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Effect of Glycerol Deprivation on the Phospholipid Metabolism of a Glycerol Auxotroph of *Staphylococcus aureus*

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A study of the effects of glycerol deprivation on the content and metabolism of the phospholipids of a glycerol auxotroph of *Staphylococcus aureus* showed that (i) there was an increase in the proportions of lysylphosphatidylglycerol (LPB) and a concomitant decrease in the proportion of phosphatidylglycerol. The total phospholipid content per sample and the proportion of cardiolipin did not change, but the phosphatidic acid increased transiently and then fell to pretreatment levels. (ii) The loss of ^{32}P from the lipids during the chase in a pulse-chase experiment was essentially the same in phosphatidylglycerol, cardiolipin, and phosphatidic acid during glycerol deprivation or growth in the presence of glycerol. LPG lost half the radioactivity in slightly more than two doubling times when grown with glycerol. In the absence of glycerol, ^{32}P accumulated in LPG for about 20 min and then stopped, after which time there was no apparent turnover. (iii) During glycerol deprivation, the initial ^{32}P incorporation decreased sixfold compared to that of the control with glycerol. The initial incorporation into LPG decreased only 2.5-fold, whereas that of PG decreased 45-fold. (iv) During glycerol deprivation, the free fatty acid content increased from 1.2 to 12.5% of the total extractable fatty acids and then slowly decreased. The increase was largely iso- and anti-iso-branched 21-carbon-atom fatty acids. In glycerol-supplemented cultures, the major fatty acids were branched 14- to 18-carbon fatty acids. The decrease in longer chain free fatty acids after 60 min represented their esterification into lipids. (v) During glycerol deprivation ribonucleic acid synthesis and cell growth continued for 40 min and protein synthesis continued for 90 min. Then synthesis and growth stopped. (vi) After the addition of glycerol to glycerol-deprived cells, ^{32}P and ^{14}C-glycerol were incorporated into the phospholipids without lag; ribonucleic acid, protein synthesis, and cell growth began after a 5- to 10-min lag at the pretreatment rate. The initial rate of lipid synthesis after the addition of glycerol was three times greater than the growth rate. This rapid rate continued for about 25 min until the lipid content and proportions of LPG and phosphatidylglycerol were restored.

The involvement of phospholipids in the structure and function of the bacterial membrane has been the subject of intensive investigation (18). Recent work has deepened the understanding of how the bacterial phospholipids are involved in the formation and function of the membrane-wall complex. Some of the phospholipids have been shown to be essential in the function of the lipopolysaccharide-forming enzymes (18) and in the activity of enzyme II in the phosphotransferase for the uptake of β-glucosides (8, 11). Currently, mutants defective in the synthesis of membrane components are being utilized to correlate changes in the chemical and physical properties of the membrane with changes in transport and in the activity of membrane-bound enzymes (5, 14, 19). Mutants of *Escherichia coli* K-12 requiring unsaturated fatty acids were utilized to show the requirement of lipid synthesis for the efficient induction of various transport proteins (3, 7). Others have shown that by changing the fatty acid composition of the phospholipids the efficiency of the lactose transport system can be modified (5, 19; M. K. Crandell et al., *unpublished data*).

Recently, Mindich has isolated glycerol auxotrophs of both *Bacillus subtilis* and *Staphylo-*

coccus aureus (12–14). By using the *B. subtilis* mutant, he has shown that the citrate transport system can be induced under conditions where de novo phospholipid synthesis is stopped although ribonucleic acid (RNA) and protein synthesis continue (27). However, with a glycerol auxotroph of *S. aureus*, it was shown that after glycerol deprivation the lactose transport system could be induced and could be integrated into the membrane but did not function efficiently. In further studies, Lillich and White (10) found that even though net phospholipid synthesis stopped after glycerol deprivation there was considerable turnover and resynthesis of both the glycerol and phosphate portions of the phospholipids of *B. subtilis*.

In this study, a glycerol auxotroph of *S. aureus* (S-2) obtained from L. Mindich was utilized to investigate the metabolism of its phospholipids so that future experiments could be concerned with the synthesis of electron transport system. Studies with inhibitors have indicated that changes in the lipid metabolism occur concomitantly with the formation of the electron transport system in *S. aureus* (9, 25). In this paper we report the effect of glycerol deprivation of the phospholipid composition and metabolism of a glycerol auxotroph of *S. aureus* (S-2) under conditions when protein synthesis continues.

