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Phospholipid Metabolism in the Absence of Net Phospholipid Synthesis in a Glycerol-Requiring Mutant of *Bacillus subtilis*

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A glycerol-requiring auxotroph of *Bacillus subtilis* showed no net synthesis of phospholipid when deprived of glycerol. Although there was no net synthesis of phospholipid, we found that: (i) fatty acids and ^{32}P were slowly incorporated into phospholipid; (ii) in pulse-chase experiments, both ^{32}P and ^{14}C in the glycerol portion of the phospholipids were lost from phosphatidlyglycerol (PG) and lysylphosphatidylglycerol and accumulated in cardiolipin (CL); (iii) the proportions of the phospholipids in the membrane changed with a loss of PG and an accumulation of CL. The addition of glycerol to the glycerol-deprived cells resulted in a rapid incorporation of glycerol and restoration to the predeprivation metabolism and PG to CL ratio.

Lipids form a significant fraction of the mass of the bacterial membrane (15), and their synthesis and catabolism appear to be correlated with the formation and function of some of the membrane components (14). Modification of the electron transport system in *Haemophilus parainfluenzae* (21) or formation of the total membrane-bound electron transport system of *Staphylococcus aureus* (4, 7) occurs concomitantly with changes in lipid metabolism. At least in the case of *S. aureus*, inhibitors which affect carotenoid and phospholipid metabolism also greatly affect the formation and stability of the membrane-bound electron transport system (7), suggesting that the changes in lipid metabolism are involved in membrane modifications. The formation of an efficient lactose permease in *Escherichia coli* (6, 24) and the efficient function, but not the incorporation, of the phosphotransferase system in *S. aureus* (11) apparently require lipid synthesis. To study lipid involvement further, a mutant of *Bacillus subtilis* which was unable to synthesize or catabolize glycerol was selected by Mindich (9). This mutant requires glycerol for net synthesis of phospholipid, and nearly all of the labeled glycerol added to the cell can be recovered in the lipid extract (9). During glycerol deprivation, the rate of fatty acid synthesis slows to 25% of the rate during exponential growth with glycerol. This mutant, when deprived of glycerol, grows at the predeprivation rate for 30 min. This is followed by a slow increase in cell density (9). The cells remain viable for 4 hr in the absence of glycerol. In elegant experiments, Mindich (9, 10) showed that, during the period of slow increase in cell mass in the absence of net lipid synthesis, the mutant maintained a slow synthesis of deoxyribonucleic acid (DNA) until a doubling of the total DNA occurred. The cells were able to initiate a new round of DNA synthesis. The deprived cells support bacteriophage growth and induced enzyme formation; however, ribosomal ribonucleic acid (RNA) synthesis is much decreased, as when bacteria are transferred from rich to poor medium (shift-down). The cells thicken the cell wall and form the membrane-bound electron transport system, as assayed by succinic dehydrogenase activity. Incorporation of leucine into the cell membrane continues at the same rate as total protein synthesis in the deprived cultures, resulting in membranes with increased density.

The present study will show that, although there was no net increase in phospholipid during glycerol deprivation, there was active metabolism of the phospholipids that resulted in a shift in the proportions of the phospholipids, much like that seen in the fatty acid auxotroph of *E. coli* deprived of oleate (5). Addition of glycerol to the deprived cultures resulted in a return to the predeprivation lipid composition.

MATERIALS AND METHODS

Materials. Materials were obtained from suppliers as noted previously (17, 18).

