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Biosynthesis of Phospholipids in *Clostridium butyricum*: Kinetics of Synthesis of Plasmalogens and the Glycerol Acetal of Ethanolamine Plasmalogen

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The biosynthesis of the plasmalogen forms of phosphatidylethanolamine (plasmenylethanolamine) and phosphatidylglycerol (plasmenylglycerol) and of the glycerol acetal of plasmenylethanolamine has been studied in cultures of *Clostridium butyricum* IFO 3852. When growing cells were pulsed with [^{32}P]orthophosphate, there was a lag of 5 to 7 min between the rapid incorporation of label into the acylphosphatides and the rapid incorporation of label into the corresponding plasmalogens. The labeling of the glycerol acetal of plasmenylethanolamine was even slower. In pulse-chase experiments with $^{32}P_i$, the kinetics of labeling indicated precursor-product relationships between phosphatidylethanolamine and plasmenylethanolamine and between the latter and its glycerol acetal. A precursor-product relationship was also seen between phosphatidylglycerol and cardiolipin, but the kinetics of labeling of the alkenyl-containing forms of these lipids were not consistent with direct precursor-product relationships with the acyl lipids. In the presence of hydroxylamine and $^{32}P_i$, both phosphatidylserine and plasmenylserine accumulated ^{32}P in a ratio of ca. 15:1. Upon release of the inhibition of phosphatidylserine decarboxylase, label appeared in the following sequence: phosphatidylethanolamine, plasmenylethanolamine, and the glycerol acetal of plasmenylethanolamine. Acyl phosphatidylglycerol was identified as a major phospholipid (17% of lipid phosphorus) in *C. butyricum* grown in low-phosphate (1.13 mM) medium with 50 mM Tris buffer. Of the acyl phosphatidylglycerol, 13% was acid labile. There appear to be two plasmalogen forms of acyl phosphatidylglycerol. One of these has a single alkenyl ether group, and the other has alkenyl ether groups on both glycerols.

Many obligately anaerobic bacteria including gram-negative and gram-positive species, spirochetes, and mycoplasma contain 1-*O*-alk-1′-enyl-2-acyl glycerol phospholipids (plasmalogens) (12, 18, 23, 42). These alkenyl ether lipids are usually present with diacylphospholipids of the same phospholipid classes and often represent more than half of the total phospholipids (12, 15, 23). Thus plasmalogen analogs of phosphatidylethanolamine (PE), phosphatidyl-*N*-methylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol (PG), and cardiolipin (CL) have been found in various procaryotes, and, as is true for the diacylphosphatides, there is species specificity in their distribution (12, 15).

It is now known that plasmalogens in animal tissues are synthesized by a pathway that culminates in the oxygen-dependent removal of two hydrogens from a saturated ether lipid precursor. This reaction involves cytochrome b_5 and a reduced pyridine nucleotide (21). We have previously summarized evidence against this aerobic pathway to plasmalogens in anaerobic bacteria (12, 15). Among the most important of these findings is the fact that dihydroxyacetonephosphate, the precursor of alkyl and alkenyl ether lipids in animal tissues, does not serve as the precursor to plasmalogens in anaerobic bacteria (19, 33). As indicated above, molecular oxygen is required for the formation of the alk-1-enyl bond in animal cells, and this is not available to strict anaerobes.

In an earlier study of the formation of the plasmalogen forms of PE (plasmenylethanolamine) and phosphatidyl-*N*-methylethanolamine (plasmenyl *N*-methylethanolamine) of *Clostridium beijerinckii* ATCC 6015 (formerly *Clostridium butyricum*), we observed a lag of 5 to 10 min between the beginning of incorporation of labeled inorganic phosphate into the diacylphospholipids and into their corresponding plasmalogens (3). This finding led to the suggestion that the backbones of the diacylphosphatides may serve as precursors of plasmalogens in this species. In a later test of this hypothesis, P.-O. Hagen performed pulse-chase experiments with $^{32}P_i$ which pointed even more strongly to a precursor-product relationship between diacylphosphatidylethanolamine and plasmenyl *N*-methylethanolamine (12). Since these experiments were performed, a major unknown phospholipid of *C. butyricum* IFO 3852 was identified as a glycerol acetal of plasmenylethanolamine (27). The corresponding glycerol acetal of plasmenyl *N*-methylethanolamine is found in *C. beijerinckii* (14, 22). More recent studies with extracts of *C. beijerinckii* ATCC 6015 have reported nearly undetectable levels of phosphatidylserine decarboxylase, raising the question of the origin of PE in this organism (37, 38). We have therefore examined the kinetics of labeling of the plasmalogens of *C. butyricum* IFO 3852 in pulse and pulse-chase experiments with radioactive inorganic phosphate. When hydroxylamine, an inhibitor of phosphatidylserine decarboxylase, was added to growing cultures, ^{32}P-labeled phosphatidylserine and plasmenylserine accumulated in a ratio of ca. 15:1. When the inhibitor was removed, the ^{32}P-specific activities of both forms of serine phosphatide declined rapidly leading to a sequential accumulation of PE, plasmenylethanolamine, and the glycerol acetal of plasmenylethanolamine.

MATERIALS AND METHODS

Material. [^{32}P]orthophosphoric acid, carrier free, was obtained from Amersham Corp., Arlington Heights, Ill. [^{33}P]

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TABLE 1. Phospholipid composition of *C. butyricum* in low-phosphate medium[a]

Phospholipid	^{33}P (% of total)			% Plasmalogen
	Diacyl	Plasmalogen	Total	
Phosphatidylethanolamine	10.7[b]	4.7	15.4	30.5
Glycerol acetal of plasmenylethanolamine		23.1	23.1	
Phosphatidylglycerol	14.1	1.0	15.1	6.6
Cardiolipin	16.9	3.0	19.9	15.1
Acyl phosphatidylglycerol	17.2	2.5	19.7	12.7
X			0.4	
Z			0.9	
Other			5.5	

[a] Average of from 11 to 17 measurements on three separate cultures grown in 1.125 mM phosphate plus 0.05 M Tris with $^{33}P_i$, as described in the text.
[b] See reference 13 for phospholipid composition of cells grown in standard BS medium.

orthophosphoric acid, carrier free, and L-[*U*-^{14}C]serine (168 mCi/mmol) were from New England Nuclear Corp., Boston, Mass. Lipid standards were purchased from Serdary Research Laboratories, London, Ontario, Canada, or Sigma Chemical Co., St. Louis, Mo. All other reagents were reagent grade or better.