MATERIALS AND METHODS

Growth of bacteria. The glycerol auxotroph S-2 was derived from the parent strain *S. aureus* U-71 by L. Mindich and was characterized as deficient in nicotinamide adenine dinucleotide-independent L-α-glycerol phosphate dehydrogenase activity (15). For all experiments, cells were grown in medium containing 19 mM KCl, 0.49 mM K_2HPO_4, 79 mM NaCl, 20 mM NH_4Cl, 1.4 mM Na_2SO_4, 0.1 mM adenine, 0.1 mM xanthine, 0.1 mM uracil, 1.8 mM alanine, 1.4 mM arginine, 0.75 mM asparagine, 1.5 mM cysteine, 0.75 mM glutamic acid, 0.3 mM glycine, 1.1 mM histidine, 2 mM isoleucine, 4 mM leucine, 3.5 mM lysine, 1.1 mM methionine, 0.09 mM phenylalanine, 4.6 mM proline, 2.8 mM serine, 1.9 mM threonine, 0.08 mM tyrosine, 2.5 mM valine, and 0.1 M tris(hydroxymethyl) aminomethane. This medium was brought to *p*H 7.4 with concentrated hydrochloride and autoclaved. After cooling, the following solutions, which were sterilized either by filtration or autoclaving, were added in the final concentrations given: 0.2 nM biotin, 8.3 nM nicotinic acid, 0.93 nM thiamine, 2 μM $FeCl_3$, 0.1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.1 mM tryptophan, 5.5 mM glucose, and 15 μg of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) per ml. Cultures were grown in liter flasks containing 400 ml of medium or in Parrot flasks containing 1,500 ml of medium in a Fermentation Design Constant Temperature Water Bath Shaker (Allentown, Pa.) at 37 C (± 1 C) and shaken at 200 rev/min. Growth was measured as the absorbance at 750 nm with a Spectronic 20 spectrophotometer. Dry weights and lipid phosphate were determined as previously described (17) and were linear with respect to optical density (Fig. 1).

Deprivation of glycerol. For studies requiring the deprivation of glycerol, cultures were grown to an absorbance between 0.2 and 0.4 in 500 ml of medium, harvested by rapid filtration on a 142-mm Millipore filter (0.4 μm pore size), washed with an equal volume of prewarmed medium without glycerol, and resuspended in warm medium with or without glycerol. There was no lag in the growth of the bacteria after harvesting, washing, and resuspending them in the prewarmed medium.

Extraction of the lipids. The total lipids of *S. aureus* were extracted by a modification of the method of Bligh and Dyer (2) as previously described (10). The cells in the growth medium were immediately added to chloroform and methanol to give a one-phase system of chloroform-methanol-water (25:1:0.8). After extraction for a minimum of 2 hr, the two-phase system was obtained by adding chloroform and methanol to give a final concentration of chloroform-methanol-water (1:1:0.9). The lipid phase was allowed to separate and was dried by filtration over Na_2SO_4 before the chloroform phase was evaporated by flash evaporation.

Chromatography of the phospholipids. The phospholipids of *S. aureus* were separated by two-dimensional chromatography on silica gel-impregnated paper (Whatman SG-81) by using the first and third solvents of Wuthier (27). The first dimension

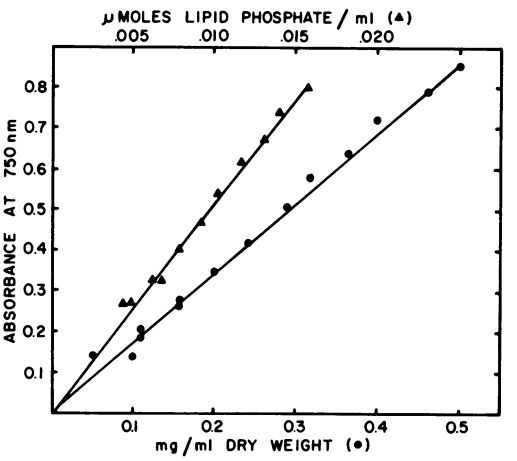

FIG. 1. *Relationship between the absorbance at 750 nm, the lipid phosphate, and the dry weight of Staphylococcus aureus S-2. Samples from a growing culture were withdrawn and the absorbance at 750 nm was measured between 0.2 and 0.6 in a Spectronic 20 spectrophotometer. The sample was centrifuged, washed, place in preweighed glass vials, and dried under vaccum at 40 C to a constant weight. An equal sample was extracted by a modification of the method of Bligh and Dyer (2), and the lipid phosphate was measured by the method of Bartlett (1).*

was run in chloroform-methanol-diisobutyl-ketone-acetic acid-water (12:5:23:13:2), and the second dimension was run in chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium chloride, pH 10.4 (15:9:13:18:3). Since lysylphosphatidylglycerol (LPG) is liable in the second solvent, the lower portion of the chromatogram was cut off as described by Short and White (20) after the first solvent. A radioautogram of the lipid separation has been published (20).

Labeling of the phospholipids. Pulse-chase experiments were done by growing 500 ml of cells to an absorbance of 0.12 in nonradioactive medium, and then pulsing the cells for two generations with 1.4 mCi of $H_3{}^{32}PO_4$. After this time, the cells were harvested by filtration, washed with an equal volume of nonradioactive medium, and resuspended in 500 ml of nonradioactive medium with or without glycerol. Localization of the glycerol moiety in this auxotroph was achieved by growing the cells in medium containing 20 μg of glycerol per ml, supplementing with 100 μCi of glycerol-*1,3-*^{14}C for 10 generations, and harvesting the cells by centrifugation. After washing twice with 50 mM phosphate buffer (pH 7.4), samples were taken and counted and the lipids were isolated as described. After mild alkaline methanolysis (25), the fatty acids, carotenoids, and vitamin K isoprenologues could be separated from the water-soluble glycerol phosphate esters. Incorporation experiments were done as described in the text. The measurement of the phospholipid composition was done by determining the percentage of ^{32}P in each phospholipid after growing the cells in constant specific-activity medium for at least 10 generations. The percentage of composition determined by the measurement of ^{32}P was consistent with the measurement of phosphate in at least two experiments. Total phosphate was determined by the method of Bartlett after digestion with 23% $HClO_4$ for 1 hr at 200 C, as adapted for the autoanalyzer (1). Samples were assayed for radioactivity on filter-paper discs in a Packard scintillation spectrophotometer model 2311 as described (10). A scintillation fluid of 9.28 mM 2,5-bis[2(5-terbutylbenzoazol)]-thiophene in toluene was used.