Growth of bacteria. A mutant of *B. subtilis* W-23

designated B-67 was isolated and generously provided by L. Mindich. This mutant requires about 0.34 mM glycerol for optimal growth and cannot divide more than once at glycerol concentrations of less than 0.02 mM. This strain also lacks the pyridine nucleotide independent L-α-glycerol phosphate dehydrogenase which is the initial step in the catabolism of glycerol in this organism (8). The mutant B-67 contains a polyribitol teichoic acid rather than one containing a glycerol polymer. Mindich (9) has shown that, in this strain, 97 $\pm$5% of glycerol-2-^3H added to the cells can be recovered in the lipid extract. The bacteria were grown at 37 C in 1,500 ml of medium in 2,500-ml low-form Ehrlenmeyer flasks which were agitated 100 times per minute on a New Brunswick "gyratory" incubator shaker. The medium was made from the following solution: KCl, 0.02 M; K_2HPO_4, 0.6 mM; NaCl, 0.08 M; NH_4Cl, 0.02 M; Na_2SO_4, 0.14 M; adenine, 0.1 mM; xanthine, 0.1 mM; uracil, 0.1 mM; and tris(hydroxymethyl)aminomethane (Tris), 0.1 M. These solutions were mixed, brought to *p*H 7.4, and autoclaved. Biotin, 0.2 nM; nicotinic acid, 8.3 nM; thiamine, 0.93 nM; tryptophan, 0.2 nM; $FeCl_3$, 2 μM; $CaCl_2$, 0.1 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 5 mM; arginine, 0.14 mM; histidine, 0.12 mM; lysine, 0.34 mM; glutamic acid, 0.06 mM; glycine, 0.31 mM; alanine, 0.18 mM; valine, 0.26 mM; isoleucine, 0.2 mM; leucine, 0.4 mM; proline, 0.46 mM; phenylalanine, 0.02 mM; serine, 0.28 mM; threonine, 0.19 mM; tyrosine, 0.08 mM; cysteine, 0.15 mM; methionine, 0.12 mM; asparagine, 0.08 mM, and bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), 50 mg per liter were filter sterilized and added in the final concentration given.

The bacteria were grown to exponential phase (0.12 mg, dry weight, per ml), harvested by filtration on membrane filters (142-mm diameter; Millipore Corp., Bedford, Mass.) with an average pore size of 0.45 μm, washed with an equal volume of medium without glycerol, and resuspended in medium with or without glycerol. All operations were carried out at 37 C, and there was no lag in the growth of the bacteria. The harvesting, washing, and resuspending took less than 3 min.

Measurement of bacterial density. The density of the bacterial cultures was measured as the absorbancy at 750 nm in 13-mm round-bottom test tubes. The absorbancy was related linearly to the dry weight (19). An absorbancy of 0.5 corresponded to a dry weight of 0.23 mg per ml.

Isolation of the lipids. Portions (25 ml) of the bacterial suspension were added directly to a glass 250-ml separatory funnel containing 62.5 ml of methanol and 31.3 ml of chloroform. The mixture was shaken vigorously and allowed to settle for at least 3 hr. A 31.3-ml amount of chloroform and an equal volume of water were then added, and the mixture was shaken. After separation of the phases, the chloroform layer was filtered through anhydrous sodium sulfate. This is a modification of the Bligh and Dyer extraction procedure (3).

Paper chromatography. The diacyl phospholipids were separated by two-dimensional chromatography with silica gel loaded paper (Whatman SG-81, Reeve Angel Co., Clifton, N.J.). Up to 0.5 μmole of lipid phosphate was applied to one corner and chromatographed in the first dimension with chloroform-methanol-diisobutylketone-acetic acid-water (23:10:45:25:4, v/v) and in the second dimension with chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium chloride buffer, *p*H 10.4 (30:17.5:25:35:6; reference 22). An autoradiogram of lipids isolated from cells grown with sodium acetate-2-^{14}C and $H^{32}PO_4$ is illustrated in Fig. 1. The lipids can be recovered quantitatively from the paper by soaking it in 3 ml of chloroform-methanol-19 mM aqueous ammonium hydroxide (20:20:1) for 1 hr, removing the paper, and repeating the soaking twice in 2-ml portions of fresh solvent.

Mild alkaline methanolysis. Mild alkaline methanolysis was performed in methanol-toluene (1:1) containing 0.1 M methanolic KOH. The methanolysis mixture was neutralized with Biorex 70 resin (BioRad Chromatography Co., Los Angeles, Calif.). The fatty acid methyl esters and glycerol phosphate esters were separated as described (18). The methanolysis of the lipids of *B. subtilis* was complete in 2 hr at 0 C.

The glycerol phosphate esters from the lipids resulting from the mild alkaline methanolysis were separated on formic acid-washed aminocellulose paper (Whatman AE-81) with the two-dimensional system described previously (19). Glycerolphosphoryl monomethylethanolamine, glycerolphosphoryl dimethylethanolamine, and glycerolphosphoryl ethanolamine (GPE) were separated on paper with the ammonium carbonate-ethanol solvent described previously (16).

Column chromatography. A column (0.8 by 10 cm) of silicic acid (60 to 100 mesh, Unisil, Clarkson Chemical Co., Williamsport, Pa.) was used to separate the glucolipids and the phospholipids with a modification of the Vorbeck and Marinetti procedure (18). The glycerol phosphate esters derived from the phospholipids by mild alkaline methanolysis were eluted with the ammonium formate-sodium borate gradient from Dowex 1 columns (200 to 400 mesh) in the formate form as previously described (16, 19).

