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Effects of Phosphonic Acid Analogues of Glycerol-3-Phosphate on the Growth of *Escherichia coli*: Phospholipid Metabolism

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The effects of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate, and 2,3-dihydroxypropyl-1-phosphonate on the metabolism of *Escherichia coli* strains 8 and 1908 were determined. These strains lack the membrane-bound glycerol-3-phosphate dehydrogenase and are constitutive for the glycerol-3-phosphate transport system. Such cells were more sensitive to growth inhibition by the four-carbon phosphonate than by glycerol-3-phosphate. The three-carbon phosphonate did not appear to inhibit cell growth. The incorporation of labeled precursors of lipid, protein, ribonucleic acid, or deoxyribonucleic acid into bacterial cells was measured in the presence of either glycerol-3-phosphate or one of its phosphonic acid analogues. The phosphonic acid analogues inhibited the uptake of labeled acetate into the lipid fraction to the greatest extent. The incorporation of [^{33}P]PO_4 into phospholipids was strongly inhibited by 3,4-dihydroxybutyl-1-phosphonate but was only slightly affected by 2,3-dihydroxypropyl-1-phosphonate. Glycerol-3-phosphate inhibited the incorporation of labeled uracil to the greatest extent during the first 20 min; however, this effect was largely reversed after 90 min. Only 3,4-dihydroxybutyl-1-phosphonate altered the distribution of labeled acetate into the phospholipids of strain 8, decreasing the percentage of counts in the phosphatidylglycerol fraction. The three-carbon phosphonate probably alters acetate incorporation by affecting the acetyl-coenzyme A pool, whereas the 3,4-dihydroxybutyl-1-phosphonate has a definite effect upon phospholipid metabolism. It is suggested that L-glycerol-3-phosphate:cytidine monophosphate phosphatidyltransferase is the probable site of action.

Chemotherapeutic and antimicrobial agents are commonly used as tools to help unravel the complex control systems that regulate and integrate biosynthetic pathways. If suitable chemical agents were available, the control systems that serve to integrate phospholipid metabolism with other biochemical processes might prove amenable to such an approach. For this reason, salts of phosphonic acid analogues of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonic acid ($CH_2OHCHOHCH_2CH_2PO_3H_2$) and 2,3-dihydroxypropyl-1-phosphonic acid ($CH_2 \cdot OHCHOHCH_2PO_3H_2$) were synthesized (4) and their effects on the growth of *Escherichia coli* were studied (10).

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The four-carbon phosphonate, 3,4-dihydroxybutyl-1-phosphonate, inhibits the cell growth of *E. coli* possessing an active glycerol-3-phosphate transport system, whereas the three-carbon phosphonate analogue does not appear to exhibit a similar effect (10). *E. coli* cells that are constitutive for the glycerol-3-phosphate transport system but which lack the membrane-bound glycerol-3-phosphate dehydrogenase (6, 13) are subject to growth inhibition by glycerol-3-phosphate (1). The inhibition of growth caused by the four-carbon phosphonate differs from that caused by the natural metabolite in at least three ways: (i) its inhibitory effect is not offset by the presence of glucose in the culture medium, (ii) it is capable of exerting its inhibitory effect on cells possessing an active, membrane-bound glycerol-3-phosphate dehydrogen-

ase (6), and (iii) its inhibitory effect is maintained in synthetic medium containing high concentrations of inorganic phosphate (10).

The present report compares some of the effects that glycerol-3-phosphate and its phosphonic acid analogues have on two strains of *E. coli* that are constitutive for the glycerol-3-phosphate transport system and that lack the membrane-bound glycerol-3-phosphate dehydrogenase (6, 13). These strains were selected because it was thought that their use would avoid complications of interpretation that might arise from the oxidation of the phosphonic acid analogues. The oxidation of the phosphonates by wild-type cells was considered to be a distinct possibility because 3,4-dihydroxybutyl-1-phosphonate is a substrate for rabbit muscle L-glycerol-3-phosphonate:nicotinamide adenine dinucleotide oxidoreductase (P. Cheng, R. Hickey, R. Engel, and B. Tropp, unpublished data; while these studies were in progress, separate investigations with purified *E. coli* membrane-bound dehydrogenase, generously provided by L. Heppel, revealed that this enzyme, unlike the oxidoreductase obtained from rabbit muscle, could not catalyze the oxidation of 3,4-dihydroxybutyl-1-phosphonate [Cheng, Engel, and Tropp, unpublished data]).