RESULTS

The glycerol auxotroph of *S. aureus* S-2 required glycerol at a concentration of 15 to 30 μg per ml for optimal growth. Below this concentration the doubling time increased, and below 4 μg per ml there was no visible growth. When cells from the exponential phase of growth were washed free of glycerol and resuspended in medium without glycerol, growth slowed after 30 min (Fig. 2). The cessation of growth, as indicated by absorbance, occurred simultaneously with the cessation of RNA synthesis (Fig. 2). If, after depriving the cells of glycerol for 90 min, glycerol was added, there was a lag before the resumption of RNA synthesis and growth. Protein synthesis was inhibited after 30 min of glycerol deprivation; however, it required about 90 min for protein synthesis to cease. When glycerol was added, even after 115 min of deprivation, protein synthesis resumed at the predeprivation rates, after a brief lag.

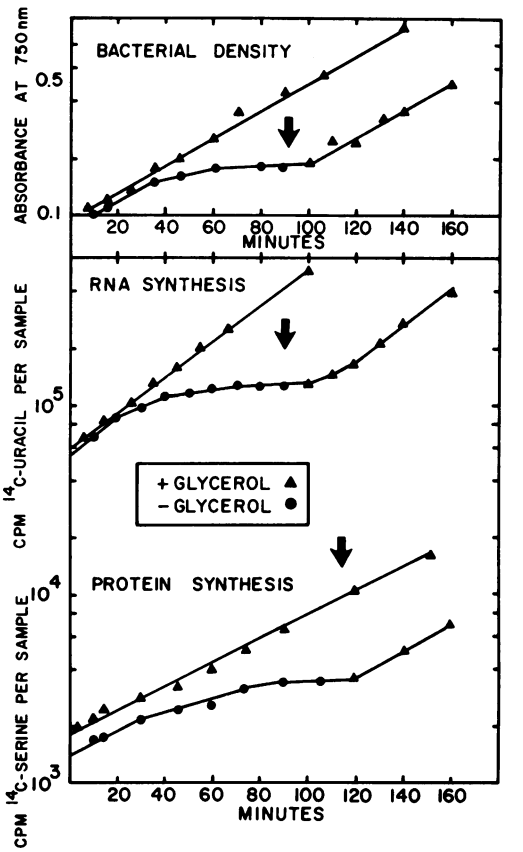

FIG. 2. *Effect of glycerol deprivation and readdition on ribonucleic acid synthesis and protein synthesis. Upper graph shows the effect of glycerol deprivation on protein synthesis and after the readdition of glycerol after starvation (marked by the arrow). The cells were grown (for seven generations), harvested, washed, and resuspended in medium containing 1 μCi of serine-2-*^{14}C *per ml. At the times indicated, 1-ml samples were withdrawn and added to 2 ml of cold 10% trichloroacetic acid, filtered on Millipore filters, washed, and counted. Middle graph shows the effect of glycerol deprivation on ribonucleic acid synthesis as determined by growing the cells in* ^{14}C*-uracil and measured as in the middle graph.*

Effect of growth phase on the phospholipid composition. The phospholipid composition of the glycerol mutant was somewhat different from that of the parent *S. aureus* U-71. In Fig. 3, the phospholipid composition of S-2 supplemented with glycerol was compared at

different stages of growth. It can be seen that during the exponential phase of growth the phospholipid composition was constant: phosphatidylglycerol (PG), lysylphosphatidylglycerol (LPG), cardiolipin (CL), and phosphatidic acid (PA) represented 92, 5, 1, and 2% of the total phospholipids, respectively. However, in early stationary phase, the proportion of PG decreased, the proportions of LPG, CL, and phosphatidylglucose (PGlu) increased, and that of PA remained constant. In the parent, *S. aureus* U-71, PG, LPG, and CL represent 80, 12, and 5% of the total phospholipids, respectively, during exponential growth (21). The metabolism of the phospholipids in the mutant were examined in the exponential phase of growth, so that any changes in the metabolism occurred under conditions when the amount of each phospholipid remained constant for those cells supplemented with glycerol.