Thin-layer chromatography. The preparation of thin-layer plates of Silica Gel G, the solvents, the methods of recovery of the lipids from the silica gel, and the reagents for detection were previously described (20).

The phospholipids and the water-soluble glycerol phosphate esters derived from each by mild alkaline methanolysis are as follows: phosphatidylglycerol (PG) and glycerolphosphoryl glycerol (GPG), phosphatidyl ethanolamine (PE) and GPE, lysyl-phosphatidylglycerol (LPG) and GPG, phosphatidic acid (PA) and α-glycerol phosphate (GP), cardiolipin (CL) and diglycerol phosphoryl glycerol (GPGPG). Diglycosyldiglyceride is abbreviated as DG.

Measurement of radioactivity. Samples were assayed for radioactivity in a scintillation spectrometer (model 2311, Packard Instrument Co.). Glycerol phosphate esters were counted on paper discs 1.5 to 2.0 cm in diameter in a scintillation fluid of 9.28 mM 2,5-bis[2(5-terbutyl benzoazol)]-thiophene (BBOT) in toluene. The efficiency of counting on these discs was 49% for ^{14}C and 90.7% for ^{32}P. When ^{32}P and ^{14}C were counted simultaneously on paper discs, the ^{14}C channel = 0.635 ^{14}C + 0.008 ^{32}P and the ^{32}P channel = 0.002 ^{14}C + 0.812 ^{32}P. The efficiency was 29% for ^{14}C and 81% for

^{32}P under these conditions. Radioautograms were prepared with Kodak no-screen X-ray film as previously described (20).

Analysis of the lipids. Lipids or glycerolphosphate esters were analyzed for phosphate, glucose, glycerol, and total fatty acid as described (19).

RESULTS

Identification of the lipids. A portion of the lipid extract of the mutant B-67 of *B. subtilis* was subjected to thin-layer chromatography in two dimensions in solvents of chloroform-methanol-pyridine-water (30:15:1:3) and chloroform-methanol-acetic acid-water (47:7:3.15:0.5), and five phosphate-containing lipids were detected with the chromatographic mobilities of CL, PE, LPG, PG, and PA with the phosphomolybdate spray. The lipids with the mobility of LPG and PE also reacted with ninhydrin. A glycolipid with the mobility of DG was detected with the diphenylamine reagent.

The phospholipids and the glucolipids were separated with a 1-g silicic acid column. A total of 45 nmoles of glucolipid (measured as glucose with the anthrone reagent) was collected after washing the column with 6 ml of chloroform acetone (1:1), 6 ml of acetone, and 10 ml of chloroform-methanol (98:2). These fractions contained less than 0.01 μmole of lipid phosphate. A total of 9.6 μmoles of lipid phosphate was eluted with 10 ml of chloroform-methanol (1:1) and 6 ml of methanol. This represented a recovery of 88% of the lipid phosphate applied to the column. Less than 0.003 μmole of lipid glucose was detected in the phospholipids.

The glucolipid isolated after silicic acid chromatography migrated with an R_F value of 0.46 in thin-layer chromatography with a solvent of chloroform-methanol-acetic acid (100:25:8). Reference DG from *S. aureus* showed the same mobility in this solvent. Glucose was the only hexose detected in the lipid after hydrolysis in 2N HCl.

The phospholipid fraction from the silicic acid column was separated on a thin-layer plate in a solvent of chloroform-methane-pyridine-water used in the initial thin-layer chromatography, into bands with R_F values: 0.80 corresponding to CL, 0.71 corresponding to PE, 0.64 corresponding to PG, and 0.45 corresponding to LPG. A portion of each fraction was deacylated by mild alkaline methanolysis, and the water-soluble glycerol phosphoryl ester was identified by chromatography on aminocellulose paper in two dimensions (18). The CL fraction gave GPGPG, the PE fraction gave GPE, the PG fraction have GPG, and the LPG fraction gave GPG as detected by reactivity with *o*-toluidine after periodate treatment (19). A portion of the PE fraction was hydrolized in 6 N HCl for 2 hr at 100 C; the HCl was removed in a stream of nitrogen and then analyzed on a short-column Technicon amino acid analyzer. Ethanolamine was the only ninhydrin-reacting material found in significant amounts in the PE. Similarly, the LPG fraction was acid-hydrolyzed and analyzed with the amino acid analyzer. The ninhydrin-reacting material isolated from the LPG had the chromatographic properties of lysine.

