PHOSPHOLIPID SYNTHESIS IN *ESCHERICHIA COLI*: STUDIES OF THE METABOLISM OF PHOSPHONIC ACID ANALOGUES OF *sn*-GLYCEROL 3-PHOSPHATE

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1976

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

2-24-76
date

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Abstract

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OF *sn*-GLYCEROL 3-PHOSPHATE

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3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate, an analogue of glycerol 3-phosphate, is incorporated into a very polar lipid material by cultures of *Escherichia coli* strain 8, and *in vitro* by CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase. These labeled lipids have been fractionated by column chromatography on DEAE cellulose, revealing that only one labeled compound is formed *in vitro*, while four are synthesized *in vivo*. The main component of the material formed by intact cells has been shown to be identical to that produced enzymatically. This species has been identified as the phosphonic acid analogue of phosphatidylglycerophosphate [(1,2-diacyl)-*sn*-glyceryl *D*-4'-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate]. Hydrolysis of this novel lipid with phospholipase C resulted in the production of diglyceride and a water soluble derivative of 3,4-dihydroxybutyl-1-phosphonate. Treatment of the latter with

alkaline phosphatase produced 3,4-dihydroxybutyl-1-phosphonate and inorganic phosphate in a molar ratio of 1.03:1. Enzymatic analysis of the phosphonate liberated in this manner showed it to be the D enantiomer, thereby confirming the proposed structure of the lipid analogue.

The analogue of phosphatidylglycerophosphate did not turn over, and appeared to have no precursor-product relationship to the other labeled lipids derived from 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate *in vivo*. Analysis of the other three labeled products revealed the tritium to be present on glycerol, and not intact phosphonate, indicating some randomization of label. Experiments with a mutant of *E. coli* lacking the anabolic glycerol 3-phosphate dehydrogenase ruled out this enzyme as a cause of the randomization.

Two phosphonic acid analogues of CDP-diglyceride, DL-2-hexadecoxy-3-octadecoxypropylphosphonyl-O-(cytidine 5'-phosphate) (analogue I), and DL-3,4-dioctadecoxybutylphosphonyl-O-(cytidine 5'-phosphate) (analogue II), have been synthesized and examined as substrates for the enzymes involved in the synthesis of phosphoglycerides in *Escherichia coli*. Both compounds were substrates for CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase. The analogues had similar K_m values (K_m of 0.060 mM for analogue II; K_m of 0.080 mM for analogue I) and a V_{max} identical to that of CDP-dipalmitin (K_m of 0.044 mM). In contrast, the analogues were poor substrates for CDP-diglyceride:L-serine phosphatidyl transferase. The analogues had lower K_m values

(K_m of 0.40 mM for analogue II; K_m of 0.80 mM for analogue I) than CDP-dipalmitin (K_m of 1.4 mM). The V_{max}, although identical for both analogues, was ten-fold lower than that observed with the natural substrate. An analysis of the products of these enzymatic reactions suggests that phosphatidylglycerophosphate phosphatase and phosphatidylserine decarboxylase may also possess a certain degree of substrate specificity.

The results with the analogues of CDP-diglyceride *in vitro* indicate that if the acylation of 3,4-dihydroxybutyl-1-phosphonate were possible *in vivo*, phospholipid metabolism in *E. coli* might be markedly altered.

To my wife and my parents.

ACKNOWLEDGEMENTS

I would like to express my gratitude to each of the following people, who in some way, aided my graduate career:

Professor Burton Tropp for his constant support and guidance as my thesis advisor.

Professor Robert Engel and Dr. Stephen Goldstein for the synthesis of labeled 3,4-dihydroxybutyl-1-phosphonate.

Dr. J.C. Tang for his gift of 3,4-dihexadecanoylbutyl-1-phosphonic acid.

Professor Arthur Rosenthal for the synthesis of the analogues of CDP-diglyceride.

Professors Maxwell Eidinoff and Julius Golubow for serving, along with professors Tropp, Engel, and Rosenthal, on my thesis committee.

Dr. Zev Leifer, Mr. David Klein, Mr. Chu-Tay Tang, Ms. Mary Connolly, Mr. Daniel Hryb, and Mr. Larry Libman, for constructive criticism, and many helpful discussions.

