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Mutants of *Escherichia coli* Defective in Membrane Phospholipid Synthesis: Macromolecular Synthesis in an sn-Glycerol 3-Phosphate Acyltransferase K_m Mutant

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sn-Glycerol 3-phosphate (G3P) auxotrophs of *Escherichia coli* have been selected from a strain which cannot aerobically catabolize G3P. The auxotrophy resulted from loss of the biosynthetic G3P dehydrogenase (EC 1.1.1.8) or from a defective membranous G3P acyltransferase. The apparent K_m of the acyltransferase for G3P was 11- to 14-fold higher (from about 90 μm to 1,000 to 1,250 μm) in membrane preparations from the mutants than those of the parent. All extracts prepared from revertants of the G3P dehydrogenase mutants showed G3P dehydrogenase activity, but most contained less than 10% of the wild-type level. Membrane preparations from revertants of the acyltransferase mutants had apparent K_m's for G3P similar to that of the parent. Strains have been derived in which the G3P requirement can be satisfied with glycerol in the presence of glucose, presumably because the glycerol kinase was desensitized to inhibition by fructose 1,6-diphosphate. Investigations on the growth and macromolecular synthesis in a G3P acyltransferase K_m mutant revealed that upon glycerol deprivation, net phospholipid synthesis stopped immediately; growth continued for about one doubling; net ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein nearly doubled paralleling the growth curve; the rate of phospholipid synthesis assessed by labeling cells with ^{32}P-phosphate, ^{14}C-acetate, or ^{3}H-serine was reduced greater than 90%; the rates of RNA and DNA synthesis increased as the cells grew and then decreased as the cells stopped growing; the rate of protein synthesis showed no increase and declined more slowly than the rates of RNA and DNA synthesis when the cells stopped growing. The cells retained and gained in the capacity to synthesize phospholipids upon glycerol deprivation. These data indicate that net phospholipid synthesis is not required for continued macromolecular synthesis for about one doubling, and that the rates of these processes are not coupled during this time period.

Investigations on the structural and functional importance of membrane phospholipid in biological membranes have been facilitated by the availability of bacterial mutants deficient in lipid synthesis (8). These investigations have utilized mutants of *Escherichia coli* defective in unsaturated fatty acid synthesis (8, 10, 14), mutants of *E. coli* (16), *Bacillus subtilis* (21, 22, 27), and *Staphylococcus aureus* (22) which require glycerol for growth, and temperature-sensitive mutants of *E. coli* defective in the sn-glycerol 3-phosphate (G3P; stereospecific numbering according to Hirschmann [15]) acyltransferase (7, 11). Investigations of macromolecular synthesis in the unsaturated fatty acid auxotrophs (8, 10, 14) and the glycerol auxotrophs (15, 21-23) revealed continued growth and macromolecular synthesis upon supplement deprival and upon cessation of net phospholipid synthesis. Recent investigations on macromolecular synthesis in the *E. coli* temperature-sensitive G3P acyltransferase mutants resulted in the suggestion that the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein is coupled with phospholipid synthesis since all of these processes shut off in parallel in these mutants at the restrictive temperature (11).

The obvious potential of mutants defective in membrane phospholipid synthesis for investigating biological membranes and the apparent coupling of phospholipid synthesis with macromolecular synthetic processes in the temperature-sensitive G3P acyltransferase mutants prompted the investigations reported here. The aims of these studies were to select G3P auxo-

trophs of *E. coli* in suitable genetic backgrounds to facilitate future investigations; to establish the enzymatic defect causing the G3P auxotrophy; and to investigate the coupling of macromolecular synthesis with phospholipid synthesis in mutants defective in membrane phospholipid synthesis because of a K_m-defective G3P acyltransferase.

In this investigation, the isolation and characterization of G3P auxotrophs of *E. coli* are reported. The G3P auxotrophy resulted from loss of the biosynthetic G3P dehydrogenase or from a defective G3P acyltransferase. Strains have been derived in which the G3P requirement can be satisfied with glycerol in the presence of glucose. The macromolecular synthesis in G3P acyltransferase K_m mutant has been investigated upon glycerol deprivation. The results, presented below, indicated that growth, net DNA, RNA, and protein synthesis are uncoupled from net phospholipid synthesis for about one doubling, and that the rates of these processes are also uncoupled during this period.

MATERIALS AND METHODS

Chemicals. DL-Glycerol 3-phosphate, streptomycin sulfate, reduced nicotinamide adenine dinucleotide phosphate (NADPH), dihydroxyacetone phosphate, and *E. coli* alkaline phosphatase (28 units/mg) were obtained from Sigma Chemical Co., St. Louis, Mo. Palmitoyl-CoA was the product of P-L Biochemicals, Inc., Milwaukee, Wis. The radioisotopes (^{32}P-phosphate, ^{3}H-leucine, ^{14}C-acetate, ^{3}H-serine, ^{3}H-uracil, ^{3}H-thymidine, and ^{14}C-G3P) and Aquasol were obtained from New England Nuclear Corp., Boston, Mass. Special noble agar, tryptone, and vitamin-free Casamino Acids were supplied by Difco, Detroit, Mich. Potassium penicillin G was obtained from Pfizer Laboratories, New York, N.Y. All other chemicals were the highest quality commercially available.

Bacterial strains. All bacterial strains are derivatives of *E. coli* strain 8 (13). This strain lacks the aerobic G3P dehydrogenase, alkaline phosphatase, and is constitutive for the transport of G3P and for glycerol kinase. A list of strains is given in Table 1.

Media. The basic growth medium was similar to the M56LP media previously described (6). The modified M56LP consisted of 100 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM KCl, 15 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 0.3 mM phosphate (K^+ salt), adjusted to pH 7.4 with HCl. Glucose was always added to 0.2 to 0.4% as indicated. Vitamin-free Casamino Acids, G3P, and glycerol were added as indicated. Medium E (25) containing 0.2% glucose, 0.2% tryptone plus 0.1% G3P, or 0.1% glycerol was employed for growth of all cells used for enzymological experiments. Solid media contained 1.5% special noble agar.

Growth of bacteria. All growth experiments used the modified M56LP medium supplemented with 0.4% glucose and 0.1% Casamino Acids (vitamin free), G3P or glycerol was added as indicated in the figure legends. All growth experiments were performed at 37 C in Nephlo culture flasks by using a New Brunswick G76 gyratory shaker bath. Growth was followed on a Klett colorimeter equipped with a number 54 filter. One Klett unit corresponded to approximately 5 $\times$ 10^8 cells/ml.