To study the metabolism of the phospholipids, a relatively simple method for separation of the complex lipids was necessary. This method involved separation of the lipids on silica gel-loaded paper with two-dimensional chromatography (Fig. 1). The high *p*H of the solvent used in the second dimension is necessary to separate the PE from the PG but causes some hydrolysis of the LPG. The lipids corresponding to the dark area in each radioautogram were eluted from the

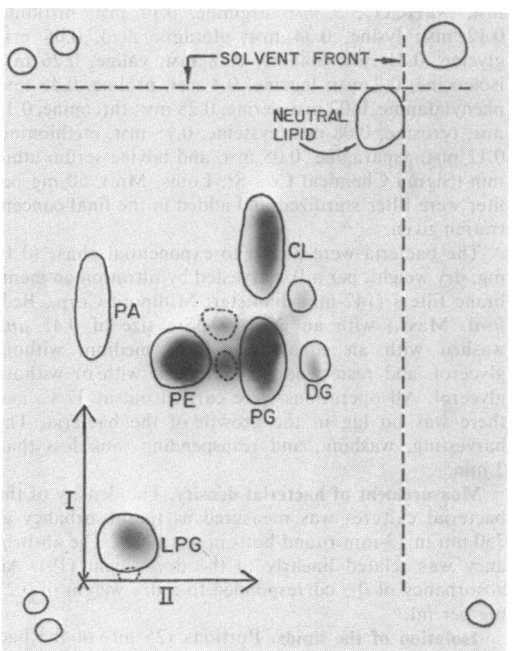

FIG. 1. *Autoradiogram of the lipids of Bacillus subtilis B-67 after chromatographic separation. Lipids were extracted from cells grown for 90 min with* $H_3{}^{32}PO_4$ *and sodium acetate-1-*^{14}C. *Approximately 0.2 μmole of phospholipid was applied to Whatman SG-81 silica gel-impregnated paper and chromatographed in the first dimension (I) with a solvent of chloroform-methanol-diisobutylketone-acetic acid-water (23:10:45:26:4, v/v). The chromatogram was then rotated 90° and chromatographed in the second dimension (II) in a solvent of chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium chloride buffer, pH 10.4 (30:17.5:25:35:6, v/v; reference 23); an autoradiogram was prepared (21).*

silica gel loaded paper and deacylated by mild alkaline methanolysis. The glycerol phosphate esters were then identified by two-dimensional chromatography on aminocellulose paper. The CL yielded GPGPG, PA yielded αGP, PG yielded GPG, LPG yielded PGP, and PE yielded GPE exclusively. The GPE did not contain detectable proportions of glycerophosphorylmonomethylethanolamine or glycerophosphoryldimethylethanolamine as determined by paper chromatography with the ethanolic ammonium carbonate solvent (16). The lysine methyl esters released from the LPG are not labeled when the bacteria are incubated with glycerol-*1,3*-^{14}C.

The proportions of GPE, GPG, GPGPG, and αGP after deacylation of the total lipid are similar after separation by two-dimensional chromatography on aminocellulose paper or by column chromatography with the ammonium formate-sodium borate gradient.

Effect of glycerol deprivation on growth and phospholipid content. The glycerol auxotroph grows with a doubling time of 38 to 40 min when 0.34 mM glycerol was present. If the glycerol-containing medium was removed by filtration and the cells were suspended in medium without glycerol, the cells continued to increase in density at the predeprived rate for about one doubling, and then the increase in density slowed abruptly (Fig. 2). Adding glycerol to the deprived culture initiated exponential growth after a 10-min lag (Fig. 2). The cells were grown with $H_3{}^{32}PO_4$ (0.1 μCi per μmole of phosphate) for nine doublings before the filtration and were resuspended in the medium without glycerol but with the same specific activity of ^{32}P. The ^{32}P content of the phospholipids remained constant through the period of glycerol deprivation, indicating no net phospholipid synthesis. During this period, however, the phospholipid per gram (dry weight) of cells fell from 48 to 30 μmoles of lipid phosphate per gram (dry weight), probably reflecting the continued synthesis of DNA and protein after cessation of net phospholipid synthesis. There was a rapid incorporation of glycerol into the deprived cells with an increase in the rate of phospholipid synthesis relative to growth, thus the lipid phosphate per gram (dry weight) of cells increased (Fig. 2).