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CHAPTER 1

THE METABOLIC FATE OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE IN *ESCHERICHIA COLI*: FORMATION OF A NOVEL LIPID, THE PHOSPHONIC ACID ANALOGUE OF PHOSPHATIDYLGLYCEROPHOSPHATE[1]

Introduction

The great interest in elucidating the molecular architecture of biological membranes, and their functional mechanisms, has led to an intensive examination of the role of lipids in these structures. *Escherichia coli* has proven to be particularly attractive as a model system in these studies. This organism has a simple phospholipid composition, consisting of three species (1), and the steps involved in their biosynthesis, outlined in Figure 1, have been well characterized (2-13). In addition, the potential exists for altering phospholipid metabolism by genetic means. Work with mutants of *E. coli* defective in phospholipid synthesis has been extensively reviewed (14,15). Such studies have permitted some understanding of the general role of lipids in membranes. However, the function of specific phospholipids in the mediation of membrane processes is not clear. Recently, mutants have been isolated in which the synthesis of a particular class of phospholipids is blocked (16-20). The study of such strains should help to clarify

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1. A portion of the work described in this chapter has been published: *J. Biol. Chem.* **250**, 1633-1639 (1975).

this situation.

As an alternative approach, phosphonic acid analogues of glycerol 3-phosphate have been examined in the hope of finding an inhibitor of a specific step in the synthesis of phosphoglycerides. One compound which appears promising is 3,4-dihydroxybutyl-1-phosphonate. This analogue inhibited the growth of E. coli, and also had a marked effect upon phospholipid metabolism (21,22). Treatment with concentrations of 3,4-dihydroxybutyl-1-phosphonate which only slightly affected growth resulted in an immediate 50% decrease in the rate of phosphatidylglycerol synthesis (23). In vitro examination of the enzymes involved in glycerol 3-phosphate metabolism in E. coli suggested that the phosphonate exerted an effect in vivo at the level of CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase. The compound was both a competitive inhibitor and a substrate for this enzyme (24).

3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate was incorporated into a highly polar lipid material in vitro by CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase, and by bacterial cultures. Preliminary experiments indicated that both materials were similar, and that they were probably phosphonic acid analogues of phosphatidylglycerophosphate. Since the presence of such an unnatural acidic lipid in the membrane of E. coli could have important effects, experiments were undertaken to fully clarify its structure. This chapter reports that the lipid material formed in vitro

and the major component synthesized *in vivo* from 3,4-dihydroxybutyl-1-phosphonate are indeed identical, and are the phosphonate analogues of phosphatidylglycerophosphate [(1,2-diacyl)-*sn*-glyceryl D-4'-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate —Figure 2]. In addition, some aspects of the metabolism of this lipid are discussed.

Materials and Methods

Chemicals: rac-3,4-Dihydroxybutyl-1-phosphonate was prepared as described previously (25). rac-3,4-Dihydroxy[3-^{3}H]-butyl-1-phosphonate (31 mCi/mmole and 480 mCi/mmole) was prepared using the method of Goldstein et al. (26). 3,4-Di-hexadecanoylbutyl-1-phosphonic acid was a gift of Dr. J.C. Tang of Queens College.

The following were obtained from the Sigma Chemical Co., St. Louis, Mo.: Bacillus cereus phospholipase C (Type III); rabbit muscle glycerol 3-phosphate dehydrogenase (L-glycerol 3-phosphate:NAD oxidoreductase, E.C. 1.1.1.8); NAD (Grade III); cabbage phospholipase D (Type I); tris-(hydroxymethyl)aminomethane (Tris); Triton X-100 (octyl-phenoxypolyethoxyethanol); palmitic acid; monopalmitin; and dipalmitin. Cytidine diphosphate-dipalmitin (CDP-dipalmitin) was purchased from Serdary Research Laboratories, London, Ontario, Canada. Bacterial phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, used as chromatographic standards, were obtained from Supelco, Inc., Bellefonte Pa. Sil-N-HR, polygram CEL 300 DEAE, and CEL 300 PEI thin layer plates were products of Brinkmann Instruments Inc., Westbury, N.Y. Anasil G thin layer plates were obtained from Analabs Inc., New Haven, Conn. Chelex 100 was a product of Bio-Rad. [1-^{14}C]Acetate (55 mCi/mmole) was purchased from New England Nuclear, Boston, Mass., and carrier free $H_3{}^{32}PO_4$ from Schwartz-Mann, Orangeburg, N.Y.

DE-52 DEAE cellulose was a product of Whatman. All other chemicals were of reagent grade. Solvents were redistilled before use.