Selection of G3P auxotrophs. Strain 8 was grown in the modified M56LP medium containing 0.2% glucose and 0.2% G3P, mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 100 μg/ml (1) and allowed to grow to stationary phase. The cells were washed two times by centrifugation to remove the G3P, and resuspended at about 2 $\times$ 10^7 cells/ml. After the cells had grown for two generations, penicillin G

TABLE 1. *Bacterial strains*

Strain	G3P dehydrogenase (biosynthetic)	G3P acyltransferase	Glycerol kinase
8 (parent)[a]	Positive	Positive	Constitutive, inhibited by fructose 1,6-diphosphate
BB2 BB20 3C3	Negative	Positive	Constitutive, inhibited by fructose 1,6-diphosphate
BB13 BB26	Positive	Defective in apparent K_m for G3P	Constitutive, inhibited by fructose 1,6-diphosphate
BB20-14	Negative	Positive	Constitutive (cells can utilize glycerol in presence of glucose presumably by loss of inhibition to fructose 1,6-diphosphate)
BB26-36 BB13-5	Positive	Defective in apparent K_m for G3P	Constitutive (cells can utilize glycerol in presence of glucose presumably by loss of inhibition to fructose 1,6-diphosphate)

[a] Hayashi et al. (13). All strains lack alkaline phosphatase and the aerobic G3P dehydrogenase and are constitutive for the transport of G3P.

was added to 10,000 units/ml. Three hours later, the cells were washed four times with the modified M56LP containing 0.2% glucose and 0.2% G3P, and were allowed to grow to stationary phase. G3P auxotrophs were identified by replica plating the survivors on glucose-modified M56LP plates with and without 0.1% G3P. After one penicillin cycle, the G3P auxotrophs constituted about 0.1% of the population. Another penicillin cycle resulted in an additional 5- to 10-fold enrichment. Since all of the mutants grow on minimal glucose plates supplemented with G3P, they have no nutritional requirements other than G3P. Spontaneous revertants to G3P prototrophy were obtained by plating cells on modified M56LP glucose plates. The reversion rate was about 10^{-8} reversions per generation per bacterium.

Selection of strains from the G3P auxotrophs that are supplementable with glycerol in the presence of glucose. The G3P requirements of these auxotrophs cannot be satisfied with glycerol in the presence of glucose. Strains BB26 and BB20 were mutagenized as described above. The cells were washed to remove the G3P, and samples were plated on modified M56LP containing 0.4% glucose and 0.1% glycerol. The colonies that formed (about one colony formed per 10^6 cells plated) were checked to see if the G3P requirement was maintained. Five of the 27 colonies derived from BB13 and one of the 36 derived from BB26 showed a requirement for G3P which could be supplemented with glycerol in the presence of glucose. Forty-two of the 90 colonies derived from BB20 showed this property. These strains presumably contain a glycerol kinase which is insensitive to inhibition by fructose 1,6-diphosphate (28, 29).

Enzyme assays. The biosynthetic G3P: NADPH dehydrogenase (EC 1.1.1.8) was assayed at 25 C on a Gilford 2400S recording spectrophotometer by using the conditions previously described (18). Cells were disrupted by sonic treatment in 50 mM Tris-hydrochloride, pH 7.4, which contained 1 mM dithiothreitol. Cell debris was removed by centrifugation at 27,000 × *g* for 30 min at 2 to 4 C by using a Sorvall RC2B certrifuge. Activity was measured in crude extracts or after a streptomycin sulfate precipitation and ammonium sulfate precipitation as described by Kito and Pizer (18). The assay was linear with protein and the time employed. One unit of G3P dehydrogenase activity corresponds to the dihydroxyacetone phosphate-dependent oxidization of 1 nmol of NADPH per min at 25 C.

The activity of the G3P acyltransferase was measured in membrane preparations prepared as described previously (20) by determining the incorporation of ^{14}C-G3P into phospholipid. The assay was performed at 25 C in screw-capped culture tubes (13 by 100 mm) in a volume of 0.2 ml. The incubation mixture contained 0.1 M Tris-hydrochloride, pH 8.5, 1 mg of BSA per ml, 50 μM Palmitoyl-CoA, 1 mM dithiothreitol, 5 mM $MgCl_2$, and ^{14}C-G3P (specific activity 3300 DPM/nmol) in amounts ranging from 75 μM to 1.5 mM, and 30 to 90 μg of membrane protein. The assay was started by adding the enzyme and was stopped 10 min later by adding 0.6 ml of 1% $HClO_4$. The assay was linear with respect to time and amount of protein employed. The quantity of G3P converted to lipid was measured by extracting the incubation mixture by the Bligh-Dyer procedure (3) by using 3 ml of MeOH/$CHCl_3$, 2:1, followed by 1 ml each of $CHCl_3$ and 1% $HClO_4$. The resulting chloroform phase was washed three times with 2 ml of 1% $HClO_4$. A 1-ml amount of the chloroform phase was transferred to a scintillation vial, evaporated under nitrogen, and then counted in 8 ml of Bray (4) solution. The method of Lineweaver and Burk was employed to estimate apparent K_m's for G3P (19).

Protein was determined by a microbiuret procedure (24).

Net synthesis of DNA, RNA, protein, and phospholipid. The net synthesis of DNA, RNA, and phospholipid was determined by using ^{32}P-phosphate as previously described (11). Net protein was determined by using ^{3}H-leucine as previously described (11).

Rates of RNA, DNA, protein, and phospholipid synthesis. Rates of synthesis have been assessed by briefly labeling cells with radioisotopes as described below. The incorporation of label was linear for the time employed. The rates of RNA, DNA, and phospholipid synthesis were determined by briefly labeling 0.7 ml of cells with 25 μCi of ^{32}P-phosphate (added carrier free to the culture medium) for 5 min as previously described (11). The rates of RNA, DNA, and protein synthesis were also determined as previously described (11) by briefly labeling 1.0-ml samples of cells with 0.2 μCi of ^{3}H-uracil (26 mCi/mmol), 10 μCi of ^{3}H-thymidine (520 mCi/mmol), and 10 μCi of ^{3}H-leucine (41 Ci/mmol). The rate of phospholipid synthesis was also estimated by measuring the incorporation of ^{14}C-acetate and ^{3}H-serine into Bligh-Dyer extractable material by labeling 0.8 ml of cells with 10 μCi of ^{14}C-acetate (59 mCi/mmol) for 5 min or with ^{3}H-serine (3.38 Ci/mmol) for 3 min. The $CHCl_3$ phase of the extraction was washed three times with 2 ml of water, and 1 ml of the $CHCl_3$ phase was transferred to a scintillation vial, evaporated under nitrogen, and counted.