Cells and culture conditions. *C. butyricum* IFO 3852 was obtained from M. Matsumoto, College of Pharmacy, Shizuoka, Japan. For labeling with radioactive phosphate, these bacteria were grown in a Casamino Acid medium (Difco Laboratories, Detroit, Mich.) (6) modified to reduce the phosphate content as follows: Casamino Acids were treated with $MgCl_2$ to reduce the phosphate content (26), and the concentration of added phosphate buffer was reduced to 1.13 mM. Tris-hydrochloride buffer, pH 7.3, at a final concentration of 0.05 M, was added. A 2-ml sample of a culture grown anaerobically overnight in the standard medium of Broquist and Snell (BS medium) (6), containing 6.75 mM phosphate and $^{33}P_i$, as indicated for individual experiments, was used to inoculate 30 ml of freshly autoclaved, low-phosphate medium containing $^{33}P_i$ in a 40-ml glass centrifuge tube. At early logarithmic phase, as judged by turbidity measurements in parallel cultures, $^{32}P_i$ was added as indicated. In experiments with hydroxylamine, 0.01 volume of 500 mM NH_2OH, pH 7.3, was added 2 min before the addition of $^{32}P_i$. In $^{32}P_i$ incorporation studies, 4-ml samples of culture were removed under a flow of N_2 at the times indicated. These samples were pipetted onto 2 g of ice in 15-ml centrifuge tubes and kept on ice until all samples were harvested. Carrier cells (20 mg wet weight) were added to each sample. In pulse-chase experiments, after 20 min of incubation with $^{32}P_i$ in the absence of hydroxylamine or 30 min in the presence of hydroxylamine, cells were collected by centrifugation, usually at 9,000 × *g* for 10 min. They were washed once at 15 to 20°C with 0.05 M potassium phosphate buffer, pH 7.3, under N_2, suspended in 60 ml of fresh, prewarmed medium containing $^{33}P_i$ at the original specific activity, and allowed to resume anaerobic growth. Samples of 7 ml each were removed at the times indicated and pipetted onto 3.5 g of ice. Samples, to which carrier cells were added, were kept on ice until they were centrifuged and washed once with 0.05 M KPO_4, as described above. Cell pellets were usually stored overnight at −20°C until the lipids were extracted. Inocula and experimental cultures were made anaerobic by the pyrogallol double-plug technique (17) until sampling was begun. In 30-ml centrifuge tubes and 65-ml bottles, the double plugs were placed in glass tubes inserted in one-hole rubber stoppers. After sampling was begun, the cells were kept under a gentle flow of nitrogen.

Lipid extraction. Cell pellets were suspended in 0.8 ml of water, and lipid was extracted by the method of Bligh and Dyer (4). The upper phase was removed, and the lower phase with interfacial precipitate was passed through a 4.5-cm Whatman 1 PS filter paper. The centrifuge tubes and filter paper were washed with a small volume of choloroform.

Separation and assay of phospholipids. Except where indicated, phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on commercial silica gel plates. Alk-1-enyl ether lipids were hydrolyzed between the first and second dimensions by exposing the plates to HCl fumes for 5 min in a glass chromatography tank, prewarmed in a 80°C oven for 5 min (43).

TLC was carried out as indicated for individual experiments. The following solvent systems were used: (i) chloroform–methenol–7 N ammonia (60:35:5, vol/vol/vol); (ii) chloroform-methanol-acetic acid (65:25:8, vol/vol/vol); (iii) chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol/vol/vol); (iv) EDTA (3.8 mM) and ammonium bicarbonate (0.7 M) in a 90 mM ammonia solution made with 67% (vol/vol) of alcohol (36); (v) isobutyric acid-water-ammonia (66:33:1, vol/vol/vol), and (vi) hexane-diethylether (3:2, vol/vol).

Purification of acyl PG. *C. butyricum* IFO 3852 was grown in 27 liters of BS medium containing 6.75 mM KOP_4 plus 0.05 M Tris-hydrochloride. Lipids were extracted with chloroform-methanol (2:1, vol/vol) (11). A portion of the lipid, 174 mg, in chloroform was applied to an 11.5-g BioSil A column (Bio-Rad Laboratories, Richmond, Calif.) (1.6 cm by 15 cm) and eluted with 300 ml of chloroform (fraction I), 600 ml of acetone (fraction II), and 300 ml each of methanol-chloroform (vol/vol), 1:19 (fraction III), 1:9 (fraction IV), and 1:4 (fraction V). Two-thirds of the acyl PG eluted in fraction IV, and 20% eluted in fraction III. Acyl PG in fraction IV was separated preparatively from CL on silica gel Prekotes (Alltech Associates, Inc., Applied Science Div., State College, Pa.) in chloroform-methanol-water-ammonia (130:60:6:3.4, all vol). It was detected by spraying with water and eluted from the scraped gel with 5 ml of chloroform-ethanol-water (3:10:2, vol/vol/vol). The yield was 61.5 μg of lipid phosphorus or 2.1 mg of lipid, assuming an average molecular weight of 1,050. ^{33}P-labeled acyl PG was purified from cells grown in 50 ml of low-phosphate Casamino Acid medium containing 843 μCi of [^{32}P]orthophosphate. The cells were extracted with chloroform-methanol (4), and the lipids were chromatographed on silica gel by two-dimensional chromatography in solvent system A followed by solvent system B. After autoradiography, lipids were eluted with chloroform-methanol-water (1:2:0.8, vol/vol/vol) followed by chloroform and water (4). The lipid was recovered from the chloroform phase after separation.