Bacteria: E. coli strain 8, originally isolated by Hayashi et al.(27), was a gift of Dr. J. Cronan Jr. of Yale University. The genotype of this strain, in terms of the symbols of Taylor and Trotter (28), and the allele numbers of the Coli Genetic Stock Center (Yale University), is: Hfr C glp R^c2, phoA8, tonA22, $T2^R$, rel-1 (λ), glp D3. Strain BB20-14, a derivative of strain 8 which contains an additional lesion (gps A) resulting in an inactive biosynthetic sn-glycerol 3-phosphate dehydrogenase (29), was obtained from Dr. R. Bell of the Duke University Medical Center. Unless otherwise noted, strain 8 was cultured on the medium of Garen and Levinthal (30) supplemented with 0.6 mM phosphate and 0.5% potassium succinate. Strain BB20-14 was cultured on the same minimal media supplemented with 0.6 mM phosphate, 0.5% glucose, and 0.1% glycerol. Cells were incubated, and growth monitored, as previously described (23).

Incorporation of 3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate Into Lipids In Vivo: E. coli strain 8 was cultured in either 20 or 40 ml of media. Upon reaching a cell density of 1.5×10^8 cells per ml (26 Klett), 0.03 mM 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate was added. For long incubation times (20 minutes and 2 hours), the specific activity of

the analogue was 31 mCi/mmole. In five minute pulse experiments, it was increased to 480 mCi/mmole. In some studies, involving double labeling, the cultures also received [1-^{14}C]acetate (10 μCi/ml) and potassium acetate (100 μg/ml) at the same time as the phosphonate. In others, [^{32}P]phosphate (2 μCi/ml) was added one generation before the phosphonate to insure equilibration of phosphate pools. After incubation with the labeled compounds, the cells were either centrifuged at 5000 x g for 10 minutes at room temperature, and washed with media lacking succinate, or collected and washed on a Millipore filter (HAWP 45 um). The cells were resuspended in a volume of distilled water equal to one tenth the original culture volume, and the lipids extracted by the method of Bligh and Dyer (31) as modified by Ames (32). During the lipid isolation, precautions were taken not to disturb the interface between the aqueous and chloroform layers. The resulting chloroform extract was washed once with 1 ml of 2 M KCl, twice with 1 ml of 1 mM 3,4-dihydroxybutyl-1-phosphonate, and twice with 1 ml of distilled water. After the last wash, enough methanol was added to form one phase, and the insoluble debris removed by filtration through glass wool.

Turnover of Lipids Derived From 3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate: 3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate (31 mCi/mmole) was added to 40 ml cultures of *E. coli* strain 8 at a concentration of 0.03 mM. At 30 minute intervals, 1

ml of culture was withdrawn, and the lipids extracted by the Ames (32) procedure described earlier. After 2 hours of incubation, the cultures were filtered on Millipore filters, washed twice with 10 ml of media lacking succinate, and resuspended in fresh media without phosphonate. The cells were cultured for an additional 3 hours, during which time 1 ml samples were removed, and extracted as above. One ml portions of lipid extract were placed in scintillation vials, and counted as previously described (22).

Preparation of CDP-diglyceride:sn-Glycerol 3-phosphate Phosphatidyl Transferase: Cells of E. coli strain 8 in the mid-logarithmic phase of growth were harvested by centrifugation, and washed once with 0.1 M tris-HCl buffer, pH 8.0, containing 10 mM β-mercaptoethanol. Approximately 1 g (wet weight) of cells was resuspended in 15 ml of the same buffer, and the cell suspension subjected to eight 15-second bursts with a Branson model W140D sonifier, at a power setting of 8. The cell debris was removed by centrifugation at 3000 x g for 10 minutes. The resulting supernatant was centrifuged for 60 minutes at 40,000 x g in a Spinco model L ultracentrifuge. The pellet was resuspended and centrifuged again at 40,000 x g for 60 minutes. After the second high speed centrifugation, the pellet, containing the particulate CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase (4), was resuspended in 5 ml of buffer.

All steps in the preparation of the enzyme were performed at 1-4°C. Protein concentrations were determined by the method of Lowry et al. (33).

Incorporation of 3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate Into Lipid In Vitro by CDP-diglyceride:sn-Glycerol 3-phosphate Phosphatidyl Transferase: The reaction mixture was similar to that described by Chang and Kennedy (4) for the assay of this enzyme. It contained (0.5 ml total volume), 0.25 M tris-HCl buffer, pH 8.5; 0.08 mM CDP-dipalmitin; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol; 1 mg Triton X-100; and 0.038 mM 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (480 mCi/mmole). The reaction was initiated by the addition of 100-200 µg of particulate enzyme. After 30 minutes at 37°C, the lipids were extracted from the reaction mixture by the method of Bligh and Dyer (31). The chloroform phase, containing the lipids, was washed as described above for cell extracts.