The experiments reported here demonstrate that the phosphonic acid analogues have quite different effects from one another and from glycerol-3-phosphate. The four-carbon phosphonic acid analogue appears to primarily affect phospholipid biosynthesis.

MATERIALS AND METHODS

Chemicals. The radioactive tracers used were as follows: [6-^3H]uracil (1 Ci/mmol) from Amersham Searle, Arlington Heights, Ill; [2-^{14}C]acetate (39 mCi/mmol) and carrier-free [^{33}P]PO_4 from Schwarz/Mann, Orangeburg, N.Y.; [*methyl*-^3H]thymine (13.6 Ci/mmol) and L[^{3}H]isoleucine (1 mCi/0.087 mg) from New England Nuclear Corp., Boston, Mass. DL-Glycerol-3-phosphate (grade X) was purchased from the Sigma Chemical Co., St. Louis, Mo. Supelcosil silica gel 12A and the "chromatographically pure" bacterial phospholipids, phosphatidylethanolamine, cardilipin, and phosphatidylglycerol, were purchased from Supelco, Inc., Bellefonte, Pa.

The dilithium salt of 2,3-dihydroxypropyl-1-phosphonate was synthesized by the procedure of Rosenthal and Geyer (9). A modification of this procedure was used to synthesize the dilithium salt of 3,4-dihydroxybutyl-1-phosphonate (4). All comments concerning glycerol-3-phosphate or one of its phosphonate analogues refer to the racemic mixtures unless a specific enantiomer is specified. All other chemicals were of reagent grade.

Bacterial strains. *E. coli* strains 8 and 1908 were kindly provided by J. Cronan, Jr. The genotypes of these two strains as expressed by the genetic symbols, described by Taylor (11), are as follows: strain 8 HfrC glpD3, glp R^{c}2, phoA8, tonA22, T2^R, rel-1 (λ) and strain 1908 F$^-$ thi-1, his-1, pyrD34, str-118, gal-6, xyl-7, mtl-2, thy-A25, glpD3, glpR^{c}2. Strain 8 was isolated by E. C. C. Lin and strain 1908 by G. N. Godson.

Growth of bacteria. Medium for the strain 8 cultures consisted of the low phosphate synthetic medium of Garen and Levinthal (3) supplemented with 0.6 mM phosphate and 0.5% potassium succinate. Cultures of *E. coli* strain 1908 did very poorly on such a low-phosphate medium supplemented with succinate and other nutritional requirements. Therefore cultures of this strain were supplemented with each of the following per liter of Davis and Mingioli synthetic medium (2): thiamine-hydrochloride, 1 mg; thymine, 4 mg; uracil, 20 mg; and histidine-hydrochloride, 40 mg. All cultures were incubated with adequate aeration at 37 C. Fully supplemented overnight cultures were diluted 30-fold into the same medium. The cultures were then incubated at 37 C in a New Brunswick Metabolyte water-bath shaker, model G77, at 200 cycles per min. Cell growth was monitored at 660 nm with a Klett-Summerson colorimeter (10). Unless otherwise stated, all experiments were initiated when the turbidity reached 25 to 40 Klett units. The cell density and type of medium appear to be significant factors when determining the effectiveness of a given concentration of 3,4-dihydroxybutyl-1-phosphonate. In all experiments in which one of the phosphonate compounds was added to cultures, LiCl was added to the control cultures so that the final Li^+ concentrations in both sets of cultures were identical. These concentrations of Li^+ do not appear to alter the bacterial growth rates.

Assay of macromolecular biosynthesis. For the assay of protein, ribonucleic acid (RNA), or deoxyribonucleic acid synthesis (8, 12), 1 ml of the culture medium was supplemented with either 0.15 μCi of L-[^{3}H]isoleucine and 15 μg of L-isoleucine, 0.20 μCi of [6-^3H]uracil and 20 μg of uracil, or 3.0 μCi of [*methyl*-^3H]thymine and 4 μg of thymine, respectively.