Scintillation counting. All samples for the determination of the rate or net synthesis of RNA, DNA, and protein were counted in 8 ml of Aquasol. All samples measuring G3P acyltransferase activity were counted in 8 ml of Bray solution. Samples were counted in either a Packard 3320 or an Intertechnique SL30 scintillation counter. The counting efficiency for ^{14}C in Bray was 80% under the conditions employed.

RESULTS

Rationale for selection of parent strain. Strain 8, a K12 derivative of *E. coli* (13) was chosen as the parent strain for deriving the G3P auxotrophs because it constitutively transports the required supplement, and because this process will not be interfered with by alkaline phosphatase since it has been lost by mutation. Further, strain 8 was selected because it cannot aerobically catabolize the required nutrient, and because the in vivo pool of G3P can be

widely modulated by adding G3P to the medium (5, 12, 13, 18, 28, 29). Strain 8 contains glycerol kinase at constitutive levels which assures that the G3P requirement can be satisfied with glycerol under appropriate conditions (12, 28).

Selection and growth properties of G3P auxotrophs. G3P auxotrophs of *E. coli* strain 8 of Hayashi et al. (13) were derived by penicillin selection, and were identified by replica plating. All of the G3P auxotrophs selected for investigation grew as well as their parent, strain 8, on plates supplemented with G3P; did not form colonies or show any growth on plates after 48 h at 37 C in the absence of G3P; and did not require any nutrients other than G3P. The properties of the bacterial strains are summarized in Table 1.

G3P dehydrogenase mutants. The G3P requirement of the independently isolated strains BB2, BB20, and 3C3 resulted from a defective biosynthetic G3P dehydrogenase. The dihydroxyacetone phosphate-dependent oxidation of NADPH was not detected in either crude extracts for BB2, BB20, and 3C3 or after two purification steps for BB2 and BB20 (cf. Table 2). Mixing crude extracts of BB2, BB20, and 3C3 with an extract of their parent, strain 8, did not result in inhibition of the parental activity. Thus, the absence of activity in the mutant extracts does not appear to result from the production of an inhibitor in the mutants. The basis for the G3P requirement in these mutants was the same as for the glycerol-requiring mutant isolated by Hsu and Fox (16). Extracts prepared from spontaneous revertants of BB2, BB20, and 3C3, which formed colonies similar in size to their parent, showed G3P dehydrogenase activity that approached parental levels for some revertants (cf. Table 2). However, most of the revertant extracts contained significantly lower G3P dehydrogenase activity than parental extracts. This suggests that wild-type cells possess excess G3P dehydrogenase activity than is actually required for normal growth.

G3P auxotrophs containing normal levels of G3P dehydrogenase. Extracts from several of the G3P auxotrophs contained nearly wild-type levels of G3P dehydrogenase activity. Data for two independently isolated strains, BB13 and BB26, are shown in Table 2. The G3P dehydrogenase activity present in these strains was inhibited by G3P to the same extent as their parent, strain 8 (cf. Table 3).

G3P acyltransferase K_m mutants. Strains BB13 and BB26 contained a defective G3P acyltransferase. Membrane preparations from the parent, strain 8, contained a G3P acyltransferase activity that had an apparent K_m for G3P of 88 μM (Fig. 1A). (All apparent K_m's were determined from Lineweaver-Burk plots (19) which are shown in Fig. 1). Membrane preparations from BB13 showed a G3P acyltransferase activity that had an apparent K_m for G3P of 1250 μM, which is 14.2-fold higher than the parent (cf. Fig. 1B). Membrane preparations from strain BB26 contained an acyltransferase activity with an apparent K_m for G3P of 1,000 μM, 11.4-fold higher than its parent (cf. Fig. 1C). The apparent K_m's for G3P of the G3P acyltransferase of strains 8, BB13, and BB26 were essentially identical to those reported in Fig. 1 in at least three other experiments (data not shown) that utilized different membrane preparations. The maximal velocities (V_{max}) of

TABLE 2. *G3P dehydrogenase activity of the G3P auxotrophs and their revertants*

Strain	Sp act[a] (units/mg)	
	Crude extract[b]	0 → 75% $(NH_4)_2SO_4$[c]
8 (wild type)	16.8	14.7
BB13		10.8
BB26		6.5
BB2	ND[d] (<0.1)	ND (<0.1)
BB2 R-1[e]	2.5	
3C3	ND (<0.1)	
3C3 R-1	1.6	
3C3 R-2	10.3	
BB20	ND (<0.1)	ND (<0.1)
BB20 R-1	3.0	
BB20 R-2	0.8	
BB26-36	32.9[f]	

[a] One unit equals 1 nmol of NADPH oxidized per min at 25 C.

[b] The crude extract and ammonium sulfate fractionations were performed on separate batches of cells grown at 37 C and frozen before breakage.

[c] Essentially step II enzyme of Kito and Pizer (18).

[d] ND, Not detected.

[e] R indicates revertant.

[f] Crude extract prepared from unfrozen cells grown at 25 C.

TABLE 3. *G3P inhibition of the G3P dehydrogenase[a] in strain 8, BB13 and BB26*

Strain	Inhibition (%)				
	25[b]	50	75	100	150
8 (parent)	11	38	63	73	93
BB13	8	31	71	79	95
BB26	8	34	54	82	94

[a] The enzyme preparation employed was the same as in Table 2 (cf. footnote c).

[b] G3P concentration (μM).