Effect of glycerol deprivation on the proportions of the phospholipids. During glycerol deprivation when there was no net increase on the total phospholipids, the proportions of the phospholipids changed (Fig. 3).

PG falls from 60 to 40%, PE increases from 21 to 32%, CL increases from 4 to 10%, PA increases from 0.4 to 1.3%, and the LPG remained about 13% of the total phospholipid. In view of the increase in cell mass without net increase in phospholipid, this represented a loss of 60% of the PG, a loss of 40% of the LPG, no change in the PE or PA, and a gain of 30% in the CL in terms of the phospholipid per gram (dry weight). When glycerol was added to the deprived culture, there was a shift in the proportions of the phospholipids to the predeprivation proportions (Fig. 3).

Effect of glycerol deprivation on the incorporation of ^{32}P and acetate-1-^{14}C into the phospholipids. Glycerol deprivation depressed the incorporation of ^{32}P and ^{14}C from acetate into the phospholipids about 10-fold (Fig. 4). The addition of glycerol to the deprived culture stimulated the incorporation of acetate into the lipid fatty acids and ^{32}P into the phospholipids. Mindich (9–11) has reported that, in the absence of glycerol, ^{14}C from glucose was incorporated into free fatty acids. In our studies, deprivation stimulated incorporation of ^{14}C from acetate into neutral lipid (labeled NA in Fig. 1). The stimulation was maximal within 5 min after glycerol deprivation. At least a portion of the neutral lipids were free fatty acids, since, after methylation, they co-chromatographed with authentic fatty acid methyl esters on gas-liquid chromatography.

The addition of glycerol to the deprived culture resulted in the incorporation of glycerol into the phospholipids (Fig. 5). The highest initial specific activity was found in PA, suggesting its precursor role.

Turnover of ^{14}C-glycerol and ^{32}P in the phospholipids during glycerol deprivation. In the first 15 min after glycerol deprivation, there was rapid incorporation and subsequent turnover of ^{32}P- and ^{14}C-glycerol in CL (Fig. 6). After this initial burst of activity, there was an accumulation of both isotopes in CL. The addition of glycerol stops the accumulation of ^{14}C into glycerol of the CL and initiates turnover of the ^{32}P. There is rapid incorporation of ^{32}P into PG followed by turnover of the ^{32}P at the same rate in the supplemented culture for 30 min. The rate of turnover then decreases. The ^{14}C-glycerol in PG in the deprived culture turns over at about the same rate as in the supplemented culture. In the presence of glycerol, both ^{32}P and ^{14}C accumulated for a short period in LPG. In LPG both isotopes turned over in the absence of glycerol. This turnover ceases when glycerol was readded to the culture. In the presence of glycerol, ^{32}P was lost and ^{14}C-glycerol was accumulated in PE. In the absence of glycerol, the accumulation of ^{14}C into glycerol and loss of ^{32}P from PE ceased after 20 min. There was little difference in PA metabolism in the presence or absence of glycerol.

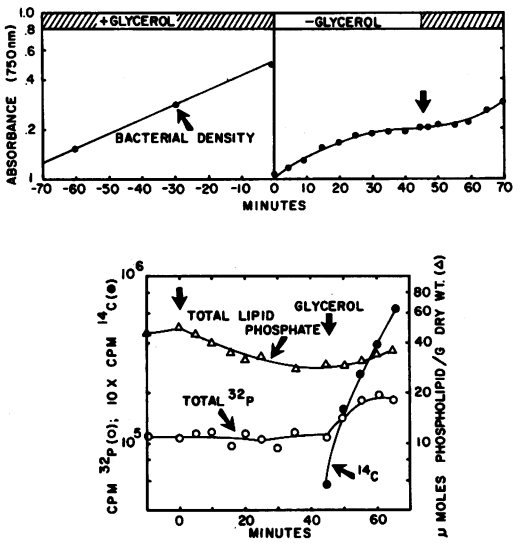

FIG. 2. *Growth and lipid content of the glycerol auxotroph of Bacillus subtilis deprived of glycerol. Cells were grown with glycerol and $H_3{}^{32}PO_4$ with a specific activity of 0.1 μCi per μmole of phosphate for 15 divisions, inoculated into fresh medium, and grown with glycerol to mid-exponential growth phase. The culture was then filtered and washed with an equal volume of medium not containing glycerol. The $H_3{}^{32}PO_4$ content of the medium was maintained at the same specific activity throughout the experiment. After 45 min in the absence of glycerol (indicated by the second arrow in the upper figure), glycerol-1,3-^{14}C (specific activity of 0.74 μCi per μmole in the culture) was added. The final glycerol concentration was 0.34 mM. In the upper graph, the bacterial density was measured as the absorbancy at 750 nm. At points before and after the filtration, samples were withdrawn and the lipids were extracted. The ^{32}P and ^{14}C in the lipids and the total lipid phosphate measured colorimetrically were determined. In the lower figure, the first arrow indicates the time of filtration and washing to remove glycerol; the second arrow indicates the time of addition of glycerol. The ^{14}C is plotted at 10 times its actual value for convenience.*