DEAE Cellulose Column Chromatography: DEAE cellulose (Whatman DE-52) was treated as described by the manufacturer to remove the fines, and the residual material suspended in glacial acetic acid to convert it to the acetate form. The cellulose was washed free of acid with chloroform-methanol-water (2:3:1). After equilibration with this solvent for 1 hour at 4°C, a 1 x 30 cm column of DE-52 was prepared. Solvent was passed through the column until a

constant bed height was obtained.

An all glass and teflon apparatus capable of producing a linear elution gradient, was constructed from two 250 ml ehrlenmeyer flasks. The flasks were connected by means of a stopcock, which also fixed the flasks in the same plane. The mixing flask was equipped with a magnetic stirrer, and was connected to the column by means of teflon tubing and an airtight teflon stopper.

Column chromatography was performed at 4°C. Lipids were placed on the column in a small volume (1 ml or less) of chloroform-methanol-water (2:3:1). The sample was washed into the column with three 0.5 ml portions of the same solvent. To insure proper mixing of the gradient, chloroform-methanol-water (2:3:1) was added until the solvent reached a height of 2-3 cm above the column bed. The gradient apparatus was then connected; 150 ml of chloroform-methanol-water (2:3:1) were placed in the mixing flask, and 150 ml of the same solvent containing 0.1 M ammonium acetate, pH 7.2, in the reservoir. After leveling the flasks, the column was eluted at a rate of 10-12 ml per hour, and fractions of 4.0 ml were collected. The gradient apparatus produced 300 ml of a linear gradient of ammonium acetate (0 - 0.1 M, pH 7.2) in chloroform-methanol-water (2:3:1). The salt concentration in the fractions was determined with a Beckman model RC 16B2 conductivity bridge equipped with a K-1 cell.

Following elution, fractions containing the desired

lipids were pooled, reduced *in vacuo*, and the residue was extracted by the method of Bligh and Dyer (31). The column was regenerated by passing 40 ml of chloroform-methanol-water-acetic acid (2:3:1:1) through it, followed by washing and re-equilibration with chloroform-methanol-water (2:3:1).

Hydrolysis With Phospholipase C and Alkaline Phosphatase: Lipids were hydrolyzed with *Bacillus cereus* phospholipase C in a two phase system similar to that described by DeHaas *et al*. (33). Samples of labeled lipid material in chloroform were placed in screw cap test tubes, and the solvent removed under a stream of nitrogen. One ml of diethyl ether, 0.2 ml of *B. cereus* phospholipase solution (10 U/ml in 0.1 M tris-HCl buffer, pH 7.2), and 0.02 ml of 1 mM $ZnCl_2$ were added. Crude *E. coli* lipids (100 μg) were also included in reaction mixtures containing the analogue of phosphatidylglycerophosphate purified by column chromatography. The tubes were tightly capped, and shaken on a vortex mixer overnight at 25°C to permit complete hydrolysis. At the end of the incubation period, the ether was removed under nitrogen, and the water and chloroform soluble products were separated by the procedure of Bligh and Dyer (31). The chloroform phase was washed as described for cell extracts, and aliquots were counted as previously described (22). The extent of hydrolysis was calculated from the loss of chloroform soluble label. In some experiments, the chloroform solution was reduced *in vacuo*, and the dissolved

hydrolysis products analyzed by thin layer chromatography.

This procedure was modified in experiments where the water soluble hydrolysis products were to be characterized. Different phospholipase C preparations contained varying amounts of phosphatase activity, as measured by the hydrolysis of p-nitrophenyl phosphate. For this reason, shorter incubation periods were used (60 minutes for lipids formed *in vivo*, and 10 minutes for lipid synthesized *in vitro*). This, along with incubation at pH 6.0, minimized the phosphatase activity, but still allowed over 80% hydrolysis. When incubations were performed at pH 6.0, 0.1 M ammonium acetate buffer was substituted for tris-HCl, and 1 mM zinc acetate for $ZnCl_2$.

Water soluble hydrolysis products were isolated by the method of Bligh and Dyer (31) as above. The chloroform layer was washed with 0.5 ml of distilled water, and the washings combined with the initial aqueous layer. The combined fraction was washed with 0.2 ml of chloroform, and heated to 100°C for 5 minutes to inactivate the phospholipase C. It was then divided in half, and each half dried *in vacuo*. The residue from one half was redissolved in a minimum amount of distilled water for chromatographic analysis. Samples which contained ammonium acetate were treated with Dowex 50 (H^+) before chromatography.