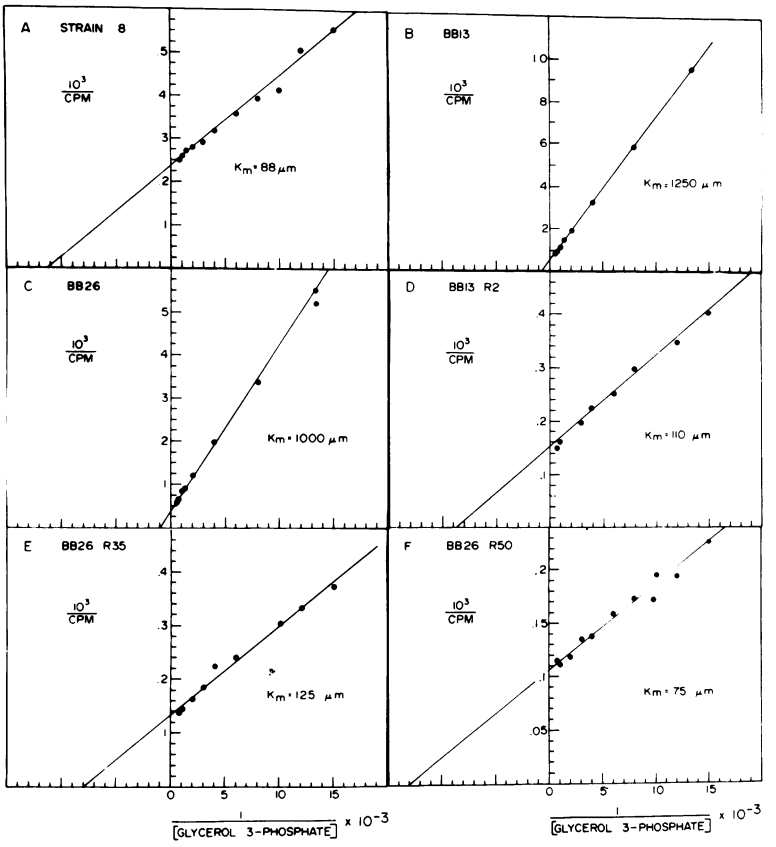


FIG. 1. *G3P dependence of the G3P acyltransferase in membrane preparations from strain 8 and the independently isolated mutant strains, BB13 and BB26, and revertants. The G3P acyltransferase was assayed as described in Materials and Methods. The data are presented as Lineweaver-Burk plots. The quantity of protein employed in each assay is shown in parenthesis. A, Strain 8 (31 μg); B, strain BB13 (55 μg); C, strain BB26 (74 μg); D, revertant of strain BB13, BB13-R2 (78 μg); E, revertant of strain BB26, BB26-R35 (66 μg); and F, revertant of strain BB26, BB26-R50 (70 μg).*

regard are similar to the mutant previously BB26 were 6.0, 2.3, and 2.6 nmol per min per mg, respectively (calculated from data in Fig. 1A–C). Membranes prepared from independently isolated revertants of BB13, BB13-R2, and of BB26, BB26-R35, and BB26-R50, contained G3P acyltransferase activity with apparent K_m's for G3P of 110, 125, and 75 μM, respectively (cf. Figure 1D–F). Therefore, all of the revertants contained membranous G3P acyltransferase activity with apparent K_m's for G3P similar to their parent. BB13 and BB26 contained a G3P acyltransferase activity defective in the apparent K_m for G3P and in this regard are similar to the mutant previously described by Kito et al. (17).

Effect of G3P deprivation and alkaline phosphatase on the growth of BB13 and BB26. The growth properties of strain 8, BB13, and BB26 are shown in Fig. 2. Strain 8 grew equally well in the presence or absence of G3P and was unaffected when *E. coli* alkaline phosphatase was added to the growth medium which contained G3P (cf. Fig. 2A). Strains BB13 and BB26 grew as well as their parent when supplemented with G3P (cf. Fig. 2B and C) and continued to grow when G3P was removed by filtration and the cells were resuspended in medium without supplement. Under these conditions the turbidity nearly doubled. Growth of BB13 and BB26 was also arrested upon addition of alkaline phosphatase to the growth medium containing G3P, presumably because the G3P was degraded to glycerol and phosphate. Glycerol cannot satisfy the G3P requirement of BB13 or of BB26 grown in medium containing glucose because glycerol kinase is inhibited by fructose 1,6-diphosphate (28, 29). The continued growth of these acyltransferase mutants upon G3P deprivation was similar to the growth

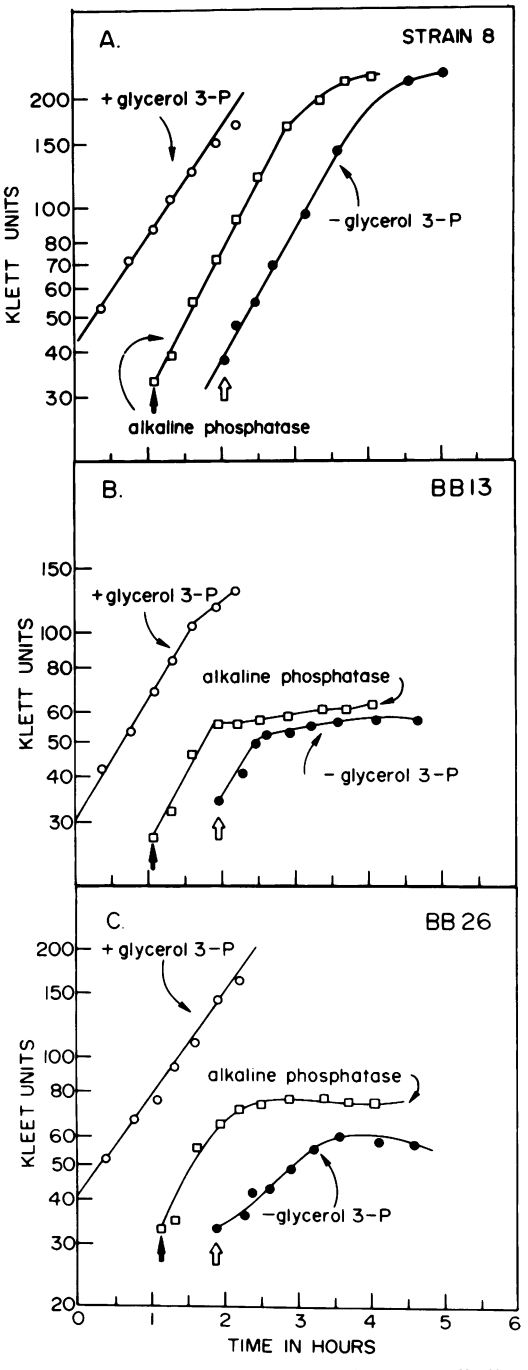
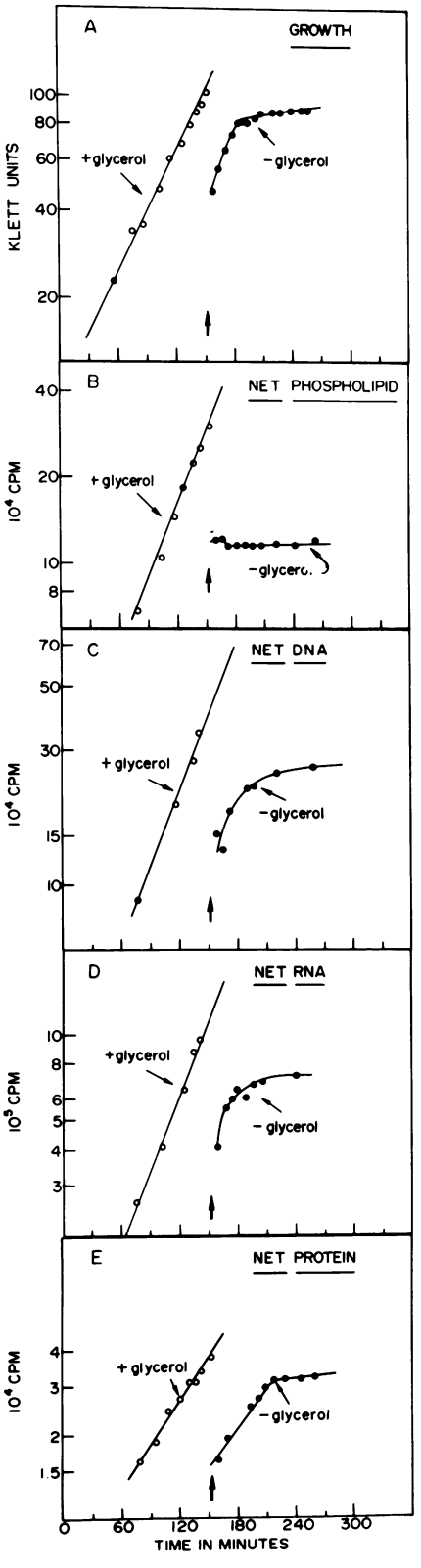