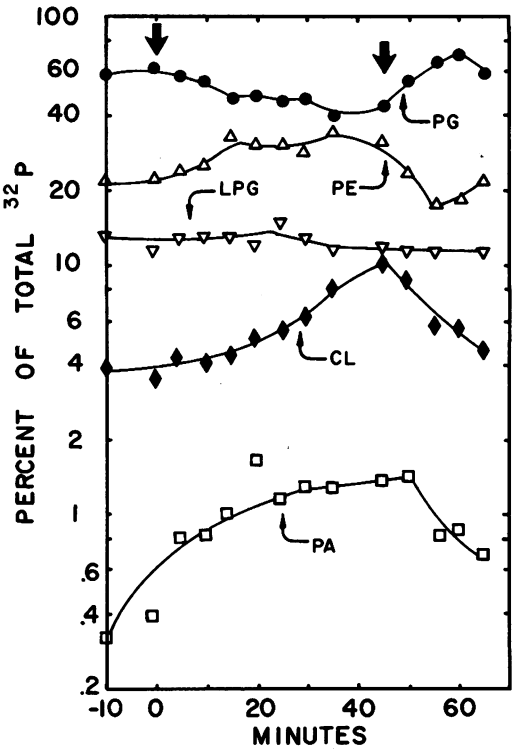

FIG. 3. *Proportions of the phospholipids in the Bacillus subtilis glycerol auxotroph during glycerol deprivation and recovery. The lipids were extracted and separated as in Fig. 1 from samples taken as indicated in Fig. 2. The percentage of the lipid ^{32}P in each lipid was then determined. The specific activity of the medium was maintained at 0.1 μCi per μmole of phosphate in the medium throughout the experiment. Arrows indicate filtration and washing to remove glycerol and addition of glycerol, as in Fig. 2.*

DISCUSSION

This study establishes that the lipid content of the glycerol auxotroph B-67 of *B. subtilis* strain W-23 very closely resembles that reported for *B. subtilis* strain 168 (2, 14). When the glycerol auxotroph was deprived of glycerol, growth slowed markedly and there was no net synthesis of phospholipid (Fig. 2). In this period of no net synthesis of phospholipid, the cells were able to incorporate both ^{32}P into the backbone and ^{14}C into the fatty acids, although at a slower rate than during normal exponential growth with glycerol (Fig. 4). Although there was no net synthesis of phospholipid, the lipid composition of the membrane changed markedly during glycerol deprivation (Fig. 3). In the 45-min period of glycerol deprivation, the cells increased in mass; thus, the lipid content per gram (dry weight) decreased about 40%. PE and PA were synthesized at a rate that maintained the content of these two phospholipids constant in the membrane. Because there was no change in the proportion of LPG, the membrane content of LPG actually fell by 40%. The most striking change was that the proportion of PG decreased by 20%, resulting in a 60% decrease per gram (dry weight). This loss of PG was coupled with an accumulation of CL, representing a 30% gain in CL per gram (dry weight) (*see below*).

Measurements of the loss of radioactivity from lipids on transfer from radioactive to nonradioactive medium represent the minimal rate of metabolism; any recycling of radioactive products would make the observed rate appear much slower than the actual rate (22). Even so, glycerol deprivation resulted in changes in the turnover