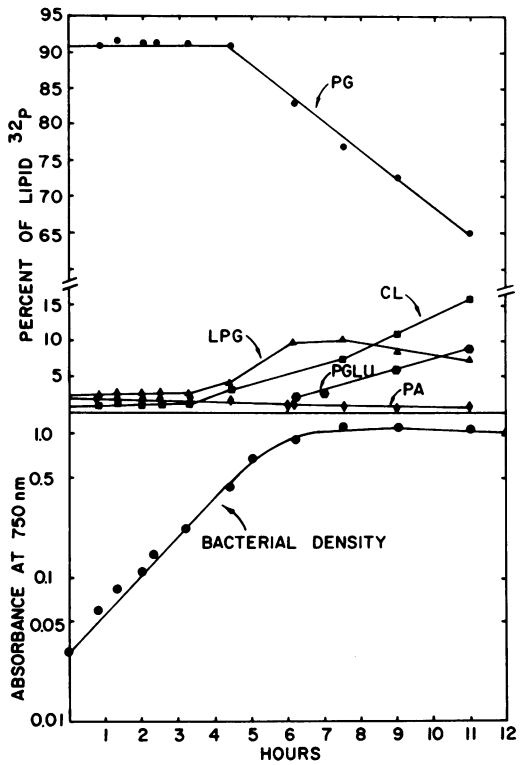

FIG. 3. *Effect of growth on the phospholipid composition of Staphylococcus aureus S-2. The cells were grown in medium containing ^{32}P at constant specific activity for at least 10 generations. At the points indicated, 25-ml samples were withdrawn from the flask and added directly to 37.5 ml of chloroform and 75 ml of methanol as described. The individual phospholipids were separated by chromatography on silica gel-impregnated paper, and the individual lipids were localized by radioautography. PG (●), LPG (▲), CL (■), PA (◆), and PGlu (⬟).*

Localization of the glycerol incorporated by the glycerol auxotroph. When cells were supplemented with ^{14}C-glycerol, the distribution of the radioactivity showed that 41% of the total ^{14}C-glycerol incorporated into the cells was found in the lipid fraction of the cells (Table 1). By mild alkaline methanolysis, it was shown that 96% of the total radioactivity in the lipid fraction was found in the water-soluble glycerol phosphate backbone. Only 4% of the labeled glycerol appeared in the fatty acids, carotenoids, or vitamin K_2 isoprenologues.

Turnover of the phospholipids in cells in the presence and absence of glycerol. When cells grown in glycerol were pulsed with $H_3{}^{32}PO_4$ for two generations and washed with medium devoid of glycerol and ^{32}P, there was no lag in growth in both cells supplemented with glycerol and devoid of glycerol. In the cultures without glycerol, it was seen that growth slowed after 36 min and completely ceased after 50 min (Fig. 4). Immediately after the deprivation of glycerol, net phospholipid synthesis ceased. The turnover of the individual phospholipids was examined for two generations after the deprivation of glycerol. The major phospholipid, PG, lost 55% of the ^{32}P in one generation; and CL and PA (not shown) lost 20% of the isotope in one generation in both the cells supplemented with glycerol and

TABLE 1. *Localization of glycerol-1,3-^{14}C incorporated by Staphylococcus aureus S-2*[a]

Fraction	Counts per min	Per cent ^{14}C
Whole cells	2.05×10^7	100
Lipid extract	8.2×10^6	41
Fatty acids[b]	2.4×10^5	3
Glycerol phosphate esters	7.8×10^6	38

[a] Cells were grown in 50 ml of medium containing 20 μg of glycerol and 2 μCi of glycerol-1,3-^{14}C per ml to an absorbance of 0.66 at 750 nm. The cells were harvested by centrifugation and washed twice with 50 mM phosphate buffer (pH 7.4). Samples were taken for total radioactivity. The lipids were extracted as described and samples were counted on filter-paper discs. Mild alkaline methanolysis (25) was performed on the lipid extract, and the fatty acids, carotenoids, and vitamin K_2 isoprenologues were separated from the water-soluble glycerol phosphate esters and counted.

[b] The fatty acid fraction includes the carotenoids and vitamin K_2 isoprenologues; however, together they represent less than 1% of the fatty acids.

those without glycerol (Fig. 4). The major difference in the turnover rates of the plus and minus glycerol-grown cells was seen in LPG. Without glycerol, LPG accumulated a twofold increase in radioactivity. In the presence of glycerol, LPG lost 25% of the radioactivity after one generation.

Effect of glycerol deprivation on the phospholipid composition. When cells were labeled for 10 generations in medium containing (constant specific activity) $H_3{}^{32}PO_4$, the percentage of composition of the individual