The other half was reconstituted to 0.2 ml with a solution of *E. coli* alkaline phosphatase (1.25 U/ml in distilled water). The sample was incubated at 25°C for 1

hour, and then heated at 100°C for 5 minutes to inactivate the enzyme. The mixture was reduced *in vacuo* and analyzed as above. When ammonium acetate buffer was used in the phospholipase C hydrolysis, water soluble products were treated with alkaline phosphatase as above, except that the enzyme was dissolved in 0.05 M $NaHCO_3$ buffer, pH 8.1. Material obtained in this manner was chromatographed after treatment with Dowex 50 (H^+). This modification was also used to treat water soluble products obtained by other degradative methods with alkaline phosphatase.

Hydrolysis with 90% Acetic Acid: Samples of lipid material in chloroform were placed in screw cap test tubes. The solvent was removed under nitrogen, and 0.5 ml of 90% acetic acid were added. The samples were heated on a boiling water bath for 20 minutes. At the end of this period, the acetic acid was removed *in vacuo*, and 0.2 ml of water were added. The chloroform and water soluble products were isolated by the method of Bligh and Dyer (31). The latter were either chromatographed directly, or treated with alkaline phosphatase and then analyzed. This procedure usually resulted in 40-50% hydrolysis of labeled lipids.

Phospholipase D Hydrolysis: Lipids were treated with phospholipase D as described by Yang (35).

Mild Alkaline Hydrolysis: Lipids were deacylated using the

procedure of Dittmer and Wells (36).

Enzymatic Determination of D-3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate: The assay, based upon the results of Cheng et al. (37), was that described by Michal and Lang (38), modified to permit the determination of the extent of oxidation of rac-3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate by radiochemical means. The reaction mixture (0.5 ml total volume) contained: glycine-hydrazine buffer (0.5 M glycine, 0.2 M hydrazine, 2.5 mM EDTA), pH 9.5; 2.5 mM NAD; and 1-6 nmoles of rac-3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (31 mCi/mmole). The reaction was initiated by the addition of 0.05 mg of rabbit muscle L-glycerol 3-phosphate dehydrogenase (145 U/mg), and the mixture incubated at 25°C. After 15 minutes, 100 nmoles of unlabeled 3,4-dihydroxybutyl-1-phosphonate were added to dilute the labeled NADH produced by the reaction. After 30 and 60 minutes, 0.2 ml samples were removed, and placed in test tubes containing 100 nmoles of unlabeled 3,4-dihydroxybutyl-1-phosphonate. Following adjustment of the pH to 7 and the volume to 1 ml, 20 mg of activated charcoal were added to each test tube to remove the coenzyme. The samples were filtered, and 0.8 ml portions of the filtrates were counted in 10 ml of Patterson-Greene scintillation fluid (39). The extent of oxidation of rac-3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate was measured as the decrease in water soluble tritium. Water soluble material produced by the sequential

treatment of labeled lipids with phospholipase C and alkaline phosphatase, was assayed for D-3,4-dihydroxy-[3-^{3}H]butyl-1-phosphonate in an identical manner, after treatment with Chelex 100 (Na^{+}) to remove Zn^{++} ions.

Under the conditions of the assay, complete oxidation of labeled material was usually obtained during the initial 30 minutes of incubation. Ninety-eight to ninety-nine per cent of the coenzyme was spectrophotometrically found to be bound to the charcoal, while less than 2% of the labeled phosphonate was nonspecifically adsorbed.

Results

Based upon the known mode of action of CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase (4), it seemed likely that the compound formed *in vitro* from 3,4-dihydroxybutyl-1-phosphonate would be the phosphonic acid analogue of phosphatidylglycerophosphate (Figure 2, reaction 1). Preliminary studies indicated that this compound was also formed *in vivo*. A thin layer chromatographic comparison of lipids synthesized *in vitro* and *in vivo* from 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate is presented in Table 1. These lipids appeared to be very similar, and much more polar than the normal phospholipids of *E. coli*. The failure to migrate in solvent systems 1 and 2, which are commonly used in phospholipid separations, would be expected of an analogue of phosphatidylglycerophosphate. However, material isolated from the cells appeared to contain more than one labeled component, while only one was formed *in vitro*. The major component of the lipids formed *in vivo* was chromatographically identical to the material synthesized by the enzyme *in vitro*.

