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Interaction of *sn*-Glycerol 3-Phosphorothioate with *Escherichia coli*: Effect on Cell Growth and Metabolism

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sn-Glycerol 3-phosphorothioate was found to be bacteriocidal to strains of *Escherichia coli* which have a functional *sn*-glycerol 3-phosphate transport system. This effect was manifest in strains 7 and 8, which are constitutive mutants for the utilization and transport of *sn*-glycerol 3-phosphate (*glpR*^{c}*2*). Strain E15, which is considered to be wild type for the glycerol phosphate functional units, was affected by the phosphorothioate analog only under conditions that are known to induce the transport system for *sn*-glycerol 3-phosphate. In addition, another strain of *E. coli*, strain 6, which is isogenic with strain E15 but has an impaired *sn*-glycerol 3-phosphate transport system (*glpT13*), was not affected by similar concentrations of *sn*-glycerol 3-phosphorothioate. Transport studies in which [^{3}H]glycerol phosphate and its phosphorothioate analog were used demonstrated that the latter compound was taken up via the specific active transport system for *sn*-glycerol 3-phosphate; the K_m values were 9 and 11 μM, respectively. The rates of macromolecular synthesis were found to be inhibited severely by *sn*-glycerol 3-phosphorothioate at a concentration at which *sn*-glycerol 3-phosphate had no effect (5 μM). At a lower concentration of the analog (0.5 μM), the rates of protein synthesis and RNA synthesis (52 and 58% below control values after 90 min, respectively) were more sensitive than the rates of DNA synthesis and cell wall synthesis (18% below control values after 3 h for DNA; transient decrease in the cell wall values after 90 min). The levels of the nucleoside triphosphates were not affected by the presence of the phospholipid precursor or its analog at a concentration of 5 μM. The phospholipid composition was significantly altered in the presence of bacteriocidal concentrations (5 μM) of *sn*-glycerol 3-phosphorothioate. The amount of phosphatidylglycerol in the membranes decreased from 13.5 to 3.5%. Concomitant with this decrease in phosphatidylglycerol content was a fourfold increase in the ^{32}P content of cardiolipin (from 6.8 to 24.2%), whereas the phosphatidylethanolamine content showed only a minor reduction (8%) after 3 h. The rates of synthesis of all of the phospholipids decreased in the presence of 5 μM *sn*-glycerol 3-phosphorothioate, with the most significant effects observed for phosphatidylglycerol (63% after 3 h). Phosphatidylglycerol showed increased rates of turnover after 90 min (21%) and 3 h (11%), with concomitant increases in the levels of cardiolipin of more than twofold. Our data suggest that a considerably greater proportion of phosphatidylglycerol turnover may be recovered in cardiolipin than is metabolized via other pathways (e.g., the membrane-derived oligosaccharide pathway).

The elucidation of the role played by phospholipids in the functioning of biological membranes is fundamental to our understanding of membrane-mediated events. Investigators have used a variety of approaches (e.g., biochemical, genetic, chemical, and physical) to study the interactions between phospholipids and proteins in biological membranes (7, 9, 26, 32). In this paper, we describe our initial biochemical approach for studying the role of the polar headgroup of phospholipids in membrane function. The approach that we have developed involves the manipulation of the membrane phospholipid population by the substitution of the phosphate moiety of the polar headgroup by its phosphorothioate analog (i.e., one of the nonbridge oxygens of the polar headgroup phosphate is replaced by sulfur). The rationale behind this replacement is based upon the observation that phosphorothioate monoesters and diesters are, in many cases, resistant to hydrolytic cleavage by phosphatases and phosphodiesterases, respectively (8, 34). We have recently demonstrated that phospholipases A_2 and C exhibit abso-

lute and opposite preference for a single diastereoisomer of 1,2-dipalmitoyl-*sn*-glycerol 3-thiophosphorylethanolamine, leaving the other isomer intact (22). Therefore, it is possible that phospholipids containing this substitution have a longer in vivo lifetime and that a block in a specific step(s) in the metabolism of phospholipids occurs, resulting in an abnormal membrane state and, consequently, the perturbation of specific membrane processes.

We focused our initial studies on the metabolism of these analogs and their effect on membrane function in *Escherichia coli*, since phospholipid biosynthesis and function in bacteria have been extensively studied and characterized by both biochemical and genetic techniques (24). The direct incorporation of phospholipids into the outer membranes of gram-negative bacteria has been achieved by fusing phospholipid vesicles with deep rough mutants of *Salmonella* spp. (14) or by culturing *E. coli* in the presence of 1- and 2-acyl lysophospholipids (13). These methods can also be exploited for the introduction of specific, chemically synthesized thiophospholipids into membranes. However, since *E. coli* possesses an active transport system for *sn*-glycerol 3-phosphate (glycerol-P) (11), the precursor of all phospholipids in *E. coli*, it is possible to manipulate metabolically polar headgroup composition by culturing cells in the presence of *sn*-glycerol 3-phosphorothioate (glycerol-phosphorothioate) rather than glycerol-P. In this paper we report that glycerol-phosphorothioate is transported into strains of *E. coli* with a functioning glycerol-P transport system and, in addition, is bacteriocidal to these organisms at concentrations that approximate its K_m for uptake.

(A preliminary report of the results was presented at the 71st Annual Meeting of the American Society of Biological Chemists, 1–5 June 1980, New Orleans, La. [Fed. Proc. **39**:1982, 1980]. The results were taken from a thesis presented by J.W.H. to the Albert Einstein College of Medicine.)

MATERIALS AND METHODS

Materials. *E. coli* phosphatidylethanolamine, egg phosphatidylglycerol, bovine heart cardiolipin, L-α-lysophosphatidylethanolamine, L-α-lysophosphatidylglycerol, rabbit muscle glycerol-3-phosphate dehydrogenase (70 U/mg), NAD^+, DL-dithiothreitol, *rac*-glycerol-P, and chloramphenicol were purchased from Sigma Chemical Co. Tryptone and Bacto-Agar were obtained from Difco Laboratories. [4,5-^{3}H]isoleucine (109 Ci/mmol), (DL-+-*meso*)-2,6-diamino-[*G*-^{3}H]pimelic acid dihydrochloride (900 mCi/mmol), and [1,3-^{3}H]glycerol (2.5 Ci/mmol) were obtained from Amersham Corp. Carrier-free [^{32}P]phosphoric acid (in HCl-free water), [*methyl*-^{3}H]thymidine (73.6 Ci/mmol), [5,6-^{3}H]uridine (38.3 Ci/mmol), and [^{35}S]ATPγS (88.8 Ci/mmol) were purchased from New England Nuclear Corp. [1,3-^{3}H]glycerol-P was synthesized enzymatically from [1,3-^{3}H]glycerol and ATP, and [1,3-^{3}H]glycerol-phosphorothioate was synthesized enzymatically from [1,3-^{3}H]glycerol and ATPγS; these syntheses were performed in the presence of glycerol kinase, and the compounds were purified by high-pressure liquid chromatography (22a). Glycerol-phosphoro[^{35}S]thioate was prepared in a similar manner by thio-phosphorylation of glycerol with [^{35}S]ATPγS. Glycerol-phosphorothioate was prepared by mild alkaline hydrolysis of 1,2-dipalmitoyl-*sn*-glycerol 3-phosphorothioate and was purified by ion-exchange chromatography on DEAE-52, as elsewhere described (22a).