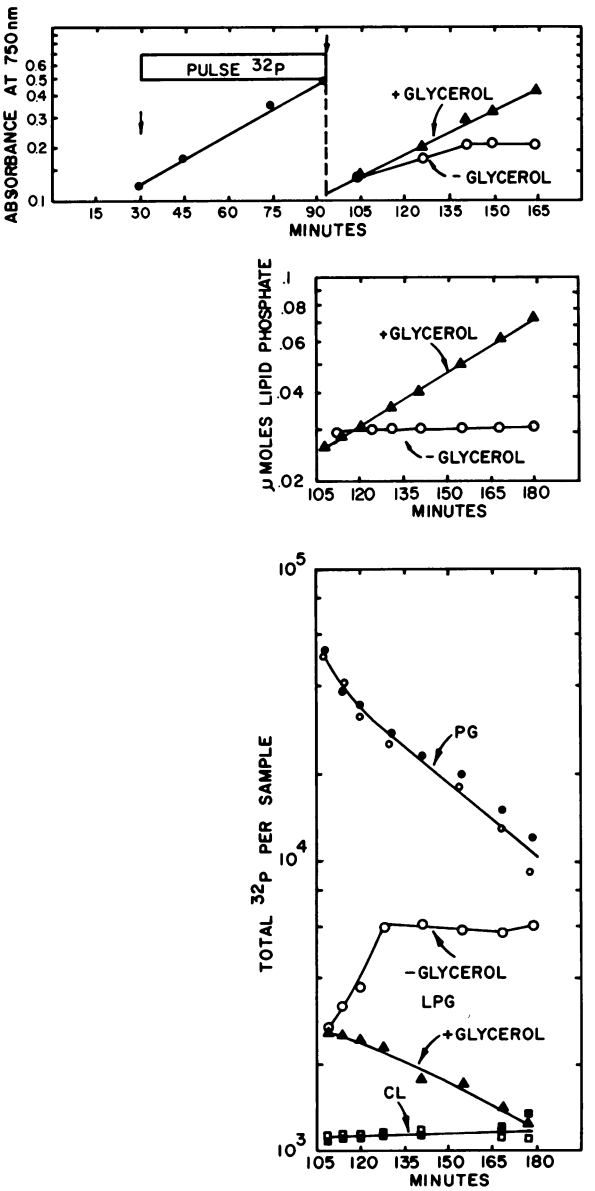

FIG. 4. *Turnover of the phospholipids of Staphylococcus aureus S-2, plus and minus glycerol. An exponentially growing culture of S. aureus was pulsed for two generations with* $H_3{}^{32}PO_4$ *(1.0 mCi/500 ml), filtered, washed with medium devoid of glycerol and radioactivity, and resuspended in medium plus glycerol (solid symbols) and minus glycerol (open symbols). Samples were withdrawn at the times indicated, and the lipids were extracted and separated as described. PG + glycerol (●), PG − glycerol (○), LPG + glycerol (▲), LPG − glycerol (△), CL + glycerol (■), and CL − glycerol (□).*

phospholipids was determined by measuring the radioactivity in the individual phospholipids. The effect of glycerol deprivation on the phospholipid composition of the mutant is seen in Fig. 5. The top graph shows the effect of glycerol deprivation on (i) the total phospholipid content and (ii) the total radioactivity in the phospholipids. It can be seen by both measurements that there is no increase in the content of lipid phosphate after the deprivation of glycerol. When glycerol was added to the deprived cells after 60 min of deprivation, there was a 5-min lag before the content of phospholipids started to increase. When phospholipid synthesis resumed, the rate of synthesis after the addition of glycerol was three times greater than the growth rate. This rapid rate of synthesis continued for about 25 min. During the time of glycerol deprivation, when the total phospholipid content remained constant, the proportions of the individual phospholipids changed drastically. Immediately after glycerol deprivation, the PG decreased from 92 to 45% of the total lipid phos-

