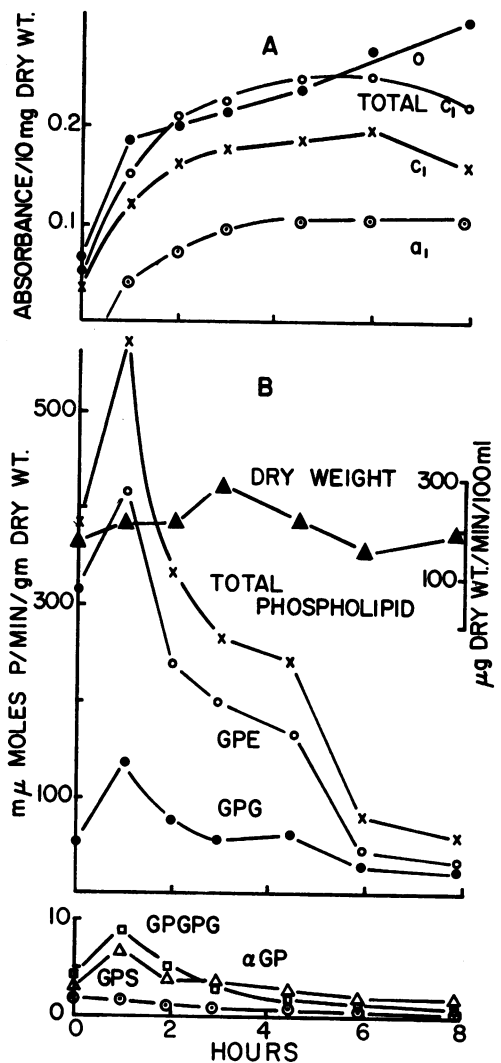
FIG. 7. *Changes in phospholipid phosphate incorporation and proportions during the terminal portion of the shift to high cytochromes. The experiment was performed as in Fig. 6, except that the period of ${}^{32}P$ incorporation was increased to 15 min.*

crease in the total cytochrome content associated with the shift to aerobic growth (Fig. 8), there is synthesis of cytochromes b_1 and o that is coupled with increased phospholipid biosynthesis. When the rate of synthesis of the membrane-associated respiratory pigments slows, there is a marked decrease in the rate of phospholipid biosynthesis.

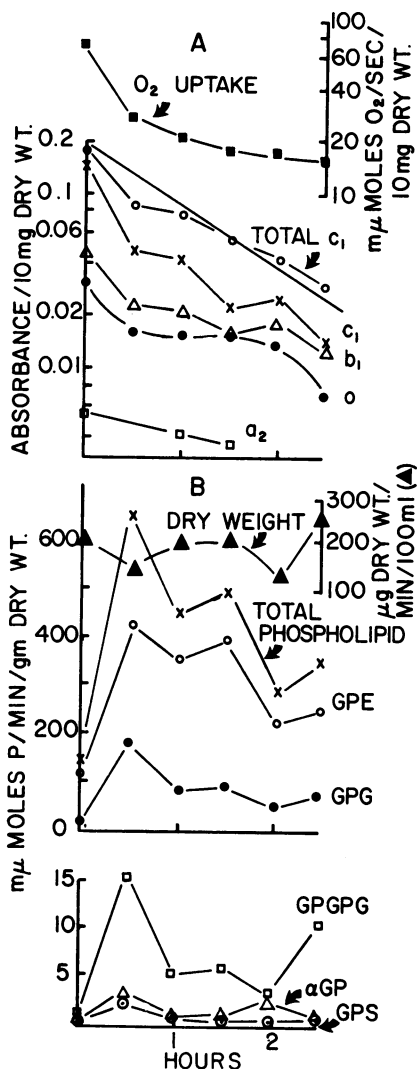plotted, as is the rate of increase in dry weight of the bacteria per 100-ml sample.

FIG. 8. *Changes in phospholipid phosphate incorporation and the proportions during a rapid decrease in cytochrome content. The experiment was performed as in Fig. 6 and 7, with the modifications described in the text.*

The rate of synthesis of phospholipid can decrease five- to sixfold under conditions where the growth rate remains constant (Fig. 7B).

These results are, at best, a qualitative measure of the trends in phospholipid biosynthesis. The assumption that the specific activity of the medium represents the specific activity of the phosphate pool is clearly wrong because the maximal rate of phospholipid synthesis, 0.6 μmoles of lipid phosphate per min per g (dry weight) of cells, could account for only 50% of phospholipid

necessary to maintain a concentration of phospholipid phosphate at 70 μmoles per g in bacteria with a 50-min doubling time (Fig. 6–8). Again, this assumed specific activity does not allow for the lag in phosphate uptake demonstrated in Fig. 5 and the demonstration of different phosphate pools for GPG and GPGPG in Fig. 2.

Total phospholipid. Early in periods of active modification of the respiratory pigments of the membrane there is a 5 to 10% increase in the total phospholipid content. When the early period is over and most of the cytochromes have reached or nearly reached the new steady-state levels, there is a 5 to 10% decrease in the total phospholipid content.