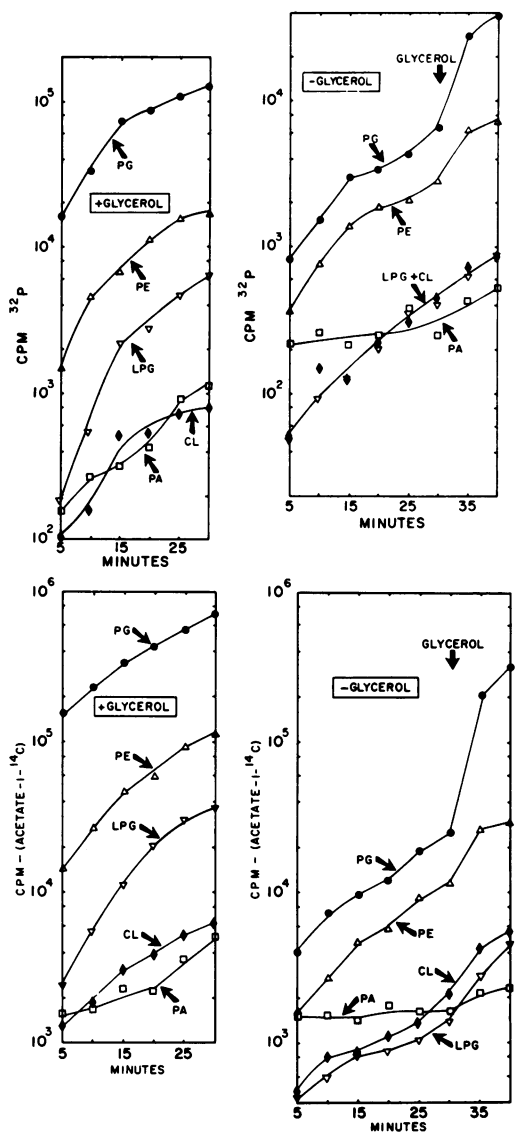

FIG. 4. *Incorporation of ^{32}P and ^{14}C during glycerol deprivation by the glycerol auxotroph of Bacillus subtilis. Cells were grown as in Fig. 2, and the culture was filtered, washed, and divided into two portions. One portion was suspended in medium containing 0.34 mM glycerol (+glycerol, left-hand graphs); and the other portion was suspended in medium not containing glycerol (−glycerol, right-hand graphs). Each portion contained 500 μCi of sodium acetate-1-^{14}C and 500 μCi of $H_3{}^{32}PO_4$ per 1,000 ml. The lipids were extracted and separated as in Fig. 1. The arrow in the right-hand graph indicates the time of addition of glycerol to the deprived culture. The upper graphs show the ^{32}P content of each lipid; the lower graphs show the ^{14}C in the fatty acids of each of the phospholipids.*

of the phospholipid phosphate and phospholipid glycerol (Fig. 6). In this deprived culture, there appeared to be an initial incorporation of ^{14}C and ^{32}P into CL and ^{32}P into PG from some cellular nonlipid pool just after the removal of glycerol. During deprivation, both ^{32}P and ^{14}C accumulated in the CL. Glycerol deprivation induced a loss of radioactivity from LPG not detected in the supplemented culture.

In *H. parainfluenzae* and *S. aureus*, during exponential growth, the concentration of the phospholipids in the membrane remain constant, yet ^{32}P and ^{14}C were removed and replaced from the phospholipid molecule at different rates (17, 22). The phosphate and free glycerol of PG had a much more active metabolism than the diacylated glycerol (17, 21). Recent studies have shown a rapid metabolism of the free glycerol in *E. coli* during a shift down (1). It was clear that

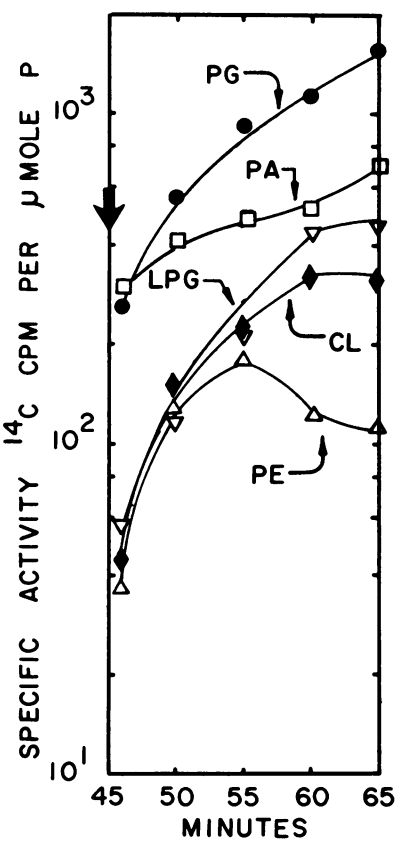

FIG. 5. *Incorporation of glycerol-1,3-^{14}C into the phospholipids of the glycerol auxotroph of Bacillus subtilis. Cells were deprived of glycerol as in Fig. 2 and then glycerol was added as indicated by the arrow. The lipids were separated as in Fig. 1 and the specific activity of each lipid was determined.*