FIG. 2. *Effect of G3P deprivation and alkaline phosphatase on the growth of the G3P acyltransferase mutants. The media was supplemented where indicated with 0.05% G3P. Growth curves are shown for each strain in the presence of G3P, or its absence (cells were filtered at the open arrow on 0.45 μm HAWP 42-mm Millipore filters, washed five times with 2 to 3 ml of growth medium, and resuspended). Growth curves are also shown after addition of 1 unit of E. coli alkaline phosphatase per ml of growth medium containing G3P (added at the solid arrow). A, Strain 8; B, strain BB13; C, strain BB26.*

properties of the G3P dehydrogenase mutants (16), BB2 and BB20 (unpublished data). Therefore, the growth properties of the auxotrophs upon G3P deprivation do not provide a diagnosis for the enzymatic defect causing the G3P auxotrophy.

Selection of strains where the G3P requirement can be satisfied with glycerol in the presence of glucose. Strains where the G3P requirement of BB20 (G3P dehydrogenase mutant) and BB26 (G3P acyltransferase K_m mutant) could be satisfied with glycerol in the presence of glucose were selected because the growth of strain 8 in the absence of glucose is strongly inhibited by addition of G3P or glycerol to the medium (5); the inhibition of growth caused by G3P is overcome with glucose (5); and supplementation with glycerol allows ^{32}P-phosphate to be utilized to investigate phospholipid metabolism. The selection of these strains were accomplished by searching for mutagen-induced variants of BB20 and BB26 capable of growth on plates containing glucose and glycerol which maintained their requirement for G3P. Many strains of this type were derived, and two of these, BB20-14 and BB26-36, were selected for further investigations (cf. Table 1). The G3P requirements of these strains can be supplemented with glycerol in the presence of glucose presumably because they contain a glycerol kinase resistant to inhibition by fructose 1,6-diphosphate. Such aberrant glycerol kinase mutants have been previously described (28, 29). As shown in Table 2, an extract from BB26-36 contained high G3P dehydrogenase activity.

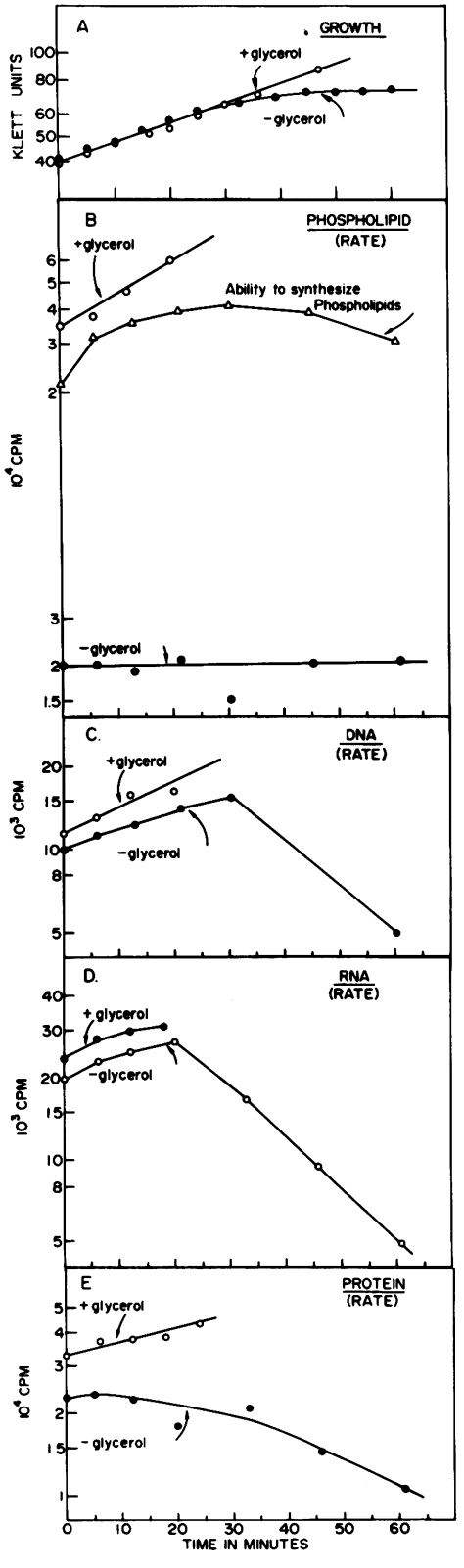
Growth and net synthesis in the G3P acyltransferase K_m mutant, BB26-36. The growth and net synthesis of phospholipid, DNA, RNA, and protein were measured in BB26-36. In Fig. 3A, the growth of BB26-36 supplemented with glycerol was identical to that of its parent (cf. Fig. 2A) and glycerol deprivation was followed by nearly a doubling of the turbidity, a result similar to that seen when BB26 was deprived of G3P (cf. Fig. 2C). ^{32}P-Phosphate incorporation was used to measure net synthesis of phospholipid (Fig. 3B), DNA (Fig. 3C), and RNA (Fig. 3D), and ^{3}H-leucine was used to measure net protein synthesis (Fig. 3E) in the presence and absence of glycerol. As expected, BB26-36 supplemented with glycerol showed net phospholipid, net DNA, net RNA, and net protein synthesis that paralleled the growth of the strain. However, BB26-36 deprived of glycerol