These observations were confirmed by the fractionation of such lipids on a column of DEAE cellulose as described in Figure 3. The major tritium labeled fraction was eluted after the normal phospholipids of *E. coli*, indicating its very anionic nature. Furthermore, while only

one labeled component was observed *in vitro*, four were found *in vivo*. A typical distribution of labeled products isolated from bacterial cultures is listed in Table 2 (Experiment 1). It is apparent that fraction IV, the major component isolated from bacterial cells, had an elution volume identical to the lipid formed *in vitro* (Figure 3A and D). Due to this chromatographic behavior, fraction IV was believed to be the analogue of phosphatidylglycerophosphate synthesized *in vivo*. The constant isotope ratio in fraction IV, doubly labeled with ^{3}H and either ^{14}C or ^{32}P, on this column (Figure 3B and C) and in other chromatographic systems, indicated that there was only one compound present.

The similarity between the lipid formed *in vitro* and fraction IV was further examined by means of mild alkaline hydrolysis. If these materials represent identical analogues of phosphatidylglycerophosphate, such treatment should reveal them to have one and the same backbone (Figure 2, reaction 2). The data presented in Table 3 indicate that this was the case.

If the lipid present in fraction IV and that synthesized *in vitro* have the proposed structure, treatment with phospholipase C should produce diglyceride and 4-phospho-3-hydroxybutyl-1-phosphonate (Figure 2, reaction 3). Alkaline phosphatase treatment of the water soluble derivative should yield inorganic phosphate and 3,4-dihydroxybutyl-1-phosphonate in a 1:1 molar ratio (Figure 2, reaction 4).

In a preliminary experiment, crude lipids synthesized *in vivo* and that formed *in vitro* form 3,4-dihydroxy[3-^{3}H]-butyl-1-phosphonate were treated with phospholipase C. Such hydrolysis produced a polar, water soluble compound, which failed to migrate when chromatographed as described in Table 4. Treatment of this material with alkaline phosphatase converted it almost quantitatively to 3,4-dihydroxybutyl-1-phosphonate. Similar results were obtained by chromatography on Whatman No. 1 paper, using ethyl acetate-formamide-pyridine (1:2:1) as the solvent system. These results were consistent with the *in vitro* and *in vivo* formation of the analogue of phosphatidylglycerophosphate.

Further evidence in support of the structure shown in Figure 2 comes from double labeling studies. When fraction IV containing ^{32}Pi and 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate was treated with phospholipase C, all the ^{32}P, and 98-99% of the ^{3}H was converted to a water soluble form. As shown in Figure 4, this material had a different R_f from either phosphate or 3,4-dihydroxybutyl-1-phosphonate, and migrated as one compound with a constant isotope ratio across the peak. Hydrolysis of this material with alkaline phosphatase completely cleaved it into a mixture of ^{32}Pi and 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate. Based upon the ^{3}H:^{32}P ratio, 3,4-dihydroxybutyl-1-phosphonate and phosphate were produced in a molar ratio of 1.03:1. Similar results were obtained by chromatography on Whatman No. 1 paper, using 1 M ammonium acetate (pH 7.1):ethanol (1:1) as the

solvent system (Figure 5). In these chromatographic systems, the water soluble product obtained by phospholipase C treatment of the lipid synthesized *in vitro*, was identical to that obtained from fraction IV.

The structure of these lipids was also probed by hydrolysis with 90% acetic acid. Such treatment would be expected to yield a water soluble product identical to that produced by phospholipase C (40). Using the chromatographic methods described in Figures 4 and 5, this was indeed found. Material obtained in this manner was quantitatively converted to 3,4-dihydroxybutyl-1-phosphonate by alkaline phosphatase.

It has already been shown that 3,4-dihydroxybutyl-1-phosphonate is a substrate for rabbit muscle L-glycerol 3-phosphate dehydrogenase (37). D-3,4-dihydroxybutyl-1-phosphonate, the enantiomer corresponding to *sn*-glycerol 3-phosphate, is the one thought to have biological activity (24, 37). Based on the fact that the dehydrogenase removes the tritium from labeled phosphonate, and transfers it to NAD, an assay has been devised which permits a determination of D-3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate. When *rac*-3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate was examined by this method, 45% of the compound was oxidized, as shown in Figure 6, corresponding to 90% of the D form. When water soluble material released by phospholipase C from fraction IV was treated with alkaline phosphatase and assayed, 86% was oxidized. A similar amount of material obtained from the *in vitro*

preparation was recognized by the enzyme. These results indicated that D-3,4-dihydroxybutyl-1-phosphonate was incorporated as an intact moiety into fraction IV and the *in vitro* product. This is in agreement with the known stereospecificity of CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase (4).