Bacterial strains. Isogenic strains derived from *E. coli* strain E15 were used in this study. Strains E15 and 6 were obtained from B. Bachmann, Coli Genetic Stock Center, Yale University. Strains 7 and 8 were provided by R. Engel and B. Tropp, Queens College of the City University of New York. The genotype of *E. coli* E15, as expressed by the genetic symbols described by Taylor (31), is as follows: *HfrC phoA8 tonA22 T2^{r}rel-1*. This strain was assumed to be wild type for the glycerol phosphate functional units. Strain 6 has an impaired glycerol phosphate transport system (*glpT13*). Strains 7 and 8 are constitutive mutants for the utilization and transport of glycerol phosphate (*glpR^{c}2*). Strain 8 has an additional lesion, *glpD3*; i.e., this strain lacks the catabolic glycerol phosphate dehydrogenase (pyridine nucleotide independent).

Assays. Glycerol-P and glycerol-phosphorothioate were assayed by the method of Bergmeyer (2), using glycerol phosphate dehydrogenase and NAD^+.

Growth of bacteria. The bacteria were cultured in Garen-Levinthal minimal medium (10) supplemented with 0.6 mM phosphate, 0.5 mM dithiothreitol, and 0.5% sodium succinate as the sole carbon source. Overnight cultures grown in supplemented medium (1% casein amino acids) were harvested and suspended in 15 to 25 ml of minimal medium at an initial density of 15 to 17 Klett units. Cell growth was monitored as previously described (28). Experiments were initiated at a cell density of 25 to 30 Klett units by inoculating a culture with the desired concentration of glycerol-P or glycerol-phosphorothioate. Cell viability was determined by plating a culture onto nutrient broth agar plates. Single colonies were counted after overnight incubation at 37°C.

Transport assays. Overnight cultures prepared as described above were suspended in 10 mM Tris-hydrochloride (pH 7.0) containing 150 mM NaCl, 0.5 mM dithiothreitol, and 40 μg of chloramphenicol per ml at a Klett reading of 75 U (corresponding to approximately 4×10^9 cells per ml). The transport assay was initiated by adding 1 ml of a bacterial suspension (pre-equilibrated at 37°C) to 10 μl of [1,3-^{3}H]glycerol-P or [1,3-^{3}H]glycerol-phosphorothioate (final concentration, 0.5 μM; 2.5 Ci/mmol). At different times, 50-μl samples were removed and processed by the method of Leifer et al. (19). The kinetics of uptake of [^{3}H]glycerol-P (106.3 μCi/μmol; 0.1 to 100 μM) and glycerol-phosphoro[^{35}S]thioate (76.8 μCi/μmol; 0.1 to 100 μM) were determined as described above.

Effect of glycerol-phosphorothioate on cellular metabolism. (i) Rates of synthesis of DNA, RNA, protein, and cell wall. Cultures of *E. coli* strain 8 were grown as described above. The rates of macromolecular biosyn-

thesis were measured by a modification of the method of Contreras et al. (4). At the appropriate cell density the cultures were inoculated with the desired concentrations of glycerol-P or glycerol-phosphorothioate (0.1 to 5 μM). At different times 5-ml samples were removed, and 0.3-ml portions of these samples were added in triplicate to vials containing either [^{3}H]thymidine (2 μCi/nmol), [^{3}H]uridine (2 μCi/nmol), [^{3}H]isoleucine (33.3 nCi/nmol), or [^{3}H]diaminopimelic acid (0.9 μCi/nmol). The solutions were incubated in a model G76 Gyrotory water bath shaker (New Brunswick Scientific Co.) at 37°C for 10 min. The labeling was terminated by adding 1.5 ml of cold 20% trichloroacetic acid, and the precipitates were collected on membrane filters (pore size, 0.45 μm; type HAWP; Millipore Corp.) for DNA and RNA studies or on glass fiber filters (Enzo) for protein and cell wall studies. The filters were washed with 25 ml of 5% trichloroacetic acid, dried, and counted in 10 ml of Liquiscint.

(ii) Rate of phospholipid synthesis. Cultures of strain 8 were grown as described above and at the appropriate cell density were inoculated with 0.5 or 5.0 μM glycerol-phosphorothioate. At different times 2-ml samples were removed and immediately added to glass vials that were preloaded with 20 μCi of $^{32}P_i$. The cells were incubated as described above, and the labeling was terminated by adding an equal volume of cold 10% trichloroacetic acid. The contents were centrifuged at 9,500 rpm for 5 min in a Sorval SS34 rotor. The pellet was washed twice with cold 5% trichloroacetic acid and then suspended in 2 ml of cold deionized water. The phospholipids were extracted from this aqueous suspension by using a modification of the method of Ames (1) for 10 min on ice in chloroform-methanol (2:1 vol/vol). The chloroform-methanol extraction solution contained phosphatidylethanolamine (74 μg/ml), phosphatidylglycerol (25 μg/ml), and cardiolipin (15 μg/ml). The chloroform phase was washed once with 2 M KCl and once with deionized water. Portions of the chloroform solution were desiccated and counted in 10 ml of Liquiscint. Samples were also dried under a vacuum, dissolved in 100 μl of chloroform-methanol (2:1, vol/vol), and chromatographed by thin-layer chromatography on Silica Gel 60 plates (EM Laboratories), using a solvent system containing chloroform, methanol, and acetic acid (65:25:8, vol/vol/vol). ^{32}P-labeled phospholipids were located by autoradiography on Kodak BB-5 X-ray film, and the appropriate areas were excised and counted in 10 ml of Liquiscint.

(iii) Total phospholipid content. Cells were suspended in minimal medium at an initial density of 5 to 10 Klett units, and $^{32}P_i$ (5 μCi/ml) was added to each culture. The cultures were grown at 32°C in a shaking incubator for several generations to reach steady-state labeling of phospholipids. The cells were pelleted, suspended in fresh minimal medium containing $^{32}P_i$ of the same specific activity, and inoculated with various concentrations of glycerol-phosphorothioate (0.5 to 5 μM). At different times 1-ml samples were removed, and the phospholipids were extracted by a modification of the method of Ames (1), as described above, except that the chloroform phase was washed twice with 2 M KCl. The distribution of ^{32}P among the various phospholipids was determined as described above. In addition, samples were also chromatographed in the two-dimensional solvent system described by Nishijima and Raetz (21) (first dimension, chloroform-methanol-water, 65:25:4, vol/vol/vol; second dimension, chloroform-methanol-acetic acid, 65:25:10, vol/vol/vol).