Assay of phospholipid synthesis. The synthesis of phospholipids was followed by measuring the incorporation of labeled acetate or phosphate. The culture medium was supplemented with either potassium acetate (100 μg/ml) and [2-^{14}C]acetate (0.04 μCi/ml) or carrier-free [^{33}P]PO_4 (0.2 μCi/ml). Samples (2.0 ml) were removed from duplicate flasks at various time intervals for the determination of radioactivity in the lipid fraction. Zero time was always designated as the time of addition of the phosphonate, glycerol-3-phosphate, or lithium chloride to the culture. The rate of shaking was not varied during any of the incubations (variations in shaking rates can complicate the interpretation of experiments on phospholipid metabolism in *E. coli* [L. Meade, W. Nunn, and B. Tropp, unpublished data]). The 2-ml samples were removed, immediately mixed with an equal volume of chilled carrier cells, centrifuged in the cold, and then extracted overnight with 4 ml of chloroform:methanol (2:1) at room temperature. The extracts were then

washed three times with 1.0 ml of distilled water, placed in scintillation vials, and dried by evaporation at temperatures below 50 C. The amount of radioactivity was determined by adding toluene based scintillation fluid to the dried extract and counting the sample in a Beckman LS200 liquid scintillation counter.

Analysis of phospholipids. Potassium acetate (100 μg/ml) and [2-^{14}C]acetate (0.33 μCi/ml) were added to the culture medium. Treated and untreated cultures were then incubated at 37 C with shaking, and at various time intervals 10 ml of culture was removed for the analysis of phospholipids. The extraction method used chloroform:methanol (2:1) but otherwise was that of Tropp et al. (12).

After extraction, the chloroform was evaporated, the residue was taken up in approximately 0.15 ml of chloroform, and the phospholipids were resolved by thin-layer chromatography. Activated Supelcosil silica gel 12A was used as the adsorbent in a two-step developing system with acetone-light petroleum (1:3) as the first solvent and chloroform-methanol-water (65:25:3) as the second solvent (8). After development of the thin-layer chromatograms, the phospholipids were detected by exposure of the plates to iodine vapors. The radioactivity in the individual spots was determined by quantitatively transferring the gel to scintillation vials. To assure the complete recovery of labeled phospholipids, all of the silica gel in a lane was routinely assayed by this procedure. A 1-ml sample of 10% glacial acetic acid in absolute ethanol followed by 10 ml of toluene-based scintillation fluid was added to each vial for counting. The identification of various lipids was established by the simultaneous chromatography of known standards (8, 12).

RESULTS AND DISCUSSION

The phosphonic acid isosteres are of particular interest as potential antimetabolites because the functional groups $—OPO_3H_2$ and $—CH_2PO_3H_2$ have approximately the same steric properties but should be metabolized in quite different ways. Some aspects of the metabolism of compounds containing the carbon-phosphorus bond were reviewed by Kittredge and Roberts (5).

Although *E. coli* strain 1908 has obvious advantages for monitoring macromolecular biosynthesis, it was not possible to compare the effects of glycerol-3-phosphate, 3,4-dihydroxybutyl-1-phosphonate, and 2,3-dihydroxypropyl-1-phosphonate on this strain. Glycerol-3-phosphate dehydrogenase negative strains do not exhibit glycerol-3-phosphate-induced growth stasis when cultured in a high-phosphate medium supplemented with glucose (1, 10) of the type required by strain 1908.

Figures 1a and b depict the effects of various concentrations of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate, respectively, on the growth of *E. coli* strain 8. The phosphonic acid isostere affected the growth rate at considerably lower concentrations than did glycerol-3-phosphate. The four-carbon analogue can cause complete inhibition of growth at concentrations that are slightly higher than those used in the present study. It was previously demonstrated that 2,3-dihydroxypropyl-1-phosphonate at a concentration of 2.5×10^{-3} M does not significantly affect the growth of strain 8 cultured in Garen-Levinthal medium (3).