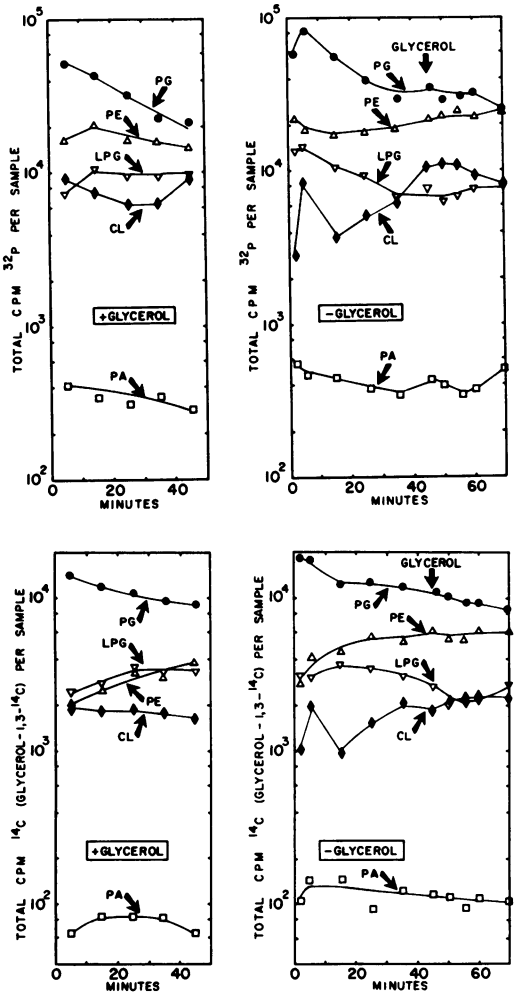

FIG. 6. *Turnover of ^{32}P and ^{14}C in the phospholipids of the glycerol auxotroph of Bacillus subtilis. The cells were grown for two doublings with 500 μCi of $H_3{}^{32}PO_4$ and 250 μCi of glycerol-1,3-^{14}C per 500 ml. The cells were filtered, washed, and resuspended in nonradioactive medium, one-half in medium with glycerol and one-half in medium without glycerol. Samples were withdrawn, and the ^{32}P and ^{14}C in the glycerols of the lipids were determined. The arrow in the right-hand graphs indicates the time of addition of glycerol to the deprived cultures. The upper graphs show the turnover of the ^{32}P in the phospholipids; the lower graphs show the turnover of the ^{14}C in the glycerol backbones of the phospholipids.*

differences between ^{14}C and ^{32}P metabolism in the phospholipids were also detectable in exponentially growing *B. subtilis* (Fig. 6). For example, one-half of the ^{32}P was lost in 26 min and one-half of the ^{14}C-glycerol was lost in 52 min from PG in glycerol-supplemented cultures. ^{32}P was lost from PE, but ^{14}C-glycerol accumulated in this lipid during exponential growth (Fig. 6, left-hand graphs). The difference between ^{32}P and ^{14}C metabolism could also be detected during glycerol deprivation in the metabolism of PG and LPG where the ^{32}P was lost faster than the ^{14}C.

Efforts to understand the differential turnover rates for parts of the PG have focused on the relationship to the metabolism of CL. CL metabolism was characterized by a rapid synthesis from two molecules of PG with the loss of glycerol (S. A. Short and D. C. White, Bacteriol. Proc., p. 151, 1971) and by the rapid hydrolysis of CL by a CL-specific phospholipase D to yield PG and PA (12, 13). The rapid metabolism of CL proved difficult to detect since CL was both made from and contributes to the much larger PG pool (Tucker and White, J. Bacteriol., *submitted for publication*). The rapid metabolism of CL appeared to be coupled to some energy-conserving process in the membrane, since agents which facilitate proton conductivity or inhibit oxidative phosphorylation inhibit rapid metabolism of CL (14). From this study, it appears that glycerol deprivation causes a distortion in the PG $\rightleftarrows$ CL $\rightarrow$ PG + PA cycle detected as a decrease in PG and an accumulation of CL. Addition of glycerol to glycerol-deprived cultures appears to stimulate incorporation into PG through PA and causes an increase in PG and a decrease in CL.

Further work will be necessary to establish whether the shift in the PG $\rightleftarrows$ CL relationship was a critical feature in efficient function of the phosphotransferase system (11) or in cellular activities that affect ribosomal RNA synthesis (9).

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