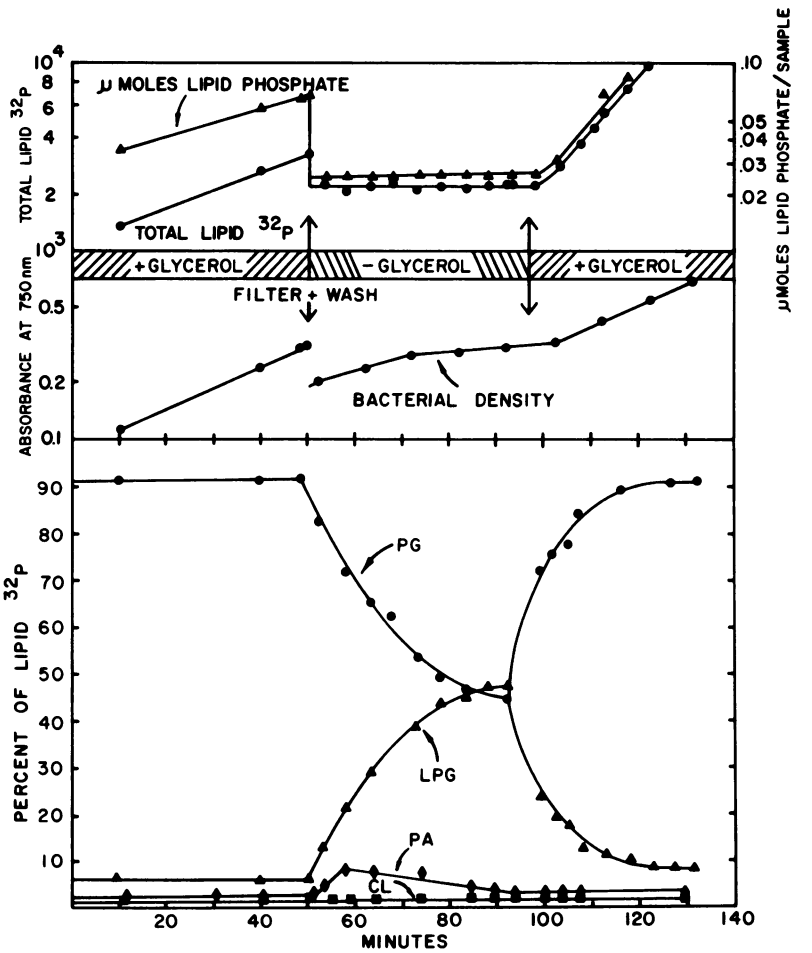

FIG. 5. *Effect of glycerol deprivation on the phospholipid composition. The cells were grown in medium containing a constant amount of* $H_3{}^{32}PO_4$ *to an optical density of 0.3, filtered, washed in medium containing radioactivity and devoid of glycerol (as indicated by the first arrow), and resuspended in medium containing* $H_3{}^{32}PO_4$ *but devoid of glycerol. At the points indicated, samples were withdrawn and added directly to 37.5 ml of chloroform and 75 ml of methanol. Top graph represents the total* ^{32}P *and micromoles of lipid phosphate per sample in cells grown with glycerol (up to the first arrow), after filtering, washing, and resuspending in deprivation medium (to the second arrow) and upon the readdition of glycerol to the medium. Middle graph shows the growth of the culture with glycerol, after filtering and depriving the cells of glycerol and upon the readdition of glycerol. Bottom graph shows the effect of glycerol deprivation on the phospholipid composition as compared to cells grown in the presence of glycerol (0 to 48 min) and to prestarved cells after the addition of glycerol. PG (●), LPG (▲), CL (■), and PA (◆).*

phate. There was a concomitant increase in the proportion of LPG from 6.5 to 45% during deprivation. Upon the readdition of glycerol, the proportions of PG started to increase immediately and that of LPG decreased. The proportions reached the predeprivation levels after about 20 min. The proportion of CL remained relatively constant both with and without glycerol. PA increased immediately after glycerol deprivation and amounted to 8% after 7 min but then decreased to the steady-state level even before the addition of glycerol. This increase in the proportion of LPG and the decrease in PG were not due to the lowering of the *p*H, since the *p*H remained constant at between 7.2 and 7.1 during the experiments.

Incorporation of ^{14}C-glycerol and ^{32}P into the phospholipids of S. aureus. After 60 min of glycerol deprivation, glycerol-*1,3-*^{14}*C* was added, and the kinetics of the incorporation of the radioactivity into the glycerol backbone of the lipids were measured (Fig. 6). Radioactive glycerol was incorporated most rapidly into PA and PG. The incorporation into LPG was somewhat slower and that into CL was even slower. This incorporation was identical to the incorporation of glycerol into cells under ordinary conditions before deprivation, except that the rate of incorporation into PG was two to three times faster. The incorporation of the labeled glycerol can be compared to the incorporation of ^{32}P into phospholipids before glycerol starvation, during starvation, and after 60 min of glycerol starvation followed by the addition of glycerol (Fig. 7). Figure 7 shows that the radioactivity first appeared in PA, and then PG, and then LPG, and finally in CL under normal growing conditions. However, during glycerol starvation, when the phospholipid composition of the cells has changed (see Fig. 5), the rate of incorporation was six- to sevenfold lower than that of the glycerol-supplemented cells. The radioactivity first appeared in PA and LPG. There was a 5-min lag before radioactivity appeared in PG (even though it is the precursor to LPG) and CL. If glycerol was added after a 60-min period of starvation, the radioactive label was first detected in PA followed by PG. There was a 5-min lag before the incorporation of ^{32}P into LPG and CL.

Effect of glycerol deprivation on the fatty acids of S. aureus. In *S. aureus* grown in medium containing constant specific-activity ^{14}C-acetate, the rate of incorporation of the acetate can be used to measure the amount of fatty acid synthesized, since (i) there was no radioactivity detected in the glycerol backbone,

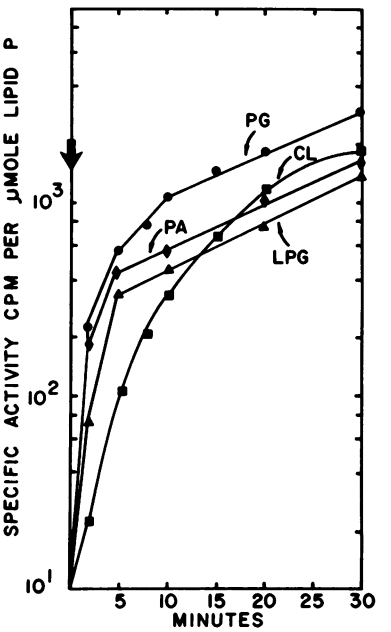

FIG. 6. *Incorporation of glycerol-1,3-*^{14}*C in Staphylococcus aureus after 45 min of glycerol deprivation. The cells were deprived of glycerol as described before; after 45 min, 20 μg of glycerol was added per ml, containing 100 μCi of glycerol-1,3-*^{14}*C, and samples were taken at the times indicated. PG (●), PA (◆), LPG (▲), CL (■).*

and (ii) the amount of neutral lipid was negligible in relation to the amount of fatty acids; the carotenoids equaled 0.1 μmole and the vitamin K_2 isoprenologues equaled 0.2 μmole per g of dry weight versus 150 μmoles of fatty acids per g of dry weight. When *S. aureus* was deprived of glycerol, fatty acid synthesis slowed almost immediately to half the rate of cells supplemented with glycerol (Fig. 8). After 75 min, glycerol was added and the rate immediately increased. In normally growing cells of S-2, the free fatty acid content was about 1 to 2% of the total extractable fatty acids. When the cells were deprived of glycerol, the content of the free fatty acids increased to 12.5% of the total fatty acids in an hour. However, even before the addition of glycerol, the content decreased. With the addition of glycerol, the free fatty acid content decreased to the predeprivation level. The fatty acid composition of this mutant was altered by the deprivation of glycerol. Under conditions where cells were supplemented with glycerol the iso- and anti-iso-branched-chain fatty acids of chain length C-14 to C-18 predominated. However, upon glycerol starvation, longer chain length, branched fatty acids appeared (mainly iso-C-