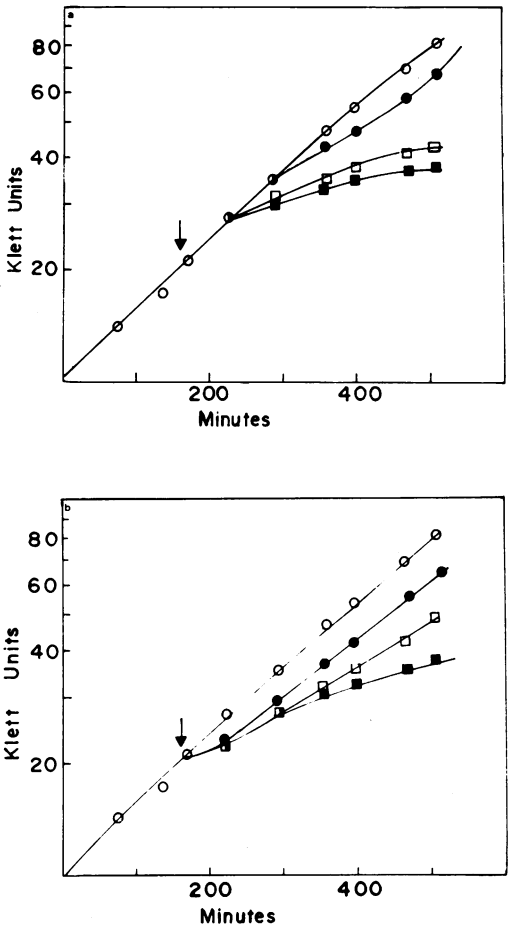

FIG. 1. *Effects of glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate on the growth of E. coli strain 8 cultured in low-phosphate synthetic medium (3) supplemented with 0.5% potassium succinate. At the time indicated by the arrow, the inhibitors were added to the final concentrations indicated. a, Glycerol-3-phosphate: O, untreated; ●, 0.12 mM; □, 0.24 mM; ■, 0.36 mM. b, 3,4-Dihydroxybutyl-1-phosphonate: O, untreated; ●, 0.02 mM; □, 0.04 mM; ■, 0.12 mM.*

The effects of glycerol-3-phosphate and its

two phosphonic acid analogues on the incorporation of radioactive precursors into protein, RNA, and lipid by *E. coli* strain 8 were determined. Figure 2 indicates that 2.5×10^{-3} M 2,3-dihydroxypropyl-1-phosphonate inhibits labeled acetate incorporation into the lipid fraction without significantly inhibiting the incorporation of either labeled uracil or isoleucine into trichloroacetic acid-precipitable material. Figure 3 shows the effect of various concentrations of 3,4-dihydroxybutyl-1-phosphonate on the incorporation of these labeled compounds. The four-carbon phosphonate inhibited the incorporation of labeled acetate into the lipid fraction more effectively than it inhibited the incorporation of either labeled isoleucine or uracil into macromolecules. 3,4-Dihydroxybutyl-1-phosphonate exhibited similar effects with strain 1908 (data not shown). It was possible to demonstrate that the incorporation of labeled thymine was not inhibited in strain 1908 (data not shown). The results of a parallel experiment with glycerol-3-phosphate are presented in Fig. 4. This compound produced a pattern of inhibition of incorporation of the labeled precursors that is markedly different from that caused by the treatment of cultures with either of its phosphonic acid analogues. Glycerol-3-phos-

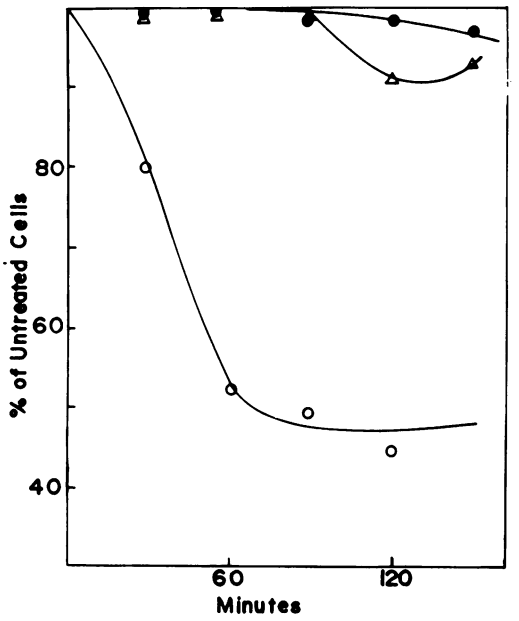

FIG. 2. *Effect of treatment with 2.5 mM 2,3-dihydroxypropyl-1-phosphonate on phospholipid, RNA, and protein synthesis by E. coli strain 8 cultured in low-phosphate synthetic medium (3) supplemented with 0.5% potassium succinate. Synthesis was measured by incorporation of [2-^{14}C]acetate into lipid (○), [6-^{3}H]uracil into RNA (△), and L-[^{3}H]isoleucine into protein (●). Isotope incorporation values are expressed as percentage of levels observed with untreated cultures at the time point of interest.*