showed no net phospholipid synthesis (Fig. 3B) but continued net synthesis of DNA (Fig. 3C), RNA (Fig. 3D), and protein (Fig. 3E) which paralleled the growth curve and nearly doubled during the period of glycerol deprivation. The ^{32}P-phosphate incorporated into phospholipid in the presence of glycerol should largely be derived by the action of glycerol kinase and to a lesser extent through glycolysis and the G3P dehydrogenase, whereas in the absence of glycerol the latter pathway should be dominant. Regardless of the route of incorporation, the ^{32}P-phosphate incorporated into phospholipid should be directly comparable since they are derived from a common ^{32}P-phosphate pool via adenosine 5'-triphosphate.

Growth and rates of synthesis in strain BB26-36. The growth and rates of synthesis in strain BB26-36 are shown in Fig. 4. The rate of phospholipid synthesis was measured by labeling cells with ^{32}P-phosphate for 5 min, and the rates of DNA, RNA, and protein were measured by labeling cells for 3 min with ^{3}H-thymidine, ^{3}H-uracil, and ^{3}H-leucine, respectively. Again the turbidity nearly doubled upon glycerol deprivation (Fig. 4A). The rate of phospholipid synthesis increased when the cells were supplemented with glycerol (Fig. 4B). Upon glycerol deprivation, the rate of phospholipid synthesis was reduced 93% compared to the control at the first time point and did not change with time for the subsequent 60 min. In two other experiments, the rate of phospholipid synthesis was depressed 94 and 95% compared to the controls and did not change significantly with time. The ability of the cell to synthesize phospholipid was assessed after glycerol deprivation by adding glycerol during the period of ^{32}P-phosphate labeling. The data, shown in Fig. 4B, indicate that BB26-36 retained high capacity for the synthesis of phospholipids at the initial time point and that the capacity increased during glycerol deprivation, nearly in parallel with the growth curve. Even after 60 min, cells deprived of glycerol retained the capacity to synthesize phospholipid comparable to control levels. The rate of synthesis of DNA and RNA (Fig. 4C and D) was initially slightly depressed compared to

FIG. 3. *Growth and net synthesis in strain BB26-36 in the presence and absence of glycerol. Net synthesis of phospholipid, RNA, and DNA were followed in one culture containing $^{32}PO_4$ at 5 μCi/ml. Net synthesis of protein was measured in a separate culture containing ^{3}H-leucine at 0.5 μCi/ml. Glycerol was removed by filtration at the arrow, and the cells were resuspended at the lower cell density indicated in prewarmed medium without glycerol which contained either $^{32}PO_4$ or ^{3}H-leucine exactly as before. A, Growth; B, phospholipid; C, DNA; D, RNA; E, protein.*



their controls and increased with time until the cells slowed in their growth. At 60 min the rate of DNA and RNA synthesis had dropped to about 50% and 25%, respectively, of their initial levels. The rate of protein synthesis (Fig. 4E) was initially about 30% lower than controls and did not change dramatically with time, dropping only to 50% of its initial rate at 60 min. Two other experiments on the rate of protein synthesis revealed little change in rate with time but that the rate was decreased 25 and 33% compared to controls at the initial time point.

Growth and rate of lipid synthesis in BB26-36 by ^{14}C-acetate and ^{3}H-serine labeling. The rate of phospholipid synthesis was measured in strain BB26-36 by briefly labeling cells with ^{14}C-acetate for 5 min, and with ^{3}H-serine for 3 min (cf. Fig. 5). Again the turbidity of the culture nearly doubled upon glycerol deprivation (Fig. 5A). The rate of phospholipid synthesis assessed by determining the incorporation of ^{14}C-acetate into Bligh-Dyer extractable material was depressed about 90% upon glycerol deprivation at the first time point (cf. Fig. 5B). The incorporation of ^{14}C-acetate had declined to about 3% of the control level at 15 min and did not change with time during the subsequent 42 min of the experiment. Identical results were obtained in another experiment. The rate of phospholipid synthesis assessed by measuring the incorporation of ^{3}H-serine into Bligh-Dyer extractable material was initially 99% lower than controls upon glycerol deprivation. The incorporation of ^{3}H-serine remained at this low level for 16 min and then gradually increased to about 10% of the control level at 52 min. Another experiment where cells were labeled for 5 min with ^{3}H-serine gave similar results. The rate of phospholipid synthesis is depressed by greater than 95% upon glycerol deprivation as assessed by briefly labeling cells with ^{32}P-phosphate (Fig. 4B), ^{14}C-acetate (Fig. 5B), and ^{3}H-serine (Fig. 5C).