Liquid scintillation counting. Radioactive lipids adsorbed to silica gel scraped from thin-layer plates were suspended with vigorous shaking in 5 ml of BioCount (Research Products International Corp.) with 0.5 ml of water. Double-labeled samples were counted under conditions that produced 0.3 to 0.4 fractional spillover of the ^{32}P counts into the ^{33}P channel, and the ^{33}P counts were corrected accordingly.

Analytical methods. Acetic acid hydrolysis of plasmalogens was performed at 37°C in 90% acetic acid (22). Mild alkaline methanolysis was done as described (24), and the resulting glycerophosphate esters were chromatographed on thin-layer plastic-backed cellulose sheets (10 by 10 cm) with

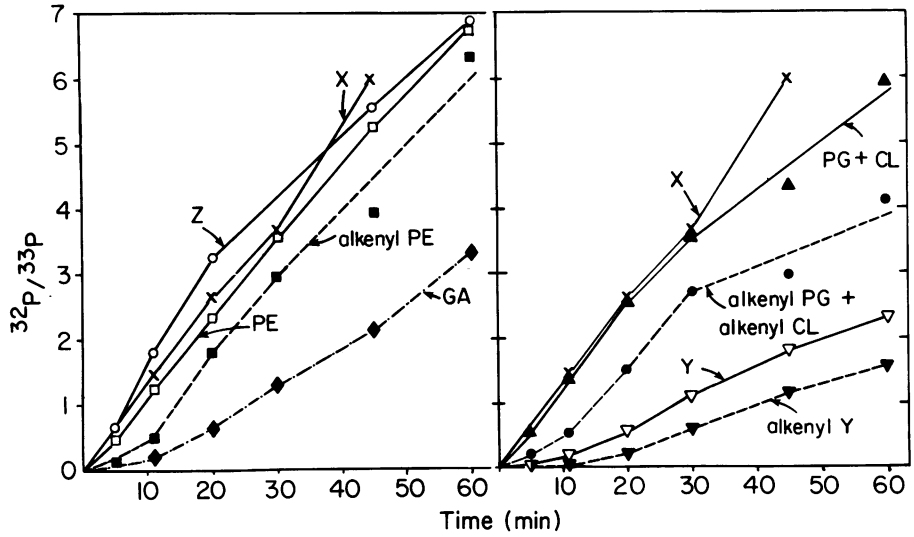

FIG. 1. Incorporation of $^{32}P_i$ into *C. butyricum* IFO 3852 phospholipids. A 32-ml culture containing low-phosphate Tris medium and $^{33}P_i$ (2.15 μCi/μmol) was inoculated with 2.0 ml of an overnight culture in standard BS medium. At the early logarithmic phase of growth (ca. 40 Klett units), $^{32}P_i$ was added at a specific activity of 19.3 μCi/μmol. At the times indicated, 4-ml samples were removed and treated as described in the text. After extraction, lipids were separated on silica gel G plates (0.3-mm thick; 20 by 20 cm). The plates were developed in both dimensions in solvent system A and treated with HCl fumes between the first and second developments. Symbols: PE, □; plasmenylethanolamine (alkenyl PE), ■; glycerol acetal of plasmenylethanolamine (GA), ◆; X, ×; Z, ○; PG + CL, ▲; plasmenylglycerol (alkenyl PG) plus CL containing an alkenyl chain (alkenyl CL), ●; acyl PG (Y), ▽; acyl PG with two alk-1′-enyl groups, ▼. The corrected values for both isotopes ranged from 100 to 17,600 cpm, except for the following: X, 100 cpm of ^{33}P and 0 to 453 cpm of ^{32}P; alkenyl PG and CL, 30 cpm of ^{32}P at 5 min; alkenyl Y, 3 and 19 cpm of ^{32}P at 5 and 11 min, respectively.

solvent systems D and E, in the first and second dimensions, respectively (36). Acetolysis was done as described by Renkonen (34), and the products of acetolysis were chromatographed on silica gel with solvent system F. Phosphate was determined by the methods of Bartlett (2) or Ames (1), and glycerol was determined by the method of Bok and Demain (5).

RESULTS

Phospholipid composition in low-phosphate medium containing Tris-hydrochloride buffer. To study the kinetics of radioactive phosphate incorporation into the phospholipids of *C. butyricum* IFO 3852, the concentration of added phosphate in BS medium (6) was reduced from 6.75 mM to 1.125 mM. The Casamino Acid solution was depleted of phosphate by treatment with $MgCl_2$ (26), and 50 mM Tris-hydrochloride was added. Growth of *C. butyricum* in this medium resulted in an increase in the proportion of a relatively nonpolar phospholipid, which represented from <1 to 2% of the total phospholipid in cells grown in BS medium (Table 1). Adding 0.05 M Tris buffer to standard BS medium containing 6.75 mM phosphate resulted in a smaller change in the content of the unknown lipid, from 2 to 4% of total lipid phosphorus in cells grown overnight (data not shown).

Characterization of acyl PG. The unlabeled lipid isolated as described above was mixed with purified ^{32}P-labeled lipid and deacylated by alkaline methanolysis. Two-dimensional chromatography of the resulting glycerophosphate esters followed by autoradiography revealed one heavily labeled spot and two minor components. The major component corresponded to standard glycerophosphorylglycerol. One of the faint spots corresponded to glycerophosphate, and the second, which migrated near the solvent fronts, may have represented alkali-stable alk-1-enyl glycerol phosphate (see below). The glycerol/phosphate molar ratio of the deacylation product was 1.89.