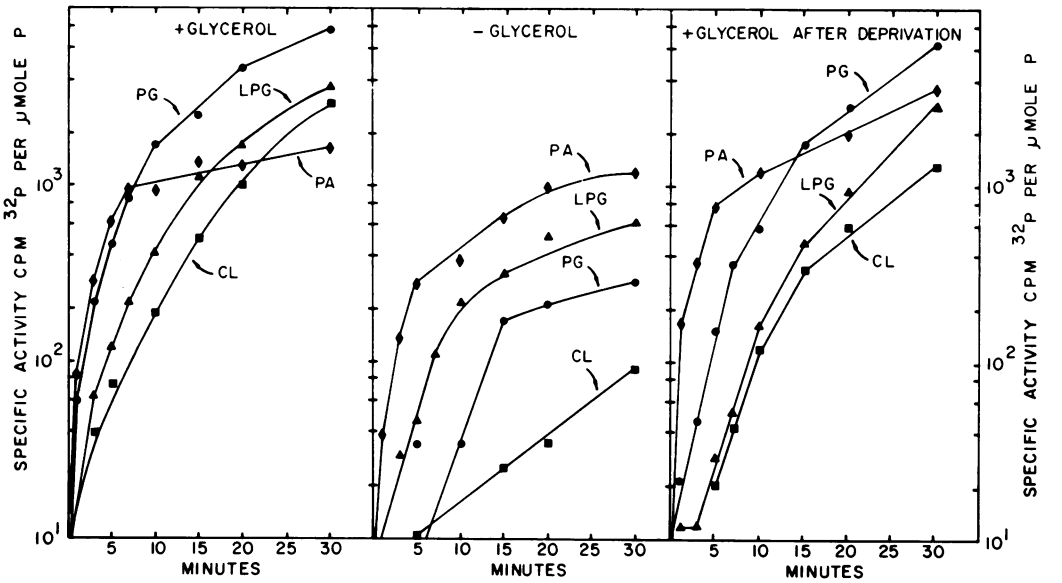

FIG. 7. *Incorporation of ^{32}P into the phospholipids of Staphylococcus aureus S-2. The incorporation of ^{32}P was measured in these lipids during normal phospholipid metabolism (left graph), in the absence of glycerol after 20 minutes of starvation, and after the addition of 20 μg of glycerol per ml to cells deprived of glycerol for 60 min. Arrow indicates the time of glycerol addition. PG (●), LPG (▲), CL (■), and PA (◆).*

21 and anti-iso-C-21, which represented 5 and 10% of the total fatty acids, respectively) and were found in the free fatty acids. After 60 min, the longer chain fatty acids were esterified into the phospholipids. The longer chain fatty acids were still present 30 min after the addition of glycerol to glycerol-starved cells.

DISCUSSION

Exponentially growing glycerol auxotrophs of *S. aureus*, when deprived of glycerol, showed an abrupt halt in the net increase in membrane phospholipids even though growth, RNA, and protein synthesis continued for 30 to 90 min (Fig. 2 and 5). During this period, there was an increase in the content of LPG and a concomitant decrease in PG (Fig. 5); however, this was not simply a conversion of old PG to LPG since ^{32}P was incorporated quite rapidly into LPG (Fig. 7). The PG in glycerol-deprived cells lost ^{32}P at the same rate as cells grown with glycerol and synthesized PG very slowly so that the fall in the proportion of PG was balanced by the increase in LPG (Fig. 3 and 4). In the period of glycerol deprivation, the total lipid phosphate and the proportion of CL remained constant (Fig. 5). Since the pathway of LPG synthesis in *S. aureus* involves PA->PG->LPG (4, 15), it is clear that during glycerol deprivation in this mutant (i) there was a rapid synthesis of LPG that involved a portion of the PG pool with a specific activity 45 times greater than that of the rest of the PG pool (Fig. 7) and (ii) there was an accumulation of ^{32}P in LPG for 20 min followed by no turnover in the absence of glycerol, in contrast to turnover of LPG during growth with glycerol (Fig. 4). This represents another example of the heterogeneity in the metabolism of the membrane lipids. Differences in the metabolism or portions of the PG and CL pools have been demonstrated in *S. aureus* (20) and *Haemophilus parainfluenzae* (22, 25). During glycerol deprivation, the striking features of phospholipid metabolism were the rapid synthesis of LPG and the cessation of its normally slow turnover. Previous work with both *B. subtilis* (16) and *S. aureus* (4, 6) suggested that the metabolism of LPG responded to the *p*H of the growth medium. During the accumulation of LPG in glycerol deprivation, the *p*H remained constant.