Fraction IV, doubly labeled with [^{14}C]fatty acids (from acetate) and 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate, was treated with phospholipase C for the purpose of characterizing the chloroform soluble hydrolysis products. This preparation was quantitatively cleaved, and the ^{14}C label converted to a form which was chromatographically identified as diglyceride (Table 5).

The results presented above show unequivocally that the analogue of phosphatidylglycerophosphate [(1,2-diacyl)-*sn*-glyceryl D-4'-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate] illustrated in Figure 2 is formed *in vitro* and *in vivo* from 3,4-dihydroxybutyl-1-phosphonate. However, the presence of three other labeled lipid products in bacterial cultures prompted an examination of the possible metabolism of this lipid analogue. Since such a highly polar lipid might prove harmful to the cell, it seemed reasonable to assume that it might be modified. The three minor components could therefore represent degradation products of the phosphatidylglycerophosphate analogue. This idea was tested by searching for a precursor-product relationship among the four labeled lipids. As illustrated in Table 2

(experiments 1-3), incubation of cells for shorter periods with labeled phosphonate resulted in similar distributions of lipid products. All four components were present, and within experimental limits, a precursor-product relationship between fraction IV and the other fractions was not found. A possible connection between the labeled lipids was also examined by increasing the concentration of 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate in the medium 10 fold (Table 2, experiment 4). Such a change did not significantly alter the product distribution. The decreased incorporation of 3,4-dihydroxybutyl-1-phosphonate observed under these conditions is consistent with the increased inhibitory effects of the phosphonate at higher concentrations (21,22).

The possibility that the analogue of phosphatidylglycerophosphate was being degraded *in vivo* was examined in another way. Figure 7 shows the results of an experiment in which the turnover of lipids labeled with tritium was investigated. As can be seen, conversion of this material into non-lipid products was not apparent. Furthermore, the distribution of labeled lipid components isolated from cells at the end of the turnover period was very similar to that obtained after two hours of incubation with 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (Table 2, experiments 5A and B). From these results, it was concluded that the analogue of phosphatidylglycerophosphate was not being catabolized.

The procedure used for the isolation of lipids in the experiment depicted in Figure 7 did not differentiate between the phosphoglycerides of intact cells and those in the medium. Since there was the possibility that such a potentially harmful lipid could be eliminated by excretion, the medium was extracted separately at various times before and during the turnover period. This revealed the presence of less than 0.5% of the total labeled lipids, indicating that the analogue of phosphatidylglycerophosphate was not being discharged into the medium.

A clue to the identity of the other labeled components derived from 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate was obtained when it was realized that they were eluting from the DEAE cellulose column coincident with the normal phospholipids of E. coli (Figure 3). This relationship was further investigated by thin layer chromatography. In two solvent systems, the labeled material in fractions I, II, and III, migrated, respectively, as phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (Table 6). This indicated the possibility that 3,4-dihydroxy[3-^{3}H]-butyl-1-phosphonate was being incorporated into these phospholipids. One way in which this might happen is by the acylation of the phosphonate to form an analogue of phosphatidic acid. However, it has already been shown that this compound is not a substrate for acyl CoA:sn-glycerol 3-phosphate acyl transferase (24). This apparent conflict was examined by chemical and enzymatic degradation of the

material in fractions I, II, and III, as illustrated in Figures 8-10, to identify the labeled moiety.

Treatment of the material in fraction I with phospholipase D resulted in no release of water soluble tritium. Chromatography of the chloroform soluble product revealed a labeled material which migrated in a manner identical to 3,4-dihexadecanoylbutyl-1-phosphonic acid (Table 7). This would be expected if 3,4-dihydroxybutyl-1-phosphonate was present as an acylated moiety in phosphatidylethanolamine. However, it has been shown that in most commonly used solvent systems, phosphonolipids and their phospholipid counterparts have similar R_f values (41). To determine unequivocally whether the phosphonate was being incorporated in this manner, the product of phospholipase D hydrolysis was deacylated, and treated with alkaline phosphatase, as illustrated in Figure 8. 3,4-Dihydroxybutyl-1-phosphonate would be expected to be impervious to this treatment, and should be recovered intact. This sequence liberated 96% of the tritium in a water soluble form. However, chromatographic analysis revealed 92% of the labeled material originally present to be glycerol, and not 3,4-dihydroxybutyl-1-phosphonate.