Chromatography of ^{32}P-labeled unknown lipid, which had been purified by preparative two-dimensional TLC (see above) and mixed with carrier crude *C. butyricum* phospholipids, on DEAE-cellulose (35), resulted in total elution of the labeled unknown lipid in the 0.02 M ammonium acetate in chloroform-methanol (4:1, vol/vol) fraction. The unknown lipid was, therefore, assumed to have a net negative charge.

When the unknown lipid was subjected to acetolysis, TLC revealed that the major products were monoacyldiacetyl glycerol, diacylmonoacetyl glycerol, and triacetylglycerol, as visualized by charring with 20% ammonium sulfate and 4% H_2SO_4 in water. The first two products are consistent with acyl PG (25), but the formation of triacetylglycerol is suggestive of the presence of PG. However, the unknown was essentially free of PG, as judged by TLC in solvent system A, and the formation of triacetylglycerol could also be explained by the presence of an alk-1-enyl ether bond on the mono-substituted glycerol. Of the unknown lipid, ca. 13% was found to be acid labile on HCl treatment, and most of the product migrated like lyso-PG, suggesting the presence of alk-1-enyl ether bonds on both the di-substituted and mono-substituted glycerols.

The unknown ^{32}P-labeled lipid was cochromatographed on silica gel by two-dimensional TLC in solvent system A followed by solvent system B with unlabeled acyl PG provided by S. Nojima, Tokyo University, Tokyo, Japan. Autoradiography and staining with iodine vapor showed that the two compounds were superimposable.

Kinetics of [^{32}P]orthophosphate incorporation into *C. butyricum* phospholipids. Pulse-labeling experiments were done to study the kinetics of incorporation of inorganic phosphate into diacylphosphatides, plasmalogens, the glycerol acetal lipid, and acyl PG. Cells were continuously labeled with $^{33}P_i$

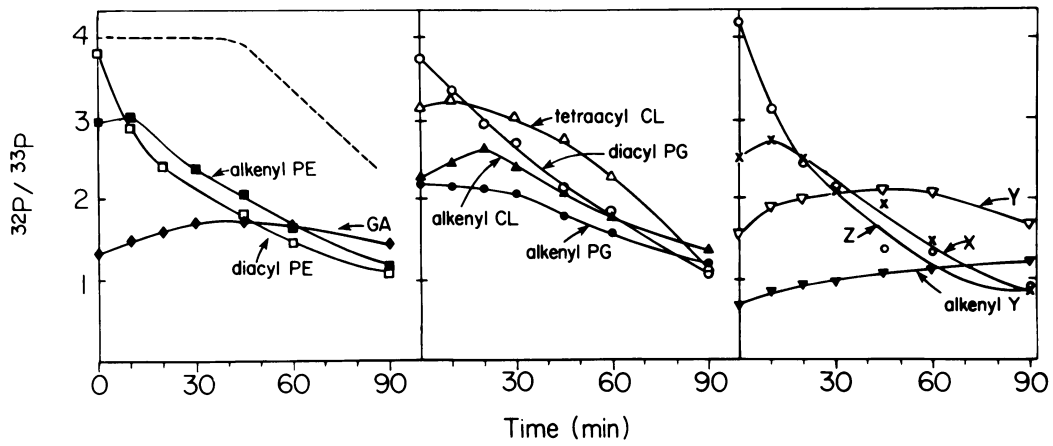

FIG. 2. Chase of ^{32}P in phospholipids of *C. butyricum* IFO 3852 after pulse-labeling. Low-phosphate medium (30 ml) containing $^{33}P_i$ (3.6 μCi/μmol) was inoculated with 1.5 ml of overnight culture labeled with $^{33}P_i$ at the same specific activity. At 50 Klett units, $^{32}P_i$ (29.6 μCi/μmol) was added, and incubation was continued. After 20 min, the culture was centrifuged and washed once in 30 ml of sterile 0.05 M KPO_4 (pH 7.3) at 15 to 20°C under N_2 atmosphere. The cells were suspended in 60 ml of low-phosphate medium with $^{33}P_i$ (3.6 μCi/μmol), and incubation was continued. Samples were removed at the times indicated and were processed as described in the text. The lipids were chromatographed on silica gel G Prekotes as described in the legend for Fig. 1. Abbreviations and symbols are as listed in the legend to Fig. 1. The dashed line indicates the expected change in ratio from dilution of the pulse-label by growth of the cultures. The corrected counts per minute for both isotopes ranged from 100 to 7,300, except for X, which was <100 for both isotopes; Z was 22 to 165 cpm for both isotopes.

and then pulsed with $^{32}P_i$ in the presence of $^{33}P_i$. Thus the ratio $^{32}P/^{33}P$ represents relative ^{32}P-specific activity. Phospholipids were separated by two-dimensional TLC in solvent system A with treatment with HCl fumes between runs, to cleave the plasmalogens. The ratio of $^{32}P/^{33}P$ in total lipid increased linearly with time for ca. 40 min after a lag period of 10 to 20 min. After 90 min, the curve was almost flat (data not shown). The initial lag reflects the slow labeling of several of the major lipids. The kinetics of $^{32}P_i$ incorporation into the separated phospholipids of *C. butyricum* IFO 3852 are shown in Fig. 1. It can be seen that ^{32}P-labeling of PE and the fraction containing PG plus CL occurred with very little lag, indicating rapid equilibration of precursor pools, but ^{32}P labeling of the corresponding plasmalogens, designated alkenyl PE and alkenyl PG plus alkenyl CL, occurred after a lag of 5 to 7 min. The lag in labeling of plasmenylethanolamine confirms the results of our earlier experiments with *C. beijerinckii* ATCC 6015 in which ^{32}P-specific activity was determined after chemical phosphate analysis. The kinetics of labeling of plasmenylglycerol and plasmalogen form(s) of CL were not studied in our earlier experiments. It can also be seen that labeling of the glycerol acetal derivative of plasmenylethanolamine became linear after a lag of ca. 10 min. As in the case of the glycerol acetal lipid, ^{32}P incorporation into acyl PG was considerably delayed, followed by a further lag before acyl PG containing two alk-1-enyl ether bonds became labeled. The phospholipids labeled X and Z represented <1% of the ^{33}P in phospholipids and have not been identified.