(iv) Phospholipid turnover. Cultures of *E. coli* strain 8 were grown as described above in the presence of 8 μCi of $^{32}P_i$ per ml. The cells were pelleted, suspended in fresh minimal medium lacking $^{32}P_i$, and inoculated with 0.5 or 5 μM glycerol-phosphorothioate. At different times 1-ml samples were removed, and the phospholipids were extracted by the method of Ames (1). The distribution of ^{32}P among the various phospholipids was determined as described above.

(v) Nucleoside triphosphate content. Cultures of *E. coli* strain 8 were grown as described above in the presence of $^{32}P_i$ (10 μCi/ml). The cells were suspended in fresh minimal medium having the same specific activity to a cell density of 25 to 30 Klett units. Samples (2 ml) were incubated in a gyratory water bath at 37°C containing glycerol-P or glycerol-phosphorothioate at the desired concentration (0.5 or 5 μM). At different times (ranging from 30 s to 60 min) 100-μl samples were removed and processed by the method of Cashel et al. (3).

RESULTS

We have investigated the stability of glycerol-phosphorothioate in aqueous solution as a function of pH. Although phosphorothioate monoesters are relatively stable compounds, under certain conditions they can undergo acid-catalyzed desulfurization reactions, giving rise to their parent "all-oxy" derivatives (23). In a typical experiment, [1,3-^{3}H]glycerol-phosphorothioate (100,000 cpm) was incubated with 100-μl portions of various buffers at pH 3 to 7 for 16 h at 30°C. After incubation the samples were cochromatographed with authentic glycerol-P and glycerol-phosphorothioate on silica gel plates. No formation of glycerol-P was detected at any pH value tested. In a similar experiment, no loss of sulfur was observed when glycerol-phosphorothioate was incubated in minimal culture medium supplemented with 0.5% succinate and 0.5 mM dithiothreitol for 15 h at 37°C. Since formation of disulfide bridges can result from oxidation of phosphorothioates, it was important to include a reducing agent in the culture medium. The effects of both 2-mercaptoethanol and dithiothreitol on the growth of *E. coli* strains 7 and 8 were investigated, and we found that, whereas 5 mM 2-mercapethanol arrested the growth of these organisms, 0.5 mM dithiothreitol had no effect (data not shown). All experiments were performed with 0.5 mM dithiothreitol.

Growth and viability. The addition of glycerol-phosphorothioate at concentrations between 2.5 and 100 μM led to rapid cessation of growth of *E. coli* strain 8 (Fig. 1A). Cell viability studies revealed that this analog was bacteriocidal at these concentrations (Fig. 1B), with the rate of cell death proportional to the analog concentra-

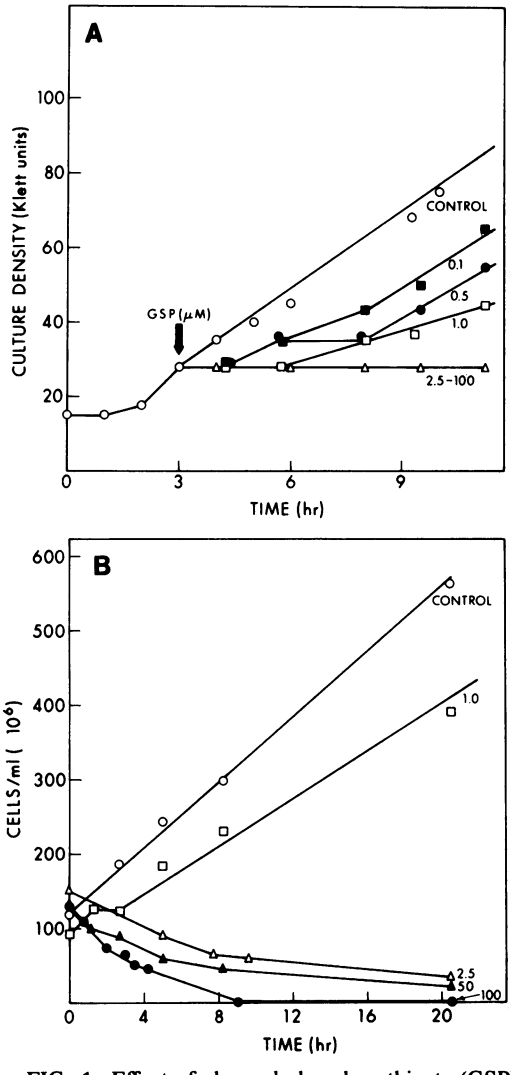

FIG. 1. Effect of glycerol-phosphorothioate (GSP) on the growth of *E. coli* strain 8. (A) Culture density. The arrow indicates when glycerol-phosphorothioate (0.1 to 100 μM) was added. (B) Cell viability. Zero time was when glycerol-phosphorothioate (0.1 to 100 μM) was added. Culture density and cell viability were determined as described in the text.

tion. A sharp transition between inhibitory and non-inhibitory growth states was observed. A short stasis period followed by growth was observed at a concentration of 1 μM, whereas at a concentration of 2.5 μM the analog was bacteriocidal. Similar effects were observed with strain 7 (data not shown), except that the minimal bacteriocidal concentration was 5 μM glycerol-phosphorothioate. The phosphorothioate analog was not toxic to the glycerol-P transport-impaired mutant (strain 6). In addition, strain E15, which is considered to be wild type for the glycerol phosphate functional units, was affected by glycerol-phosphorothioate only when it was cultured in the presence of glycerol (an inducer of the glycerol-P transport system) (Table 1).

The addition of 56 mM glucose to the culture medium 1 h after the addition of 10 or 50 μM glycerol-phosphorothioate alleviated the toxicity of the phosphorothioate analog in strains 7 and 8 (data not shown). Furthermore, when strain 7 was grown in the presence of 54 mM glycerol as the sole carbon source, it was able to overcome the bacteriocidal effects of 25 μM glycerol-phosphorothioate (data not shown). In addition, glycerol-phosphorothioate toxicity (10 μM) was not observed in the presence of high concentrations of glycerol-P (100 μM) in these cultures (data not shown), possibly because of competitive inhibition of uptake into the cells. However, when strain 7 was grown in the presence of bacteriocidal concentrations of glycerol-phosphorothioate (5, 10, 15, and 100 μM) for 4 h and then plated onto agar containing 2.3 mM glycerol-P, no reversal of the toxic effect was observed.

Transport. Both [^{3}H]glycerol-P and [^{3}H]glycerol-phosphorothioate were transported into strain 8, and the presence of high concentrations of unlabeled glycerol-P (150 μM) completely inhibited the uptake of [^{3}H]glycerol-phosphorothioate (0.5 μM) (Fig. 2). Transport of [^{3}H]glycerol-P was similarly inhibited by high concentrations of nonradioactive glycerol-phosphorothioate (data not shown). In addition, strain 6 showed an impairment of glycerol-phosphorothioate uptake. Previous studies (11) have revealed that glycerol-P is actively transported into *E. coli* when 2,4-dinitrophenol or sodium azide is added, resulting in an efflux of the internalized glycerol-P. We observed similar effects during glycerol-phosphorothioate uptake (data not shown). Kinetic studies indicated that the K_m values for the transport of glycerol-phosphorothioate and glycerol-P were 11 and 9 μM, respectively; the latter value is in close agreement with the values obtained by other workers (11, 19).