Growth and rate of DNA and RNA synthesis in BB26-36 by ^{32}P-phosphate labeling. The rate of DNA and RNA synthesis was also measured by pulsing cells with ^{32}P-phosphate

FIG. 4. *Growth and rates of synthesis in strain BB26-36. The rates of synthesis of phospholipid, DNA, RNA, and protein were measured with ^{32}P-phosphate, ^{3}H-thymidine, ^{3}H-uracil, and ^{3}H-leucine, respectively, in the presence and absence of glycerol. In addition, the ability to synthesize phospholipid was measured as a function of glycerol deprivation by adding glycerol to 0.2% during the 5-min period of ^{32}P-phosphate labeling. A, Growth; B, rate of phospholipid synthesis and ability for phospholipid synthesis; C, rate of DNA synthesis; D, rate of RNA synthesis; E, rate of protein synthesis.*

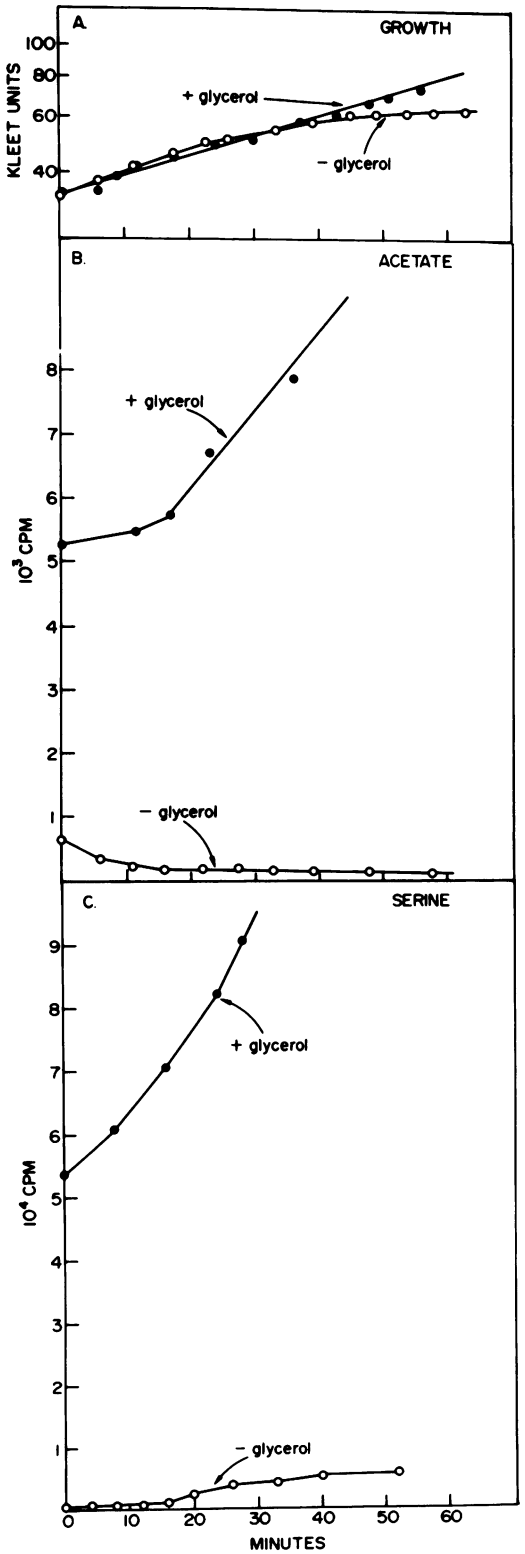


FIG. 5. *Growth and rate of lipid synthesis in strain BB26-36. The rate of lipid synthesis was assessed by briefly labeling cells with ^{14}C-acetate and with ^{3}H-serine. A, Growth; B, ^{14}C-acetate incorporation; C, ^{3}H-serine incorporation.*

for 5 min (cf. Fig. 6). Upon glycerol deprivation, the rate of DNA synthesis increased with time during the period of cell growth and then decreased to 50% of the initial rate at 50 min. The rate of RNA synthesis also increased with

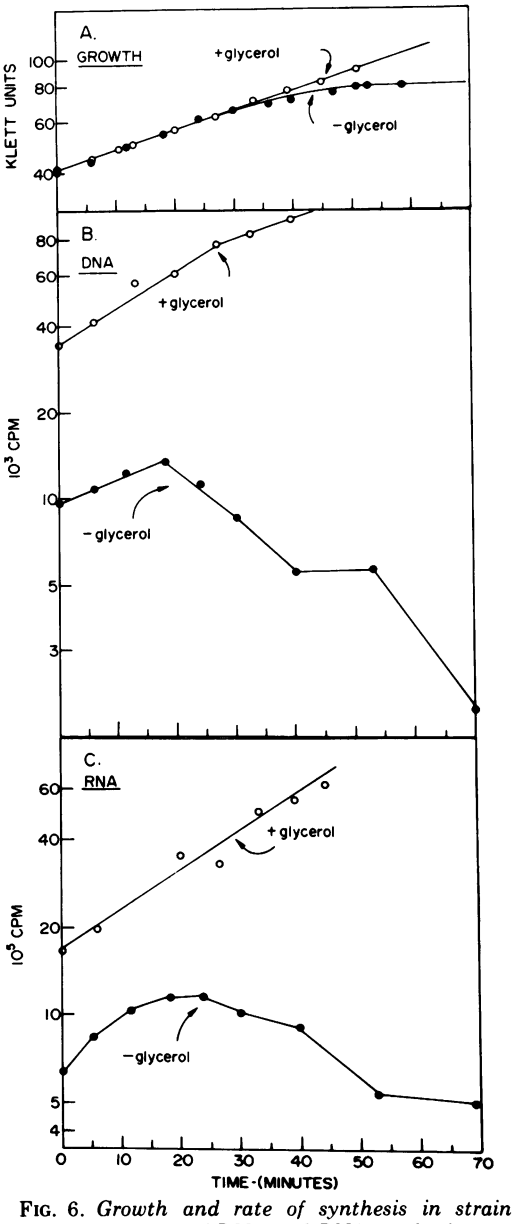


FIG. 6. *Growth and rate of synthesis in strain BB26-36. The rates of DNA and RNA synthesis were measured by briefly labeling cells with ^{32}P-phosphate. A, Growth; B, DNA; C, RNA.*

time during glycerol deprivation and then decreased after 50 min to about 80% of its initial rate. The assessment of the rates of DNA and RNA synthesis by ^{32}P-phosphate labeling agrees qualitatively with the pattern shown in Fig. 4 using specific labels. However, the rates of RNA and DNA synthesis were initially about threefold lower than controls upon glycerol deprivation. This depression of the rate of RNA and DNA synthesis upon glycerol deprival has been verified in two other experiments where the rates were about twofold lower than controls. No satisfactory explanation can be offered to account for the poor quantitative agreement between ^{32}P-phosphate labeling and the specific labeling assessments of the rates of RNA and DNA synthesis.

DISCUSSION

Strains of *E. coli* have been derived which are defective in membrane phospholipid synthesis as a result of G3P auxotrophy. These strains appear suitable for further investigations on the involvement of phospholipid in biological membranes because (1) the strains are biochemically well defined; (2) the strains are tight in their requirement for G3P; (3) investigations will not be plagued with problems of interpretation caused by the aerobic catabolism of the required supplement; and (4) glycerol supplementation facilitates the use of ^{32}P-phosphate for investigations of phospholipid metabolism.