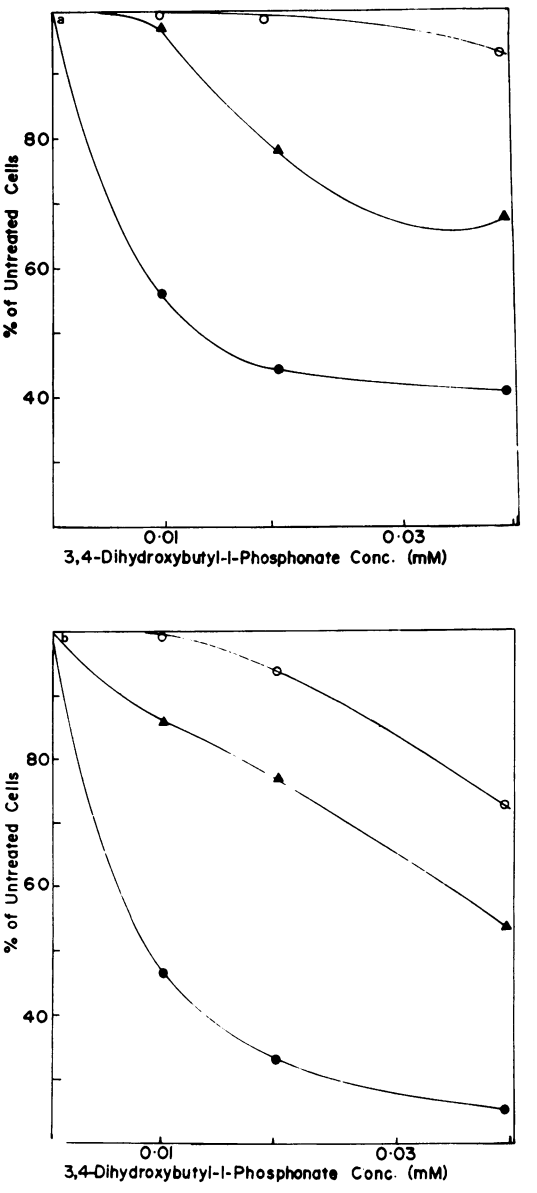

FIG. 3. *Phospholipid, RNA, and protein synthesis by E. coli strain 8 cultured in low-phosphate synthetic medium (3) supplemented with 0.5% potassium succinate as a function of 3,4-dihydroxybutyl-1-phosphonate concentration. Synthesis was measured by incorporation of [2-^{14}C]acetate into phospholipid (●), [6-^{3}H]uracil into RNA (▲), and L-[^{3}H]isoleucine into protein (○). Isotope incorporation values are expressed as percentages of the levels observed with untreated cultures at that time point. a, 20 min; b, 90 min.*

phate treatment inhibited the incorporation of labeled uracil more strongly than it inhibited incorporation of label into phospholipid or protein. *E. coli* strain 8 recovered from inhibition caused by the concentrations of glycerol-3-phosphate studied. Hence Fig. 4b (90-min incubation) shows less inhibition than does Fig. 4a (20-min incubation). The recovery phenomenon was probably due to the eventual consumption of the external glycerol-3-phosphate through the processes of phospholipid biosynthesis and turnover.