Cellular metabolism. We found that the rates of RNA synthesis and protein synthesis were more sensitive to the phosphorothioate analog than the rates of DNA synthesis and cell wall synthesis (Fig. 3). At 0.5 μM glycerol-phosphorothioate, significant decreases in the rates of RNA synthesis and protein synthesis were observed after 90 min (52 and 58% below control values, respectively). Under similar conditions, the rate of DNA synthesis was barely affected after 90 min but did decrease to 18% below control values after 3 h. Even at 1 μM glycerol-

TABLE 1. Effects of glycerol-P and glycerol-phosphorothioate on the growth of isogenic strains of *E. coli*

Strain	Glycerol-P transport phenotype	Catabolic glycerol-P dehydrogenase	Growth condition	Effect of:			
				100 μM glycerol-P	5 μM glycerol-P	100 μM glycerol-phosphorothioate	5 μM glycerol-phosphorothioate
E15	Inducible	+	Induced[a]	NE[b]	NE	Stasis[c]	NE
			Not induced[d]	NE	NE	NE	NE
6	Impaired[e]	+	Induced	NE	NE	NE	NE
7	Constitutive[f]	+	Present	NE	NE	Cidal	Cidal[g]
8	Constitutive	− (*glpD*3)	Present	Stasis[h]	NE	Cidal	Cidal[i]

[a] Grown in the presence of 0.5% glycerol as the sole carbon source.
[b] NE, No effect on growth.
[c] Transient inhibition of cell growth. The cells started to divide after 7 to 8 h.
[d] Grown in the presence of 0.5% glucose as the sole carbon source.
[e] The locus of the mutation is *glpT13*. This organism has 10% of the activity found in strain E15.
[f] Constitutive mutant for the utilization and transport of glycerol phosphate (*glpR*^{c}*2*).
[g] Minimum bacteriocidal concentration, 5 μM.
[h] No increase in cell number after 24 h.
[i] Minimum bacteriocidal concentration, 2.5 μM.

phosphorothioate, the rate of DNA synthesis was barely affected after 90 min. At 0.5 μM glycerol-phosphorothioate, cell wall biosynthesis underwent transient inhibition at 90 min but recovered by 3 h. The rates of biosynthesis of all four macromolecules were strongly inhibited by 5 μM glycerol-phosphorothioate. At 90 min, the following percentages of inhibition below control levels were observed: cell wall, 92%; protein, 88%; RNA, 61%; and DNA, 32%. Our experiments revealed inhibition of synthesis at this analog concentration as early as 45 min, which is consistent with the inhibitory trend observed after 90 min and 3 h (data not shown). Under the same conditions, 5 μM glycerol-P had no effect on the rates of synthesis of these macromolecules.

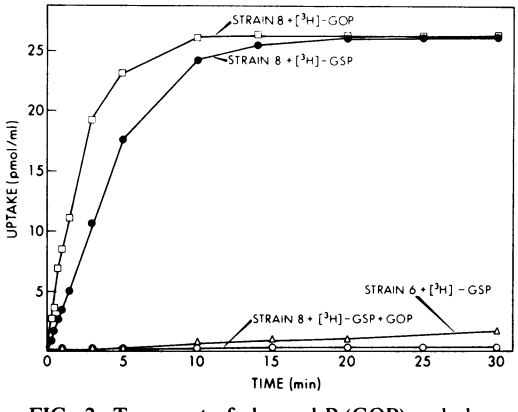

FIG. 2. Transport of glycerol-P (GOP) and glycerol-phosphorothioate (GSP) into *E. coli*. Symbols: □, [^{3}H]glycerol-P, strain 8; ●, [^{3}H]glycerol-phosphorothioate, strain 8; △, [^{3}H]glycerol-phosphorothioate, strain 6; ○, [^{3}H]glycerol-phosphorothioate, 150 μM glycerol-P, strain 8. Transport assays were performed as described in the text. Initial rates were measured by processing samples within the first 3 min of uptake, and the results of triplicate assays were averaged. The initial rates are expressed as the amount transported per milliliter of cell suspension per minute at 37°C.

Phospholipid composition, turnover, and synthesis. Our experiments indicated that significant changes occurred in the polar headgroup composition of uniformly labeled phospholipids in the presence of 5 μM glycerol-phosphorothioate. The amount of phosphatidylglycerol in the membranes decreased from 13.5 to 3.5% (Table 2) after 3 h. This represents a 70% drop in the amount of this phospholipid compared with untreated cells. Concomitant with this change in phosphatidylglycerol level was an approximately fourfold increase in the ^{32}P content of cardiolipin (from 6.8 to 24.2%) (Table 2). Under the same conditions the percentage of phosphatidylethanolamine in the membranes decreased from 78.7 to 70.6% (Table 2). To demonstrate conclusively that what appeared to be cardiolipin in the one-dimensional thin-layer chromatography system was not a result of poor resolution between cardiolipin and phosphatidic acid, ^{32}P-labeled phospholipids isolated from control and glycerol-phosphorothioate-treated cells were mixed and subjected to two-dimensional thin-layer chromatography, as described by Nishijima and Raetz (21). The putative ^{32}P-labeled cardiolipin isolated from treated cells comigrated with cardiolipin from control cells. Changes in phospholipid composition were also observed with 0.5 μM glycerol-phosphorothioate, but these changes were much less striking. The concentration of phosphatidylglycerol decreased from 15 to 9% after 90 min and to 10.7% after 3 h; this was concurrent with an increase in the concentration of phosphatidylethanolamine from 80.3 to 86.4% after 90 min and to 84.4% after 3 h. The cardiolipin contents of the cultures were not