Labeling of phospholipids during a chase period following pulse-labeling with [^{32}P]orthophosphate. These results, along with earlier findings in pulse-chase experiments with *C. beijerinckii* ATCC 6015 (12), suggested that the glycerol phosphate backbone of the plasmalogens passed through a large pool of lipid precursor. We, therefore, carried out pulse-chase experiments in which [^{32}P]orthophosphate was incorporated during a 20-min pulse under conditions of continuous labeling with [^{33}P]orthophosphate. The turnover of PE during the initial period of the chase was relatively rapid, with a half time of ca. 20 min. (Fig. 2). The cells resumed growth 45 min after suspension in fresh medium, as determined by measurements of turbidity. The doubling time in this medium was 55 min. During the first 10 min of the chase, the relative ^{32}P-specific activity of plasmenylethanolamine remained almost constant, followed by a decline from 30 to 90 min after the chase, at a rate which closely matched that of PE, with $t_{1/2}$ values of 68 and 62 min, respectively. The ^{32}P-specific activity of the glycerol acetal of plasmenylethanolamine increased for 45 min after the chase after which it began to decline slowly. A relationship similar to that of PE and plasmenylethanolamine can be seen between the relative ^{32}P-specific activities of PG and CL (Fig. 2). The two curves cross at the apogee of the CL curve, but the decline in relative specific activity of CL is slower for ca. 30 min compared with that of PG and the two ethanolamine phosphoglycerides. The ^{32}P-specific activity of CL containing an alk-1-enyl ether bond is seen to rise for 20 min after the chase and then to decline relatively slowly, whereas that of plasmenylglycerol declined throughout the period of measurement, slowly at first and then more rapidly, with a slope similar to that of the alkenyl ether bond-containing CL. The curve for acyl PG (Fig. 2) is similar to that for the glycerol acetal of plasmenylethanolamine, whereas that for the alkenyl acyl PG shows a continuous, slow rise in specific activity.

Labeling of phosphatidylserine in the presence of hydroxylamine. *Escherichia coli* (10), *Bacillus megaterium* (30), and *Megasphaera elsdenii* (32) accumulate phosphatidylserine during incubation with hydroxylamine, an inhibitor of phosphatidyserine decarboxylase. When *C. butyricum* was incubated in the presence of $^{32}P_i$ and 5 mM hydroxylamine, we initially observed inhibition of PE synthesis and accumulation of a polor phospholipid which migrated more slowly (R_f = 0.05) on TLC on Prekote silica gel G plates (Alltech Associates, Inc., Applied Science Div.) in solvent system A than did standard phosphatidylserine (R_f = 0.12) (38). However, on TLC with 30 μg of carrier phosphatidylserine in this solvent system, the unknown phospholipid was shown to

comigrate with the carrier. ^{32}P-labeled lipid without added carrier did not show this anomolous behavior in solvent systems B and C or in solvents A on Prekote Adsorbosil-5 (Alltech Associates, Inc., Applied Science Div.) or on Whatman K5 silica gel 80A plates. Deacylation of the unknown lipid plus carrier phosphatidylserine by alkaline methanolysis followed by two-dimensional TLC of the resulting water-soluble glycerol phosphate ester and autoradiography showed cochromatography of ^{32}P and ninhydrin-positive glycerylphosphorylserine. The unknown ^{32}P-labeled phospholipid was eluted from a DEAE-cellulose column (35) by acetic acid, as expected for phosphatidylserine. Both phosphatidylserine and plasmenylserine accumulated in *C. butyricum* in the presence of hydroxylamine (Fig. 3). In this experiment, 4.8% of the total serine phosphoglyceride was plasmenylserine based on counts per minute of ^{33}P. The plasmenylserine was labeled almost as rapidly as the diacyl lipid, but its relative specific activity was consistently lower at all time points.

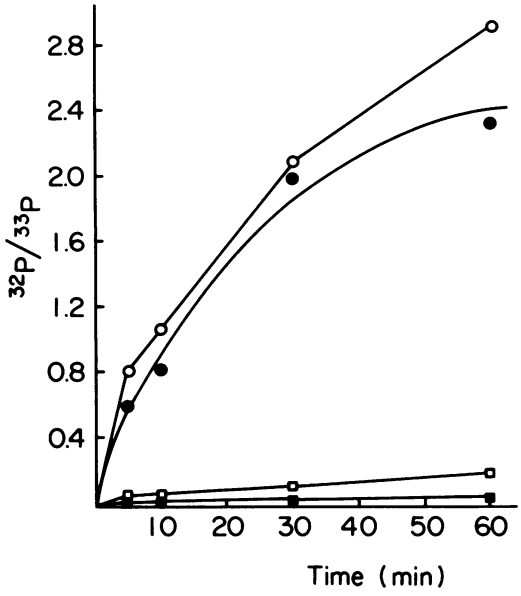

FIG. 3. Incorporation of $^{32}P_i$ into phosphatidylserine and plasmenylserine during inhibition with hydroxylamine. Cells were grown with $^{32}P_i$ (4 μCi/μmol) as described in the legend for Fig. 1. During the logarithmic growth phase, neutral hydroxylamine (5 mM) was added followed 2 min later by $^{32}P_i$ (44.4 μCi/μmol). At the times indicated, samples of 7 to 7.5 ml were removed and processed as described in the text. The lipids were separated by one-dimensional TLC on Prekotes Adsorbosil-5 in solvent system A. After autoradiography, the lipid spots corresponding to PE and phosphatidylserine were scraped off the plates and put in small glass columns, and the lipids were eluted with 3, 2, and 1 ml of chloroform-methanol-water (10:10:1, vol/vol/vol). The extracts were centrifuged for 10 min at 1,500 rpm in a desk top centrifuge to remove any remaining silica gel. The lipids were treated with 90% acetic acid overnight at 37°C after addition of 212 μg of *Megasphaera elsdenii* phospholipids as carrier (33). The hydrolysate was lyophilized, and the lipids were separated on the same type of thin-layer plate in solvent system C. The lipids were stained with I_2 vapor, and the spots corresponding to the diacylphosphatides and the lysophosphatides derived from the plasmalogens were scraped off and counted. The R_f values were as follows: PE, 0.90; lyso-PE, 0.59; phosphatidylserine (PS), 0.75; and lyso-PS, 0.46. Symbols: phosphatidylserine (○), plasmenylserine (●), PE (□), and plasmenylethanolamine (■).