The G3P auxotrophy resulted from loss of the biosynthetic G3P dehydrogenase in strains BB2, BB20, and 3C3 (Tables 1 and 2). A glycerol-requiring strain of *E. coli* has previously been shown to lack this activity (16). The G3P auxotrophy of strains BB13 and BB26 resulted from a G3P acyltransferase activity defective in its apparent K_m for G3P (Table 1, and Fig. 1). A "leaky" G3P auxotroph of *E. coli* was isolated by Kito et al. (17) from strain 9 of Hayashi et al. (13) which lacks glycerol kinase. This strain, K956, doubled in 300 min in the absence of G3P (17). The G3P acyltransferase of K956 appeared to have a higher K_m for G3P than its parent, but quantitative evaluation was quite difficult because the mutant gave nonlinear Lineweaver-Burk plots (17). Strains BB13 and BB26 contained a G3P acyltransferase activity with apparent K_m's (derived from linear Lineweaver-Burk plots, Fig. 1) for G3P 14.2 and 11.4 higher, respectively, than their parent. BB13, BB26, and BB26-36 essentially stopped growing after about one doubling upon G3P or glycerol deprivation (Fig. 2–5) and did not grow on plates without supplement. Strains BB26 and BB13 appear to be "tight" G3P acyltransferase K_m mutants. The intracellular G3P pool is regulated by G3P feedback inhibition of the G3P dehydrogenase (18). Therefore, BB13 and BB26 (both contain normal biosynthetic G3P dehydrogenase activity [Tables 2 and 3]) can grow only when the intracellular pool is elevated by adding G3P to the growth medium. In strain 8, the intracellular pool of G3P can exceed 20 mM when G3P is added to the growth medium (12). The accumulation of G3P can lead to growth stasis in the absence of glucose (5). Investigations using BB13 and BB26 must, therefore, be performed in a growth medium containing glucose which made it impossible to satisfy the G3P requirements with glycerol since the glycerol kinase is inhibited by fructose 1,6-diphosphate. This problem was overcome by selecting variants capable of utilizing glycerol in the presence of glucose (Table 1) which presumably contain a glycerol kinase desensitized to fructose 1,6-diphosphate inhibition.

The phenotype of BB13 and BB26 appeared to be the result of a single mutation since revertants were obtained at a rate of about 10^{-8} reversions per generation per bacterium, and since all revertants contained G3P acyltransferase activity similar to their parent (Fig. 1). The lesion in BB13 and BB26 is thought to be in the structural gene for the acyltransferase but since this activity is associated with the inner cytoplasmic membrane (2, 26) it is possible that mutation of another membrane component could result in a similar phenotype.

Temperature-sensitive mutants of the G3P acyltransferase have been isolated from strain 8 (7), studied genetically (9), and the regulation of macromolecular synthesis has been investigated (11). Strains BB13 and BB26 continue to grow when deprived of G3P (Fig. 2) as does BB26-36 when deprived of glycerol (Fig. 3–5). This is in sharp contrast to the temperature-sensitive acyltransferase mutants which stop growing immediately upon shift to the restrictive temperature (7, 11). Net phospholipid synthesis stopped immediately upon glycerol deprivation in BB26-36 but net DNA, net RNA, and net protein synthesis continued (Fig. 3). These results are unlike those observed for the temperature-sensitive acyltransferase mutants which immediately stop net phospholipid, protein, RNA, and DNA synthesis at the restrictive temperature (11). The rates of synthesis of phospholipid, protein, DNA, and RNA were determined in BB26-36 (Fig. 4 and 5) (for problems concerning interpretation see Glaser et al. [11]). In BB26-36, glycerol deprivation immediately decreased the rate of phospholipid synthesis 93 to 95% as assessed by ^{32}P-phos-

phate labeling, and the rate remained at this level for 60 min (Fig. 4B); further, the rate of phospholipid synthesis was decreased greater than 95% as assessed by ^{14}C-acetate and ^{3}H-serine labeling upon glycerol deprivation; the rates of DNA and RNA synthesis measured with ^{3}H-thymidine and ^{3}H-uracil, respectively, were initially decreased about 15%, increased with time until the cells stopped growing, and then declined (Fig. 4C-D); the rate of protein synthesis was initially decreased by 30% and changed little with time (Fig. 4E). These findings are similar to the studies on glycerol (16) and unsaturated fatty acid auxotrophs (10, 14), and unlike those for the temperature-sensitive G3P acyltransferase mutants, where the rates of phospholipid, DNA, RNA, and protein synthesis rapidly declined in parallel (11).

BB26-36 retained and even gained in the capacity to synthesize phospholipids during the period of glycerol deprivation (Fig. 4B) which suggests that the membrane-bound enzyme system responsible for phospholipid synthesis increased during the period of glycerol deprivation. This observation as well as continued DNA, RNA, and protein synthesis suggests that no serious energy crisis is occurring during the doubling after glycerol deprivation, yet the cells stop growing during this time period as a consequence of the cessation of phospholipid synthesis. The factors that lead to cessation of cell growth and of DNA, RNA, and protein synthesis cannot be evaluated from the present study.

The apparent coupling of phospholipid and macromolecular synthesis in the temperature-sensitive G3P acyltransferase mutants (11), and the data presented in this investigation on G3P acyltransferase K_m mutant may appear to be contradictory. The residual phospholipid synthesis in BB26-36 may account for these dramatic differences (11). Experiments are in progress to test this possibility by constructing a double mutant which is devoid of G3P dehydrogenase and contains a K_m-defective acyltransferase. In this double mutant, the rate of residual phospholipid synthesis should be abolished. Toward that end, the G3P dehydrogenase mutants have been mapped near mannitol at 71 min on the *E. coli* genetic map (J. E. Cronan, Jr., and R. M. Bell, manuscript in preparation) and mapping of the G3P acyltransferase k_m mutants is in progress. Finally, the intriguing possibility suggested by Glaser et al. (11) that the membranous G3P acyltransferase might contain a thermolabile subunit(s) in the temperature-sensitive mutants which is shared by other proteins in other biosynthetic pathways is supported by these investigations since the acyltransferase K_m mutants may be defective in a different subunit than the temperature-sensitive mutants.

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