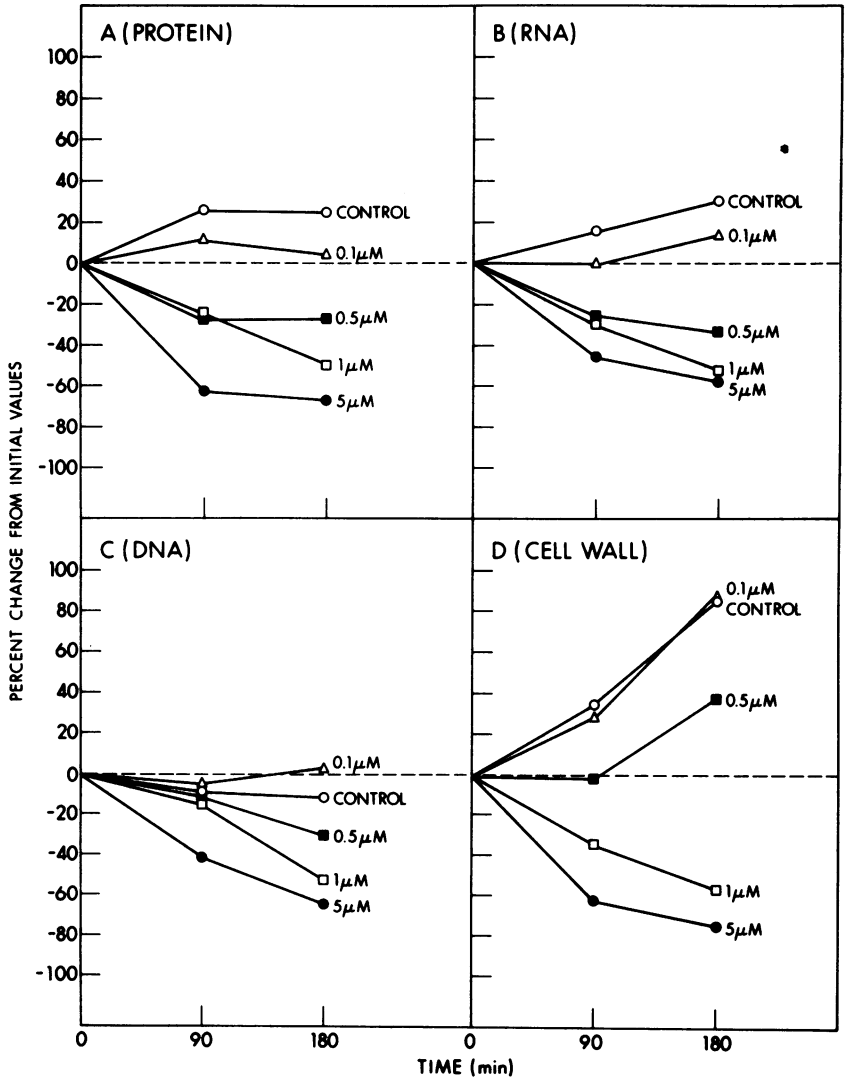

FIG. 3. The effect of glycerol-phosphorothioate on the rate of macromolecular biosynthesis in *E. coli* strain 8. The rates of macromolecular biosynthesis were measured by briefly labeling cells (10 min) with the appropriate precursor in the presence of 0.1 to 5 μM glycerol-phosphorothioate over a 3-h time period, as described in the text (DNA, [^{3}H]thymidine; RNA, [^{3}H]uridine; protein, [^{3}H]isoleucine; cell wall, [^{3}H]diaminopimelic acid). All results were normalized to constant cell number (i.e., 10^9 cells) and are expressed as percent change from initial values. The rates of synthesis of the macromolecules were determined in triplicate from the same cell samples, and the experiments were repeated several times; the results in this figure are representitive of all experiments. Symbols: ○, control; △, 0.1 μM glycerol-phosphorothioate; ■, 0.5 μM glycerol-phosphorothioate; □, 1 μM glycerol-phosphorothioate; ●, 5 μM glycerol-phosphorothioate. (A) Rate of protein synthesis. The initial values (per 10^9 cells) as follows were: control, 370 cpm; 0.1 μM glycerol-phosphorothioate, 430 cpm; 0.5 μM glycerol-phosphorothioate, 420 cpm; 1 μM glycerol-phosphorothioate, 390 cpm; 5 μM glycerol-phosphorothioate, 310 cpm. (B) Rate of RNA synthesis. The initial values (per 10^9 cells) were as follows: control, 5,900 cpm; 0.1 μM glycerol-phosphorothioate, 7,900 cpm; 0.5 μM glycerol-phosphorothioate, 7,900 cpm; 1 μM glycerol-phosphorothioate, 8,100 cpm; 5 μM glycerol-phosphorothioate, 5,800 cpm. (C) Rate of DNA synthesis. The initial values (per 10^9 cells) were as follows: control, 460 cpm; 0.1 μM glycerol-phosphorothioate, 480 cpm; 0.5 μM glycerol-phosphorothioate, 450 cpm; 1 μM glycerol-phosphorothioate, 440 cpm; 5 μM glycerol-phosphorothioate, 290 cpm. (D) Rate of cell wall synthesis. The initial values (per 10^9 cells) were as follows: control, 1,300 cpm; 0.1 μM glycerol-phosphorothioate, 1,400 cpm; 0.5 μM glycerol-phosphorothioate, 1,300 cpm; 1 μM glycerol-phosphorothioate, 1,600 cpm; 5 μM glycerol-phosphorothioate, 1,400 cpm.

TABLE 2. Phospholipid composition of *E. coli* strain 8 grown in the presence and absence of glycerol-phosphorothioate

Time (min)	% of ^{32}P in phospholipids[a]											
	Control				0.5 μM glycerol-phosphorothioate				5 μM glycerol-phosphorothioate			
	PE[b]	PG	CL[c]	Others	PE	PG	CL	Others	PE	PG	CL	Others
0	79.8	16.1	3.1	1.0	80.3	15.1	3.6	1.0	78.7	13.5	6.8	1.0
90	80.9	13.3	4.7	0.1	86.4	9.0	3.7	0.9	76.8	8.2	13.9	1.1
180	80.3	13.3	5.5	0.9	84.4	10.7	4.0	0.9	70.6	3.5	24.2	1.7

[a] The assay and extraction procedures used are described in the text. The total counts after 0, 90, and 180 min were as follows: control, 63,100, 92,700, and 185,600 cpm, respectively; 0.5 μM glycerol-phosphorothioate, 58,200, 80,100, and 111,100 cpm, respectively; 5 μM glycerol-phosphorothioate, 62,000, 72,100, and 73,000, respectively.

[b] PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; others, mainly lysophosphatidylethanolamine and lysophosphatidylglycerol.

[c] The amount of phospholipid was calculated as the percentage of the total ^{32}P in phospholipids. Since cardiolipin contains two phosphates per molecule, the percentage of cardiolipin is two times the molar quantity.

altered under these growth conditions. The composition of the total and component phospholipids was not affected by the presence of 5 μM glycerol-P. The amount of total phospholipid per cell mass was not affected at either glycerol-phosphorothioate concentration.

The rates of $^{32}P_i$ incorporation into both total phospholipid and individual phospholipid species decreased significantly after the addition of 5 μM glycerol-phosphorothioate (Fig. 4). The rate of phosphatidylglycerol biosynthesis appeared to be most sensitive to the presence of the phosphorothioate, with reductions of 52 and 63% after 90 min and 3 h, respectively, compared with untreated cultures. The rate of phosphatidylethanolamine synthesis decreased by 49 and 37% after 90 min and 3 h, respectively. Cardiolipin synthesis was least affected, with decreases of 32 and 38% after 90 min and 3 h, respectively. In the presence of 0.5 μM glycerol-phosphorothioate the rates of total and individual phospholipid biosynthesis increased slightly compared with untreated cells (Fig. 4A and B).