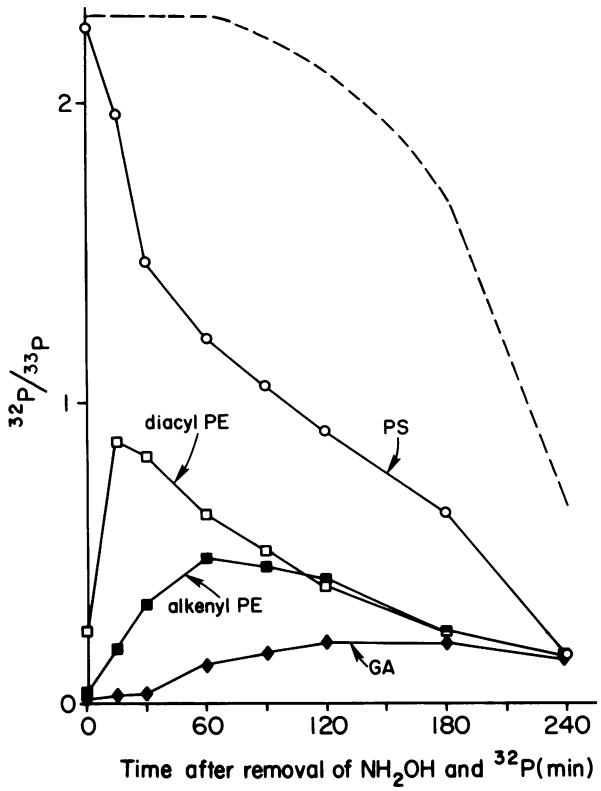

FIG. 4. Turnover and labeling of phospholipids in *C. butyricum* IFO 3852 after a pulse of $^{32}P_i$ in the presence of hydroxylamine. Cells were cultured as described in the legend for Fig. 2. $^{33}P_i$ (4.0 μCi/μmol) was present continuously, and $^{32}P_i$ (29 μCi/μmol) was added 2 min after the addition of neutral hydroxylamine to a final concentration of 5 mM. After 30 min, the cells were centrifuged and suspended as described in the legend for Fig. 2, except that $^{33}P_i$ was present at 4.0 μCi/μmol. The samples were processed as described in the legend for Fig. 2. Abbreviations: PS, phosphatidylserine; GA, glycerol acetal of plasmenylethanolamine. The dashed line indicates the expected change in ratio from dilution of the pulse-label by growth of the cultures. The corrected values for both isotopes ranged from 95 to 6,400 cpm, except for alkenyl PE, which was 46 cpm of ^{32}P at 0 min.

Kinetics of labeling of ethanolamine phosphatides from serine phosphatides accumulated during hydroxylamine treatment. After a 30-min incubation of cells with $^{32}P_i$ in the presence of 5 mM hydroxylamine, the serine phosphoglycerides contained 30% of the lipid ^{32}P. When hydroxylamine was removed, there was a rapid turnover of total serine phosphoglyceride concomitant with an increase in the ^{32}P-specific activity of PE (Fig. 4). There was a slower appearance of label in plasmenylethanolamine, and the appearance of ^{32}P in the glycerol acetal of plasmenylethanolamine was delayed for at least 15 min. In a subsequent experiment, the turnover rates of phosphatidylserine and plasmenylserine were measured separately. As noted above, there was about 15- to 20-fold more of the diacyl species, and the relative specific activity of phosphatidylserine was 1.8-fold that of plasmenylserine, in the initial sample. Both serine phosphoglyceride species were rapidly metabolized, and the relative specific activity curves declined essentially in parallel (Fig. 5). As in the preceding experiment, the relative specific activity of PE increased more rapidly than that of plasmenylethanolamine and began to decline during the rising portion