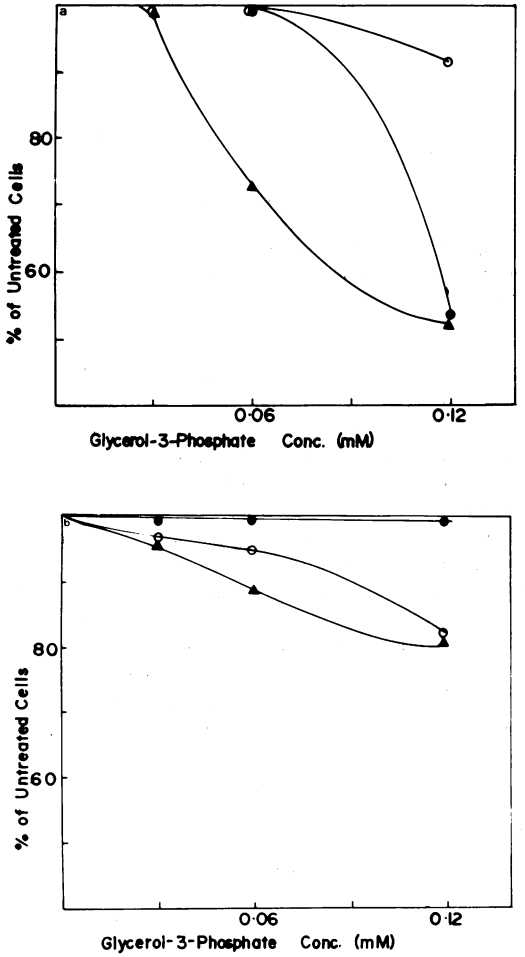

FIG. 4. *Phospholipid, RNA, and protein synthesis by E. coli strain 8 cultured in low-phosphate synthetic medium (3) supplemented with 0.5% potassium succinate expressed as a function of glycerol-3-phosphate concentration. Synthesis was measured by incorporation of radioactive precursors. See legend to Fig. 3 for a description of the symbols and experimental procedure. Incorporation values are expressed as percentages of the level observed with an untreated culture at that time point. a, 20 min; b, 90 min.*

The results thus far indicate that all three of the compounds investigated inhibit the incorporation of labeled acetate into the phospholipid fraction and, furthermore, that 2,3-dihydroxypropyl-1-phosphonate and 3,4-dihydroxybutyl-1-phosphonate inhibit the incorporation of label into the phospholipid fraction more effectively than they do the incorporation of label into protein or RNA. For further comparisons of the mode of action of these compounds, experiments summarized in Table 1 were undertaken. Only 3,4-dihydroxybutyl-1-phosphonate caused a marked change in the distribution pattern of the incorporation of labeled acetate into phospholipids. The 0.03-mM concentration of four-carbon phosphonate utilized slowed down the rate of cell growth but did not completely inhibit it (Fig. 1b). The percentage of radioactivity was considerably reduced in the phosphatidylglycerol fraction and increased in cardiolipin and at the origin of the chromatogram. Treatment of cultures of *E. coli* strain 8 with concentrations of glycerol-3-phosphate or 2,3-dihydroxypropyl-1-phosphonate that inhibited incorporation of labeled acetate into the phospholipid fraction by 50 to 80% caused only minor changes in the pattern of distribution of the label into phospholipids.

The data obtained thus far might be explained in part by effects of the phosphonic acid analogues on the acetyl-coenzyme A pool rather than on phospholipid biosynthesis. This question was investigated by culturing cells of strain 8 in medium containing [^{33}P]PO_4 for several generations before the addition of glycerol-3-phosphate or one of its phosphonic acid analogues. Labeling of the phospholipid fraction was then determined as a function of time. These measurements would account for all of the phospholipid of the bacteria since the cells are labeled to constant specific activity at the time of inhibitor addition. The data presented in Fig. 5 indicate that glycerol-3-phosphate and 3,4-dihydroxybutyl-1-phosphonate inhibit the incorporation of labeled phosphate into phospholipids. Only slight inhibition is caused by 2,3-dihydroxypropyl-1-phosphonate. It should be noted that the phospholipid content of the untreated cells increases by approximately twofold during a 2.5-h period. This result would be predicted from the bacterial growth rate. Glycerol-3-phosphate would be expected to cause inhibition since it is a direct precursor of phospholipid biosynthesis. The difference in results obtained with 2,3-dihydroxypropyl-1-phosphonate-treated cells labeled with acetate as compared with phosphate may be caused by changes in the internal acetyl-coen-