Studies of the rate of phospholipid turnover indicated that although no major effects were observed with respect to total phospholipid turnover (data not shown), significant changes occurred among the individual components (Table 3). The turnover of phosphatidylglycerol and the turnover of cardiolipin were found to be the most sensitive to the phosphorothioate analog, whereas the rate of turnover of phosphatidylethanolamine remained unchanged. Phosphatidylglycerol showed a small increase in the rate of turnover after 90 (21%) and 180 min (11%) compared with untreated cultures. However, at the same time the ^{32}P content in cardiolipin increased approximately twofold compared with untreated cells. However, our data do indicate that a significantly greater proportion of phosphatidylglycerol turnover was directed toward the synthesis of cardiolipin in treated cultures than in untreated cultures (72% of the net loss of radioactivity from phosphatidylglycerol in treated cells, compared with 31% in untreated cells after 3 h).

Nucleoside triphosphate levels. The levels of the nucleoside triphosphates in *E. coli* strain 8 were not affected by the presence of either 5 μM glycerol-P or 5 μM glycerol-phosphorothioate (data not shown).

DISCUSSION

We studied the effects of glycerol-phosphorothioate on a series of cellular functions in *E. coli*. Since it has been shown previously that all of the glycolytic enzymes except phosphoglycerate mutase can handle their respective phosphorothioate analogs (23), we used a strain of *E. coli* which lacks the catabolic glycerol-P dehydrogenase (i.e., strain 8) in an effort to minimize complications arising from nonphospholipid metabolism of the phosphorothioate analog. We also compared the effects of the phosphorothioate on an isogenic strain possessing this dehydrogenase activity (i.e., strain 7).

Previous studies have revealed that glycerol-P is bacteriostatic to certain strains of *E. coli* which are constitutive for the glycerol-P transport system (*glpT*) (20) and which lack the catabolic glycerol phosphate dehydrogenase (*glpD*) (i.e., strain 8) (5, 12). In addition, the phosphonate analog of glycerol-P, 3,4-dihydroxybutyl-1-phosphonate (DHBP), has been shown to be bacteriostatic to strains 7 and 8 (28, 30). We have found that the phosphorothioate analog is bacteriocidal to strains of *E. coli* which contain a functional glycerol-P transport system and that the toxic effects are not dependent on

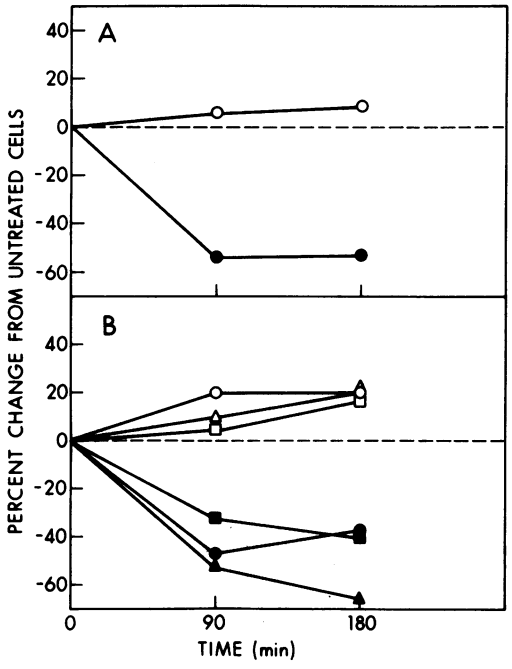

FIG. 4. Effect of glycerol-phosphorothioate on the rate of phospholipid biosynthesis. The rate of phospholipid synthesis was measured by briefly labeling cells with $^{32}P_i$ (10 μCi/ml) in the presence of 0.5 or 5 μM glycerol-phosphorothioate, as described in the text. All results were adjusted to a constant cell number (i.e., 10^9 cells), were normalized to initial values, and are expressed as percent change from untreated cells. The data were selected from experiments performed in duplicate and repeated several times. (A) Rate of total phospholipid synthesis. The initial values (per 10^9 cells) with 0.5 μM glycerol-phosphorothioate (○) were as follows: control, 8,700 cpm; glycerol-phosphorothioate, 8,200 cpm. The initial values (per 10^9 cells) with 5 μM glycerol-phosphorothioate (●) were as follows: control, 3,100 cpm; glycerol-phosphorothioate, 3,000 cpm. (B) Rate of synthesis of phospholipid components. Symbols: ○, phosphatidylethanolamine synthesis, 0.5 μM glycerol-phosphorothioate; ●, phosphatidylethanolamine synthesis, 5 μM glycerol-phosphorothioate; △, phosphatidylglycerol synthesis, 0.5 μM glycerol-phosphorothioate; ▲, phosphatidylglycerol synthesis, 5 μM glycerol-phosphorothioate; □, cardiolipin synthesis, 0.5 μM glycerol-phosphorothioate; ■, cardiolipn synthesis, 5 μM glycerol-phosphorothioate. The rates of synthesis of phosphatidylethanolamine and phosphatidylglycerol in the membranes under the conditions used were approximately equivalent, with only 1 to 2% of the total radioactivity recovered in the cardiolipin fraction.

the absence of the dehydrogenase in strain 7. Since glycerol-phosphorothioate toxicity was observed only in cells that have a functioning glycerol-P (*glpT*) transport system, the toxic effects of this analog cannot be due to some interaction with the outer cell surface, but must be the result of interference with some intracellular process. We found that the presence of glucose in the medium alleviated the toxic effects of the phosphorothioate analog on both strain 7 and strain 8. This is in agreement with the findings of Cozzarelli et al. (5) for glycerol-P-induced stasis in *E. coli* strain 8. However, the growth-inhibiting effects of the glycerol-P analog DHBP are not reversed in the presence of glucose (28). This suggests that partial catabolic repression of the glycerol-P transport system by glucose is not a factor in the rescue effect of *E. coli* from the toxicity of glycerol-phosphorothioate (17). Our results indicate that the presence of high levels of intracellular glycerol-P in *E. coli* strain 7 (achieved by culturing cells in glycerol as the sole carbon source), may protect the cells from the bacteriocidal effects of glycerol-phosphorothioate. Since the free diffusion of glycerol into the cells should not have interfered with the transport of glycerol-phosphorothioate via the glycerol-P transport system (11, 19), the protective effect must be due to the accumulations of glycerol-P inside the cells. Kinetic studies have indicated that the phosphorothioate analog is a better substrate for the glycerol-P transport system (11 μM) than DHBP (200 μM) is (19), which may explain why the toxic effects of glycerol-phosphorothioate were observed at lower concentrations than the toxic effects of the phosphonate (2.5 and 60 μM, respectively). We observed no effect on the levels of the nucleoside triphosphates at bacteriocidal concentrations of glycerol-phosphorothioate (5 μM) and exclude the possibility of depleted energy stores as a primary factor in the toxic response of the cells to the phosphorothioate analog (5, 12).