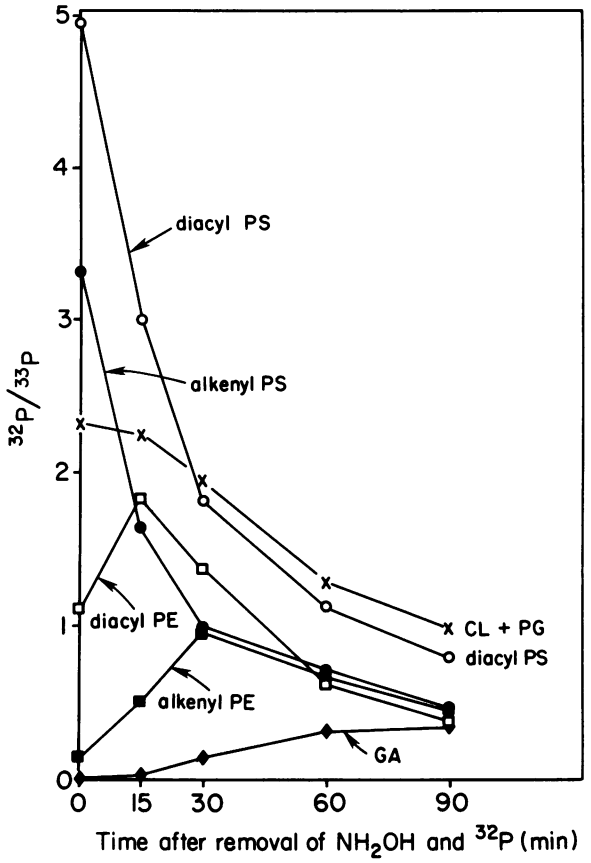

FIG. 5. Turnover of phosphatidylserine and plasmenylserine in *C. butyricum* IFO 3852 after a pulse of $^{32}P_i$ in the presence of hydroxylamine. Cells were cultured as described in the legend for Fig. 2 except that volumes were halved. The specific activities of $^{33}P_i$ and $^{32}P_i$ were 4.0 μCi/μmol and 59 μCi/μmol, respectively. The cells were washed and suspended as described in the legend for Fig. 2, and 6-ml samples were taken at the times indicated. The samples were processed as described in the legend for Fig. 3 except that carrier *M. elsdenii* lipid was added to the hydrolyzed samples after they had been hydrolyzed with 0.025 N HCl in chloroform-methanol-water (20:19:1, vol/vol/vol) at 37°C for 30 min. Abbreviations are as listed in the legends to Fig. 1 and Fig. 4. The corrected values for both isotopes ranged from 100 to 12,270 cpm, except for alkenyl PS, which was 35 to 90 cpm of ^{33}P and 16 to 299 cpm of ^{32}P.

of the plasmenylethanolamine curve. There was again a significant lag before the relative specific activity curve of the glycerol acetal of plasmenylethanolamine began its rise.

DISCUSSION

The anaerobic biosynthesis of plasmalogens in bacteria has been studied in this (3, 12, 37, 38) and other laboratories (19, 32, 33), and it is agreed that the mechanism of formation of ether phospholipids in bacteria must differ from that existing in higher organisms, which involves an oxygen-dependent desaturation of a saturated ether precursor arising from dihydroxyacetone phosphate (16, 21, 39).

Our earlier work with *C. beijerinckii* ATCC 6015 demonstrated a significant lag in the labeling of the plasmalogen forms of PE and phosphatidyl-*N*-methylethanolamine when growing cells were pulsed with [^{32}P]orthophosphate (3). When cells were labeled with [^{32}P]orthophosphate and chased with nonradioactive phosphate, total radioactivity in PE decreased, with a concomitant increase in radioactivity in the plasmalogen form of phosphatidyl-*N*-methylethanolamine (12). These experiments suggested precursor-product relationships between the diacylphospholipids and the corresponding plasmalogens.

Prins et al. (32) studied the biogenesis of plasmalogens in *Megasphaera elsdenii*, in which the two major phospholipids are plasmenylserine (26%) and plasmenylethanolamine (57%). There are smaller amounts of phosphatidylserine (10%) and PE (7.0%). When phospholipids were labeled by adding $^{32}P_i$ to growing cultures, the most rapidly labeled phospholipid was phosphatidylserine. There appeared to be a lag in the accumulation of label in plasmenylserine, but the extent of the lag phase could not be measured since the first time point, 15 min, showed significant labeling of this fraction. PE and plasmenylethanolamine labeling showed lags of almost 15 min. When cells were grown in $^{32}P_i$ and transferred into nonradioactive medium, a redistribution of label between phosphatidylserine and PE was evident, and a similar reciprocity of the curves of ^{32}P in plasmenylserine and plasmenylethanolamine was also observed. These authors (32) concluded that plasmenylserine gave rise to plasmenylethanolamine by decarboxylation. When the decarboxylase was inhibited in vivo by the addition of 2 mM hydroxylamine, there was a strong inhibition of plasmenylethanolamine formation and a concomitant accumulation of label in plasmenylserine. The data for lower concentrations of hydroxylamine were strongly suggestive of the postulated precursor-product relationship between plasmenylserine and plasmenylethanolamine.

The situation of *C. butyricum* differs fundamentally from that of *M. elsdenii* because the pool of serine phosphatides is very small. The findings that phosphatidylserine and plasmenylserine accumulate in hydroxylamine-treated cells (Fig. 3) and that ^{32}P flows from the accumulated ^{32}P-labeled serine phosphoglycerides into the ethanolamine phosphoglycerides after removal of the inhibitor (Fig. 4) indicate that, in *C. butyricum* as in other bacteria, the serine-phospholipid decarboxylation pathway is involved in ethanolamine phospholipid synthesis. Previous work in our laboratory with membrane particles from extracts of *C. beijerinckii* ATCC 6015 showed that the level of phosphatidylserine decarboxylase activity was barely detectable (37, 38). Recent studies in this laboratory (J. Verma and H. Goldfine, unpublished data) have demonstrated significant activity of phosphatidylserine decarboxylase in membranes obtained from lysed protoplasts of both *C. beijerinckii* ATCC 6015 and *C. butyricum* ATCC 19398. Thus, both whole-cell and in vitro experiments now support the role of serine-phosphoglyceride decarboxylase in the formation of ethanolamine phosphoglycerides in *C. butyricum*.

Major unresolved questions concern the origin of plasmenylserine and plasmenylethanolamine in *C. butyricum*. When cells were inhibited with hydroxylamine and labeled with $^{32}P_i$, incorporation of ^{32}P into both phosphatidylserine and plasmenylserine was rapid, but the specific activity ($^{32}P/^{33}P$) of plasmenylserine was consistently slightly lower (Fig. 3). More diacyl (10- to 20-fold) form than plasmalogen accumulated during the period of inhibition. When the inhibitor was removed, both the diacyl and plasmalogen forms of serine phosphoglyceride were rapidly metabolized with ^{32}P appearing most rapidly in PE followed by its appearance in plasmenylethanolamine (Fig. 4 and 5). Since the pool size of phosphatidylserine was 11-fold that of plasmenylserine at the end of the period of hydroxylamine treatment in this experiment and since their respective