The glycerol auxotroph not only accumulates LPG and slows incorporation of ^{32}P into the lipids when deprived of glycerol but when grown with glycerol it showed differences from the parental type. The mutant grown with glycerol has roughly half the total phospholipid per g dry weight (30 to 35 μmoles) as the wild type (60 to 65 μmoles/g dry weight, reference 21). The mutant forms much less total CL, and the CL has a much slower metabolism than the wild type (Fig. 4, reference 20). During the

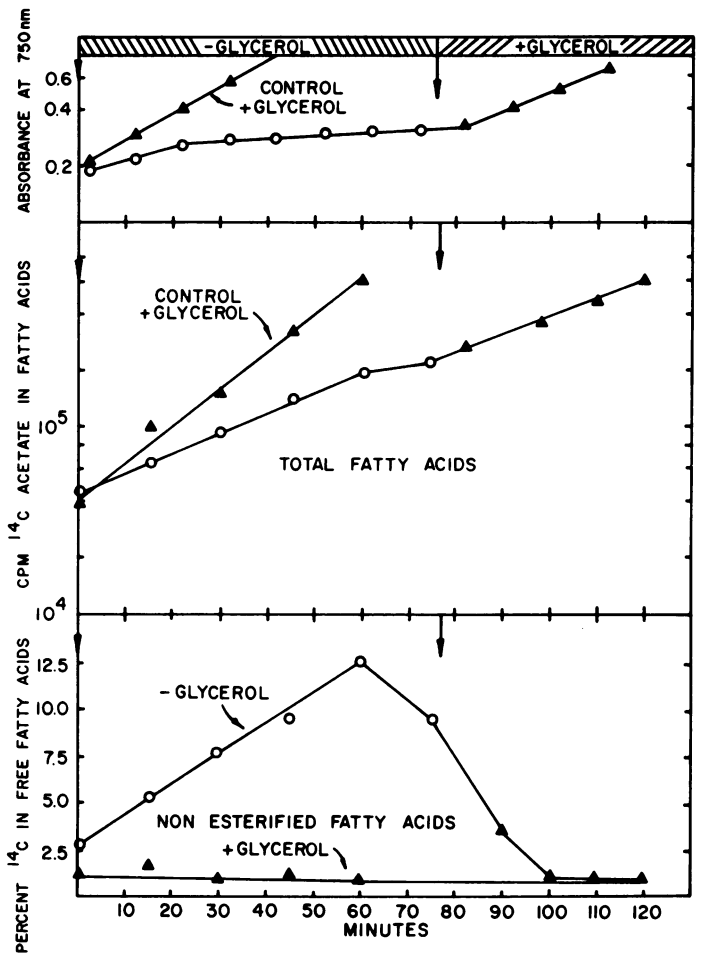

FIG. 8. *Effect of glycerol deprivation on fatty acid synthesis. Upper graph illustrates the effect of glycerol deprivation on bacterial growth* (○) *and the effect of the addition of glycerol after 76 min of deprivation* (▲). *Time zero represents the time at which the cells were resuspended in medium devoid of glycerol after harvesting and washing. Middle graph represents the effect of glycerol deprivation on the incorporation of* ^{14}*C-acetate into the fatty acids. The cells were grown (at least 10 generations), washed, and resuspended in medium containing constant specific-activity* ^{14}*C-acetate. Bottom graph represents the per cent of the radioactivity found in the free fatty acids during glycerol deprivation* (○) *and after the addition of glycerol* (▲). *Arrow at 76 min indicates the time when the glycerol was added. Free fatty acids were extracted and measured by the method of White and Cox (23).*

stationary phase, both the mutant and wild type accumulate PGlu and CL. The mutant apparently forms no phosphatidylethanolamine (Fig. 3, reference 20). In both the mutant grown with glycerol and the wild type, half the ^{32}P was lost in one doubling from PG and four doublings from LPG during exponential growth (Fig. 4, reference 20).

A second feature of glycerol deprivation was first noted by L. Mindich. He found that these mutants synthesize free fatty acids of longer chain lengths than usual (L. Mindich, *personal communication*). In this mutant the free fatty acids that accumulated were considerably longer. After about 60 min of glycerol deprivation, they were slowly esterified into the lipids (Fig. 8). The accumulation of fatty acids of longer chain lengths suggests that in the absence of a glycerol-3-phosphate acceptor the control systems that stop fatty acid elongation after seven to nine cycles involve the esterification of the nascent fatty acids into PA. The fact that these long chain fatty acids were eventually incorporated indicated that there may be slow fatty acid transacylation activity with the longer chain fatty acids. The process of phosphalipase A hydrolysis, catabolism of free fatty acids, and transacylation of newly

synthesized fatty acids has been postulated to explain the fatty acid differences in the phospholipids of *S. aureus* (24), and the cycle has been demonstrated in *H. parainfluenzae* when the wall-membrane complex is damaged (22).

When glycerol was added to the deprived culture, both glycerol and ^{32}P were rapidly incorporated into the lipids (Fig. 6 and 7). This occurred in the presence of 0.1 mM chloramphenicol, which suggested that the phospholipid-synthesizing enzymes were present during the deprivation. The major features of the recovery were the rapid synthesis of PG and the slower synthesis of LPG.

A shift in lipid metabolism was also detected in a glycerol-requiring mutant of *B. subtilis* (10). In this mutant, the free fatty acid accumulation was less pronounced and the lipid that accumulated during deprivation was CL. In both these glycerol auxotrophs, glycerol deprivation stopped net phospholipid synthesis and shifted the proportions of the phospholipids, but allowed some de novo lipid synthesis and catabolism. Perhaps this limited metabolism explains the normal induction of the lactose (14) and citrate operons (26) but the poor function of these membrane systems.

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