Our studies on phospholipid metabolism indicated that glycerol-phosphorothioate has a significant effect on the composition of the membrane phospholipids. In the presence of 5 μM glycerol-phosphorothioate, the proportion of phosphatidylglycerol in the membrane decreased substantially, with a concurrent increase in the amount of cardiolipin and a small decrease in the phosphatidylethanolamine content. These changes were due primarily to overall reductions in the rates of total and individual phospholipid biosynthesis, with only a small increase in turnover observed for phosphatidylglycerol. Reductions in the rate of phosphatidylglycerol synthesis have also been observed with the phosphonate analog of glycerol-P (30 μM) (29). van Golde et al. (33), Kennedy et al. (16), and Schulman and Kennedy (27) present evidence which suggests that the *sn*-glycerol 1-phosphate groups of membrane-derived oligosaccharides are obtained from phosphatidylglycerol (and possibly cardiolipin). The observed

TABLE 3. Turnover of phospholipids in *E. coli* strain 8 grown in the presence and absence of glycerol-phosphorothioate

Time (min)	% of ^{32}P in phospholipids[a]											
	Control				0.5 μM glycerol-phosphorothioate				5 μM glycerol-phosphorothioate			
	PE[b]	PG	CL[c]	Others	PE	PG	CL	Others	PE	PG	CL	Others
0	77.0	18.9	3.4	0.7	76.0	19.7	3.6	0.7	79.1	17.3	3.0	0.6
90	81.1	11.1	7.1	0.7	81.3	10.3	7.7	0.7	80.6	5.7	12.1	1.6
180	82.1	7.3	9.5	1.1	80.5	7.3	11.1	1.1	78.7	3.9	15.3	2.1

[a] The assay and extraction procedures used are described in the text. The total counts after 0, 90, and 180 min were as follows: control, 127,000, 109,000, and 97,300 cpm, respectively; 0.5 μM glycerol-phosphorothioate, 132,000, 116,600, and 104,900 cpm, respectively; 5 μM glycerol-phosphorothioate, 117,800, 104,000, and 98,900, respectively.

[b] See Table 2, footnote *b*.

[c] See Table 2, footnote *c*.

twofold increase in the cardiolipin content of glycerol-phosphorothioate-treated cells, which occurs despite only a small increase in net phosphatidylglycerol turnover, could be explained if glycerol-phosphorothioate treatment resulted in the inhibition of the membrane-derived oligosaccharides but not the cardiolipin pathway. This would have the effect of directing the majority of phosphatidylglycerol turnover toward cardiolipin. A decrease in cardiolipin turnover could also contribute to the increased levels of this phospholipid.

We have shown elsewhere (22a) that glycerol-phosphorothioate is an effective glycerol-P surrogate. In vitro studies with a cell-free particulate membrane preparation revealed that glycerol-phosphorothioate is a substrate for the acyl-coenzymeA:*sn*-glycerol-3-phosphate acyltransferase. The product of the reaction has been identified as thiophosphatidic acid. However, chemically synthesized thiophosphatidic acid was not a substrate for the CTP:phosphatidic acid cytidylyltransferase in the same cell-free preparation, suggesting that the formation of this phosphorothioate-containing phospholipid may be a dead-end reaction. Glycerol-phosphorothioate is also a substrate for the CDP-diglyceride:*sn*-glycerol-3-phosphate phosphatidyltransferase, and the product of this reaction was tentatively identified as phosphatidylglycerol phosphorothioate. No formation of phosphatidylglycerol was detected. These results suggest that the specific phosphatase required for the synthesis of phosphatidyl-glycerol does not catalyze the hydrolysis of the phosphorothioate monoester linkage under our in vitro conditions. In vivo studies have shown that *sn*-[^{3}H]glycerol 3-phosphoro[^{35}S]thioate is incorporated into phospholipid in *E. coli* strain 8 cells. The major phosphorothioate-containing phospholipid synthesized under these conditions was identified as thiophosphatidic acid. Interestingly, no formation of phosphatidylglycerol phosphorothioate was detected. We found no evidence for the liberation of [^{35}S]thiophosphate after *E. coli* strain 8 was incubated in the presence of 3 μM glycerol-phosphoro[^{35}S]thioate for 1 h. This appears to exclude the possibility that the toxicity of the phosphorothioate analog is due to the accumulation of high intracellular concentrations of inorganic thiophosphate by the action of a phosphatase.

The results of the in vitro and in vivo studies described above suggest possible mechanisms whereby glycerol-phosphorothioate could affect endogenous phospholipid biosynthesis. For example, the presence of high intracellular concentrations of glycerol-phosphorothioate might lead to reductions in (i) total phospholipid biosynthesis via inhibition of endogenous glycerol-P production, (ii) phosphatidic acid and consequently CDP-diglyceride biosynthesis by inhibition of the acylation reaction, and (iii) phosphatidylglycerol biosynthesis by inhibition of phosphatidylglycerol phosphate production. In addition, the presence of thiophosphatidic acid in *E. coli* membranes may also have a direct inhibitory effect on the enzyme responsible for activating phosphatidic acid to CDP-diglyceride. Our observed inhibition of phosphatidylethanolamine biosynthesis in the presence of glycerol-phosphorothioate could be explained by a reduction in the levels of CDP-diglyceride, with the greater reduction in the biosynthetic rate of phosphatidylglycerol related to reduced production of both CDP-diglyceride and phosphatidylglycerol phosphate.

Although on the basis of our studies we are not able to provide specific mechanisms for the glycerol-phosphorothioate-induced toxicity in *E. coli*, explanations for the bacteriocidal effects can be offered. Cell death may have been brought about through alterations in the structure and permeability properties of the bacterial membrane as a result of changes in phospholipid composition, as observed in this study, or

through the accumulation of abnormal phosphorothioate-containing phospholipids (i.e., the formation of negatively charged thiophosphatidic acid) (22a). Preliminary electron microscopic studies have indicated a separation of the inner and outer membranes, and a distortion of cell shape occurs when *E. coli* is grown in the presence of 5 μM glycerol-phosphorothioate, suggesting osmotic instability of the cells. Reductions in the levels of phosphatidylglycerol may have interfered with the functions of membrane-bound enzymes and transport systems; e.g., enzyme II of the membrane-based phosphotransferase system has a functional requirement for phosphatidylglycerol (18). Previous investigations have shown that cell growth and division are associated with changes in phospholipid metabolism. During the transition from exponential to stationary phase the amount of cardiolipin increases, and the amount of phosphatidylglycerol decreases (6, 15, 25). In our studies, similar changes in the proportions of these phospholipids were observed in the presence of toxic levels of glycerol-phosphorothioate. However, since a loss of viability was also observed at these concentrations, the changes in phospholipid composition may have been simply a reflection of cell death.

Our overall goal is to explore the possibility of manipulating the polar headgroup composition of *E. coli* membranes by culturing cells in the presence of the phosphorothioate analog of glycerol-P, the precursor of all phospholipids in *E. coli*. An interesting and significant finding has been the bacteriocidal nature of glycerol-phosphorothioate on strains of *E. coli* that possess a functional glycerol 3-phosphate transport system. Examination of a number of cellular metabolic functions has revealed significant inhibition of macromolecular synthesis and changes in the membrane phospholipid composition. Investigations are currently being directed toward determining the effect of the phosphorothioate analog on membrane structure and function.