turnover times (Fig. 5) are essentially identical (30.3 and 27.0 min, respectively), the turnover rate of the former is therefore ca. >11-fold the latter (44). Under these conditions, the rate of conversion of plasmenylserine to plasmenylethanolamine is much slower than that of phosphatidylserine to PE. In normal growing cultures, the apparent turnover rate (R_t) of PE to plasmenylethanolamine determined graphically from a semilog plot of the data from Fig. 2 is 0.011 mg/min per liter of culture. Since we cannot measure the pool sizes or specific activities of the serine phosphoglycerides in growing cells, comparable calculations cannot be made. Nevertheless, our data support the concept that the major flow of labeled phosphate in *C. butyricum* is from phosphatidylserine to PE and that plasmenylethanolamine arises from a large pool of precursor lipid, presumably PE. Direct conversion of plasmenylserine to plasmenylethanolamine is unlikely to account for a significant fraction of plasmenylethanolamine synthesis because passage through the very small pool of plasmenylserine in uninhibited cells should result in no lag in the incorporation of labeled phosphate into plasmenylethanolamine, but a significant lag was observed (Fig. 2). Attempts were made to analyze these results quantitatively by computer modeling. Although no one model was found that gave a satisfactory fit to all of the post-inhibition data, all acceptable models supported the qualitative arguments made above (P. Renshaw and M. Pring, unpublished data).

Our findings with *C. butyricum* and *C. beijerinckii* and those with *M. elsdenii* can be reconciled by consideration of the respective pool sizes of their intermediates. In *M. elsdenii*, both phosphatidylserine and plasmenylserine accumulate. If the latter is derived from the former and both are decarboxylated at approximately equal rates, then the bulk of the plasmenylethanolamine will be derived from plasmenylserine, as concluded by Prins et al. (32). In *C. butyricum*, as noted above, the formation of the bulk of plasmenylethanolamine from plasmenylserine cannot be reconciled with our observations, whereas the results do support its formation from PE. From our experiments, conclusions can only be made about the polar head groups, and the possibility that the polar head groups transfer to preexisting alk-1-enyl ether lipids cannot be ruled out.

Although, in labeling experiments with $^{32}P_i$, lags were also seen in the labeling of the alk-1-enyl ether bond-containing forms of PG (plasmenylglycerol) and CL (Fig. 2), their formation from the corresponding diacyl and tetraacyl lipids cannot be assumed. The specific activity curves in the pulse-chase experiment without hydroxylamine (Fig. 2) clearly indicate a precursor-product relationship between PG and CL. Precursor-product relationships are not fulfilled in the cases of PG and plasmenylglycerol or CL and its plasmalogen analog(s). The curves of PG and plasmenylglycerol and of CL and its plasmalogen analog intersect at a time point considerably past the apexes of the curves for the plasmalogens. Passage of phosphate through other pools of lipid precursors is still a possibility in these cases. The curves for PG and acyl PG do follow precursor-product relationships. Nojima et al. have presented evidence for the formation of acyl PG in *E. coli* by transfer of acyl groups from 2-acyl lyso lipids to PG (20, 28).

The glycerol acetal of plasmenylethanolamine appears to be formed from plasmenylethanolamine. A precursor-production relationship was observed in pulse-chase experiments (Fig. 2), the synthesis of the glycerol acetal was inhibited in hydroxylamine-treated cells (data not shown), and labeling with ^{32}P was delayed beyond the slow appearance of label in plasmenylethanolamine, when hydroxylamine inhibition was relieved (Fig. 4 and 5). Recent studies of phospholipid asymmetry in the plasma membrane of *C. butyricum* IFO 3852 show that ca. 80% of the glycerol acetal lipid and 70% of diacyl and plasmalogen forms of PE are in the outer monolayer (13).

The accumulation of acyl PG in *C. butyricum* grown in low-phosphate medium containing Tris was unexpected. We have grown both *C. butyricum* and *C. beijerinckii* in various media, including BS medium (6), a richer medium containing peptone and yeast extract, and media containing fatty acids in place of biotin, and have not observed major changes in the proportion of this phospholipid although the ratios of other phospholipids were affected (13, 14, 22). Kaplan et al. (7, 9) have reported an analogous situation in *Rhodopseudomonas sphaeroides*, *Rhodopseudomonas capsulata*, and *Paracoccus denitrificans*, which accumulate *N*-acylphosphatidylserine in Tris-supplemented medium. In 20 mM Tris, *N*-acylphosphatidylserine represented 28 to 40% of total lipid phosphorus in these species. We have not studied the effects of concentrations of Tris above 50 mM on the phospholipid composition of *C. butyricum*. At this concentration of Tris, acyl PG only accumulated when the added phosphate concentration was lowered from 6.8 mM to 1.125 mM. It is of interest that the photosynthetic gram-negative bacteria and *C. butyricum* accumulate a negatively charged lipid with an acyl chain on the polar head group in response to Tris addition. In *C. butyricum*, PG appears to be the precursor of acyl PG, based on the kinetics of labeling we observed. Further studies are needed to determine the effects of varying the concentration of Tris and lowering the concentration of phosphate independently. Acyl PG has been observed in enteric bacteria (8, 29), in a sterol nonrequiring mycoplasma (31), and in *Pseudomonas* BAL-31 (40). In the enteric bacteria and in *Pseudomonas* BAL-31, acyl PG is a minor phospholipid containing <2% of total lipid phosphorus. In the *Mycoplasma* strain, acyl PG appeared to represent ca. 10 to 15% of the total phospholipid. As noted above, the formation of acyl PG in *E. coli* from PG (8) with 2-acyl glycerophosphorylglycerol or 2-acyl glycerophosphorylethanolamine serving as the acyl donor has been proposed (20, 28).

Our finding of significant amounts of this unusual phospholipid in *C. butyricum* grown in low-phosphate Tris medium will permit future studies of its stereochemistry, the mechanism and topology of its biosynthesis, and the regulation of its formation in a gram-positive organism. The presence of alk-1-enyl ether bonds in this lipid is novel, and the mechanism of their formation in this phospholipid and the other lipids of the butyric acid-producing clostridia is of considerable mechanistic and evolutionary interest.

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