Proportions of the phospholipids. Early in periods of rapid cytochrome synthesis, there is a steady increase in the proportions of GPG (from 17 to 23% of the total lipid phosphate) and GPGPG (from 2 to 4%) and a decrease in the proportion of GPE (from 80 to 75%). When the levels of cytochromes are relatively constant, there is a decrease in the total lipid phosphate, the proportions of GPG and GPGPG decrease, and the proportion of GPE increases to the level characteristic of log phase cells.

DISCUSSION

The limiting membrane of *H. parainfluenzae* contains phospholipids and cytochromes in a complex. Disturbances of the lipids in the complex by solvents lead to loss of electron transport (15). One of the principal advantages of using *H. parainfluenzae* is that this bacterium has a great capacity to modify the composition of the electron transport system in response to various growth conditions (12, 13, 16).

Under conditions where there is a twofold to 50-fold increase in the functional cytochrome per bacterium, the following changes in the metabolism of the phospholipids occur. (i) There is a 1.6-fold to twofold *decrease* in the turnover of lipid phosphate of all the lipids during rapid cytochrome formation. (ii) The incorporation of phosphate into the lipids has a higher initial rate, shorter lag, and more rapid approach to steady-state specific activities than is seen in bacteria maintaining low steady-state levels of cytochromes (Fig. 5). (iii) Using the incorporation of phosphate in 10 min as a relative measure of trends in phospholipid biosynthesis, there is an increased rate of synthesis during the early part of the change in the composition of the electron transport system. This increased synthesis of phospholipid is reversed once the new steady state level of respiratory pigments is achieved (Fig. 6–8, part B). (iv) The proportions of GPG

and GPGPG increase; the proportion of GPE decreases and the total phospholipid increases 5 to 10% during early periods of change in the respiratory pigments. These changes are reversed as the cytochrome content approaches the new steady-state level. This suggests that there must be modification of the phospholipids in the membrane associated with the early phases of changes in the composition of the membrane-bound respiratory pigments. A similar situation has been documented in *S. aureus* where formation of the functional electron system involves synthesis of protoheme, cytochromes, cytochrome oxidase, isoprenologues of vitamin K_2, glucolipids, and a doubling of the phospholipid (5).

There is a possibility that the changes in phospholipid composition may involve more than biosynthesis from cytidine-5'-diphosphate-diglyceride (CDP-diglyceride). Phosphatidic acid (PA), measured in this study as αGP, reacts with CDP to form CDP-diglyceride, which in turn forms phosphatidylserine and phosphatidylethanolamine from L-serine and phosphatidylglycerol and cardiolipin from αGP (3, 4, 8, 11). The specific activity of the phosphate of GPG is twice that of the αGP all through an experiment of 1.5 divisions (Fig. 5). There is a longer lag in the incorporation of ^{32}P into αGP than in the other lipids. The turnover of the phosphate in GPG is 1.2 to 1.5-times faster than the phosphate of αGP in cells grown with either high or low cytochrome levels (Fig. 3, 4). These experiments indicate that the phosphate of GPG cannot all have come from the αGP. Either the αGP is grossly contaminated by PA that is not involved in the formation of CDP-diglyceride or there is a possibility that phosphate can be incorporated into the lipids via some transferase reactions not involving PA. It is also possible that the αGP isolated after mild alkaline methanolysis is contaminated by breakdown products from the methanolysis of other lipids, although the proportions of phosphate in PA determined after separation of the intact lipids and in αGP after separation of the deacylated lipids are very nearly equal (17). In an incorporation experiment of 0.4 divisions, the specific activity of the phosphate of the PA was less than the specific activity of the phosphate in phosphatidylethanolamine and phosphatidylglycerol. In this experiment, the diacyl phospholipids were separated by thin-layer chromatography (*unpublished data*). The fatty acid composition of PA is much different from the phosphatidylglycerol and phosphatidylethanolamine (17). Transferase activities involving fatty acids have been postulated to account for fatty acid differences in the lipids of *H. parainfluenzae* (17) and *S. aureus* (20).

E. coli is much like *H. parainfluenzae* in many aspects of its metabolism. In *E. coli* grown in peptone medium, phosphatidic acid has a higher specific activity and a greater rate of incorporation of phosphate than the other lipids (6), but if the organism is grown in defined medium, the turnover of the phosphate in phosphatidic acid is slower than in phosphatidylglycerol (1).

The fact that both turnover and incorporation of phosphate into the phospholipids can change markedly when the growth rate remains constant suggests that these postulated transferase reactions are more sensitive to changes in the concentration of the terminal electron acceptor for the electron transport system than is the overall growth rate. This suggests that energy yielding reactions could be involved in the transferase activities of phospholipid phosphate. Evidence presented in this study indicates that these types of reactions are very likely involved in the modifications of the membrane that appear necessary when the proportions of components of the electron transport system are changed.

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