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LITERATURE CITED

1. **Ames, G. F.** 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. J. Bacteriol. **95**:833–843.
2. **Bergmeyer, H. U.** 1974. Glycerol-3-phosphate dehydrogenase, p. 468. *In* Methods of enzymatic analysis, 2nd English ed., vol. 1. Verlag Chemie, New York.
3. **Cashel, M., R. A. Lazzarini, and B. Kalbacher.** 1969. An improved method for thin-layer chromatography of nucleotide mixtures containing ^{32}P-labeled orthophosphate. J. Chromatogr. **40**:103–109.
4. **Contreras, I., L. Shapiro, and S. Henry.** 1978. Membrane phospholipid composition of *Caulobacter crescentus*. J. Bacteriol. **135**:1130–1136.
5. **Cozzarelli, N. R., J. P. Koch, S.-I. Hayashi, and E. C. C. Lin.** 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. **90**:1325–1329.
6. **Cronan, J. E., Jr.** 1968. Phospholipid alterations during growth of *Escherichia coli*. J. Bacteriol. **95**:2054–2061.
7. **Cronan, J. E., Jr.** 1978. Molecular biology of bacterial membrane lipids. Annu. Rev. Biochem. **47**:163–189.
8. **Echstein, F.** 1979. Phosphorothioate analogues of nucleotides. Acc. Chem. Res. **12**:204–210.
9. **Fourcans, B., and M. K. Jain.** 1964. Role of phospholipids in transport and enzymatic reactions. Adv. Lipid Res. **12**:147–224.
10. **Garen, A., and C. Levinthal.** 1960. A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. Biochim. Biophys. Acta **38**:470–483.
11. **Hayashi, S.-I., J. P. Koch, and E. C. C. Lin.** 1964. Active transport of L-α-glycerophosphate in *Escherichia coli*. J. Biol. Chem. **239**:3098–3105.
12. **Hennen, P. E., H. B. Carter, and W. D. Nunn.** 1978. Changes in macromolecular synthesis and nucleoside triphosphate levels during glycerol-induced growth stasis of *Escherichia coli*. J. Bacteriol. **136**:929–935.
13. **Homma, H., M. Nishijima, T. Kobayashi, H. Okuyama, and S. Nojima.** 1981. Incorporation and metabolism of 2-acyl lysophospholipids by *Escherichia coli*. Biochim. Biophys. Acta **663**:1–13.
14. **Jones, N. C., and M. J. Osborn.** 1977. Translocation of phospholipids between the outer and inner membrane of *Salmonella typhimurium*. J. Biol. Chem. **252**:7405–7412.
15. **Kanfer, J., and E. P. Kennedy.** 1963. Metabolism and function of bacterial lipids. I. Metabolism of phospholipids in *Escherichia coli*. J. Biol. Chem. **238**:2919–2922.
16. **Kennedy, E. P., M. K. Rumley, H. Schulman, and L. M. G. van Golde.** 1976. Identification of *sn*-glycerol-1-phosphate and phosphoethanolamine residues linked to the membrane-derived oligosaccharides of *Escherichia coli*. J. Biol. Chem. **251**:4208–4213.
17. **Koch, J. P., S.-I. Hayashi, and E. C. C. Lin.** 1964. The control of dissimilation of glycerol and L-α-glycerophosphate in *Escherichia coli*. J. Biol. Chem. **239**:3106–3108.
18. **Kundig, W., and S. Roseman.** 1971. Sugar transport. II. Characterization of constitutive membrane-bound enzymes II of the *Escherichia coli* phosphotransferase system. J. Biol. Chem. **246**:1407–1418.
19. **Leifer, Z., R. Engel, and B. E. Tropp.** 1977. Transport of a 3,4-dihydroxybutyl-1-phosphonate, an analog of *sn*-glycerol 3-phosphate. J. Bacteriol. **130**:968–971.
20. **Lin, E. C. C.** 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. **30**:535–578.
21. **Nishijima, M., and C. R. H. Raetz.** 1979. Identification of genetic loci for phosphatidylglycerophosphate synthetase and construction of mutants lacking phosphatidylglycerol. J. Biol. Chem. **254**:7837–7844.
22. **Orr, G. A., C. F. Brewer, and G. Heney.** 1982. Synthesis of the diastereoisomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoryl-ethanolamine and their stereospecific hydrolysis by phospholipases A_2 and C. Biochemistry **21**:3202–3206.
22a. **Orr, G. A., J. W. Hammelburger, and G. Heney.** 1983. Interaction of *sn*-glycerol 3-phosphorothioate with *Escherichia coli*: in vitro and in vivo incorporation into phospholipids. J. Biol. Chem. **258**:9237–9244.
23. **Orr, G. A., J. Simon, S. R. Jones, G. J. Chin, and J. R. Knowles.** 1978. Adenosine 5-*O*-([γ-^{18}O]γ-thio)triphosphate chiral at the γ-phosphorus: stereochemical consequences of reactions catalyzed by pyruvate kinase, glycerol kinase and hexokinase. Proc. Natl. Acad. Sci. U.S.A. **75**:2230–2233.
24. **Raetz, C. R. H.** 1978. Enzymology, genetics and regulation of membrane phospholipid synthesis in *Escherichia*

coli. Microbiol. Rev. **42**:614–659.
25. **Randle, C. L., P. W. Albro, and J. C. Dittmer.** 1969. The phosphoglyceride composition of gram-negative bacteria and the changes in composition during growth. Biochim. Biophys. Acta **187**:214–220.
26. **Sandermann, H., Jr.** 1978. Regulation of membrane enzymes by lipids. Biochim. Biophys. Acta **515**:209–223.
27. **Schulman, H., and E. P. Kennedy.** 1977. Relation of turnover of membrane phospholipids to synthesis of membrane-derived oligosaccharides of *Escherichia coli*. J. Biol. Chem. **252**:4250–4255.
28. **Shopsis, C. S., R. Engel, and B. E. Tropp.** 1972. Effects of phosphonic acid analogues of glycerol 3-phosphate on the growth of *Escherichia coli*. J. Bacteriol. **112**:408–412.
29. **Shopsis, C. S., R. Engel, and B. E. Tropp.** 1974. The inhibition of phosphatidylglycerol synthesis in *Escherichia coli* by 3,4-dihydroxybutyl-1-phosphonate. J. Biol. Chem. **249**:2473–2477.
30. **Shopsis, C. S., W. D. Nunn, R. Engel, and B. E. Tropp.** 1973. Effects of phosphonic analogues of glycerol 3-phosphate on the growth of *Escherichia coli*: phospholipid metabolism. Antimicrob. Agents Chemother. **4**:467–473.
31. **Taylor, A.** 1970. Current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155–175.
32. **van Deenen, L. L. M.** 1981. Topology and dynamics of phospholipids in membranes. FEBS Lett. **123**:3–15.
33. **van Golde, L. M. G., H. Schulman, and E. P. Kennedy.** 1973. Metabolism of membrane phospholipids and its relation to a novel class of oligosaccharides in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **70**:1368–1372.
34. **Yount, R. G.** 1975. ATP analogs. Adv. Enzymol. Relat. Areas Mol. Biol. **43**:1–89.