TABLE 1. *Effects of glycerol-3-phosphate, 2,3-dihydroxypropyl-1-phosphonate, and 3,4-dihydroxybutyl-1-phosphonate on the distribution of labeled acetate into the phospholipids of E. coli strain 8 after 60 min of incubation*[a]

Treatment	Total counts per min per ml of cells	Percentage of total counts/min				
		Origin	Phosphatidylglycerol	Phosphatidylethanolamine	Cardiolipin	Neutral lipid
Expt 1						
0.06 mM LiCl	3,131	4	27	51	13	4
0.03 mM 3,4-dihydroxybutyl-1-phosphonate	871	12	10	44	24	7
0.3 mM glycerol-3-phosphate	721	4	25	45	17	6
Expt 2						
10 mM LiCl	4,338	4	25	55	12	2
2.5 mM 2,3-dihydroxypropyl-1-phosphonate	1,860	4	26	50	13	3
5.0 mM 2,3-dihydroxypropyl-1-phosphonate	1,375	5	25	50	12	4

[a] Bacteria were cultured in low-phosphate synthetic medium (3) supplemented with 0.5% potassium succinate. Phospholipids were extracted and chromatographed, and the radioactivity was determined as described in Materials and Methods. The turbidities of the cultures at the beginning of these experiments were: experiment 1, 26 Klett units; experiment 2, 33 Klett units.

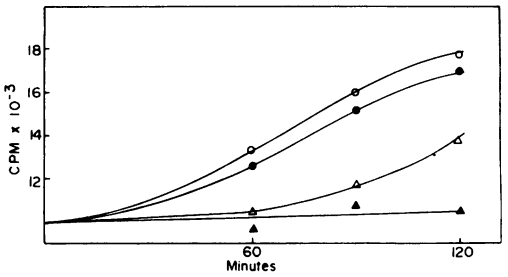

FIG. 5. *Effect of treatment with 3,4-dihydroxybutyl-1-phosphonate, 2,3-dihydroxypropyl-1-phosphonate, and glycerol-3-phosphate on the incorporation of [^{33}P]PO_4 into phospholipids of E. coli strain 8 cultured in low-phosphate synthetic medium (3) supplemented with 0.5% potassium succinate and 0.2 μCi of carrier-free [^{33}P]PO_4 per ml. Incorporation was measured as described in Materials and Methods. The bacteria were cultured in the radioactive medium for four generations before the addition of the inhibitors to the final concentrations indicated. Symbols:* ○, *untreated;* ●, *2.5 mM 2,3-dihydroxypropyl-1-phosphonate;* △, *0.03 mM 3,4-dihydroxybutyl-1-phosphonate;* ▲, *0.3 mM glycerol-3-phosphate.*

zyme A pools induced by treatment with the analogue. This interpretation is consistent with the observations that the three-carbon phosphonate does not inhibit cell growth (10) or affect the distribution of acetate incorporation into phospholipids (Table 1) and that 2,3-dihydroxypropyl-1-phosphonate can be degraded by *E. coli* (7).

In vitro investigations involving L-glycerol-3-phosphate acyltransferase have thus far tended to indicate that the four-carbon phosphonate is not a substrate and is at best an extremely poor inhibitor of the acylation reaction (P. Cheng, R. Engel, and B. Tropp, unpublished data). The data presented in Table 1 implicate L - glycerol - 3 - phosphate:cytidine monophosphate phosphatidyltransferase as a site of action. Work still in progress involving the incorporation of labeled phosphate into the phospholipid fraction and in vitro studies of the inhibition of L-glycerol-3-phosphate:cytidine monophosphate phosphatidyltransferase provide additional support for this hypothesis.

It is clear that 3,4-dihydroxybutyl-1-phosphonate has a profound effect on phospholipid metabolism under the conditions studied. The specific effects on phospholipid metabolism may prove helpful in future investigations concerning the role of phospholipids in membrane function and in studies of the regulation of phospholipid biosynthesis. In this respect, the four-carbon phosphonate may serve as a supplemental approach to the genetic one. 3,4-Dihydroxybutyl-1-phosphonate has the additional potential of possible use with a wide range of organisms. The permeability barrier may prove to be a formidable obstacle to such an approach, but this matter must be explored further before an unambiguous determination of future utility can be reached.

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