Similar results were obtained with fractions II and III. Phospholipase C hydrolysis resulted in the conversion of 59% and 43%, respectively, of the tritium in fractions II and III to a water soluble form. This observation was inconsistent with the incorporation of 3,4-dihydroxy-

butyl-1-phosphonate into the acylated backbone of phosphatidylglycerol and cardiolipin, since such material should not be susceptible to phospholipase C. Subsequent treatment and analysis of the phospholipase C hydrolysis products, as described in Figures 9 and 10, showed that 92% and 97% of the total labeled material in fractions II and III, respectively, was glycerol.

These results indicated that 3,4-dihydroxybutyl-1-phosphonate was not being acylated, but that the tritium label was being removed in a manner which permitted its appearance in the phospholipids of E. coli as a glycerol 3-phosphate moiety. In considering enzymes which could be responsible for this action, the catabolic glycerol 3-phosphate dehydrogenase was ruled out as a possibility, since E. coli strain 8 has lost this enzyme by mutation. Furthermore, 3,4-dihydroxybutyl-1-phosphonate was found not to be a substrate for this dehydrogenase (24). However, the anabolic glycerol 3-phosphate dehydrogenase did seem to be a likely candidate, since it does recognize the phosphonate in vitro (24). In order to examine the role of the biosynthetic enzyme in randomizing the tritium label, strain BB20-14, a derivative of strain 8 which has also lost the anabolic dehydrogenase (29), was cultured in the presence of 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate. The lipids were analyzed and compared to those of strain 8 under the same culture conditions (Table 2, experiments 6 and 7). Four labeled components were present in the lipids of strain

BB20-14. Moreover, the distribution was almost identical to that of the parent strain. This indicated that the anabolic glycerol 3-phosphate dehydrogenase was not the cause of the randomization. Furthermore, the experiment also showed that a change in the medium to one containing glucose and glycerol had no observable effect on the distribution of labeled lipids derived from the phosphonate. The decreased incorporation of 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate under these culture conditions can apparently be attributed to the increased availability of sn-glycerol 3-phosphate.

Discussion

Previous studies indicated that 3,4-dihydroxybutyl-1-phosphonate inhibits two enzymes of E. coli: sn-glycerol 3-phosphate:NAD oxidoreductase (The anabolic glycerol 3-phosphate dehydrogenase), and CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase (24). These in vitro observations helped to explain the effects of the analogue on phosphoglyceride metabolism (22,23). The delayed inhibition of phosphatidylethanolamine synthesis may be due to a scarcity of glycerol 3-phosphate caused by inhibition of the anabolic dehydrogenase. The effect on this biosynthetic enzyme, however, cannot be the sole cause of growth inhibition, because cells cultured in the presence of glycerol (24), and a mutant containing a product insensitive anabolic dehydrogenase (42), are susceptible to the phosphonate.[2] For this reason, interest has focused upon various aspects of the CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase reaction, although the dehydrogenase is a sensitive target when wild-type cells are cultured on succinate.

The analogue may inhibit by blocking phosphatidylglycerol formation, or by being incorporated into a novel anionic lipid, or both. It is possible that the inhibition of phosphatidylethanolamine synthesis is a consequence of generalized membrane alteration, and not due to a

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2. Unpublished results of Dr. Z. Leifer.

glycerol 3-phosphate limitation. In this regard, it is interesting to note that the growth of cells treated with low levels of analogue continues at the inhibited rate even after the compound is removed from the medium, and that there is a longer recovery time for treated than untreated cultures (Figure 7). During this period, the analogue of phosphatidylglycerophosphate is neither degraded nor excreted. Further studies designed to measure the rate of recovery of phosphatidylglycerol synthesis should prove useful in clarifying the mode of action of 3,4-dihydroxybutyl-1-phosphonate. Such work will be reinforced by the characterization of mutants of E. coli resistant to this compound.

The anabolic glycerol 3-phosphate dehydrogenase does not appear to be the enzyme responsible for the randomization of tritium from labeled analogue. E. coli also possesses an anaerobic glycerol 3-phosphate dehydrogenase (43,44). However, this enzyme should not have been of physiological significance under the aerobic culture conditions employed (44). Furthermore, its ability to recognize 3,4-dihydroxybutyl-1-phosphonate is unknown. An activity which could oxidize the analogue was detected in crude extracts of E. coli K-12, cultured under conditions different from the present ones (24). This uncharacterized enzyme may be responsible for the observed distribution of labeled lipids. The possibility of a phosphonatase must also be considered.

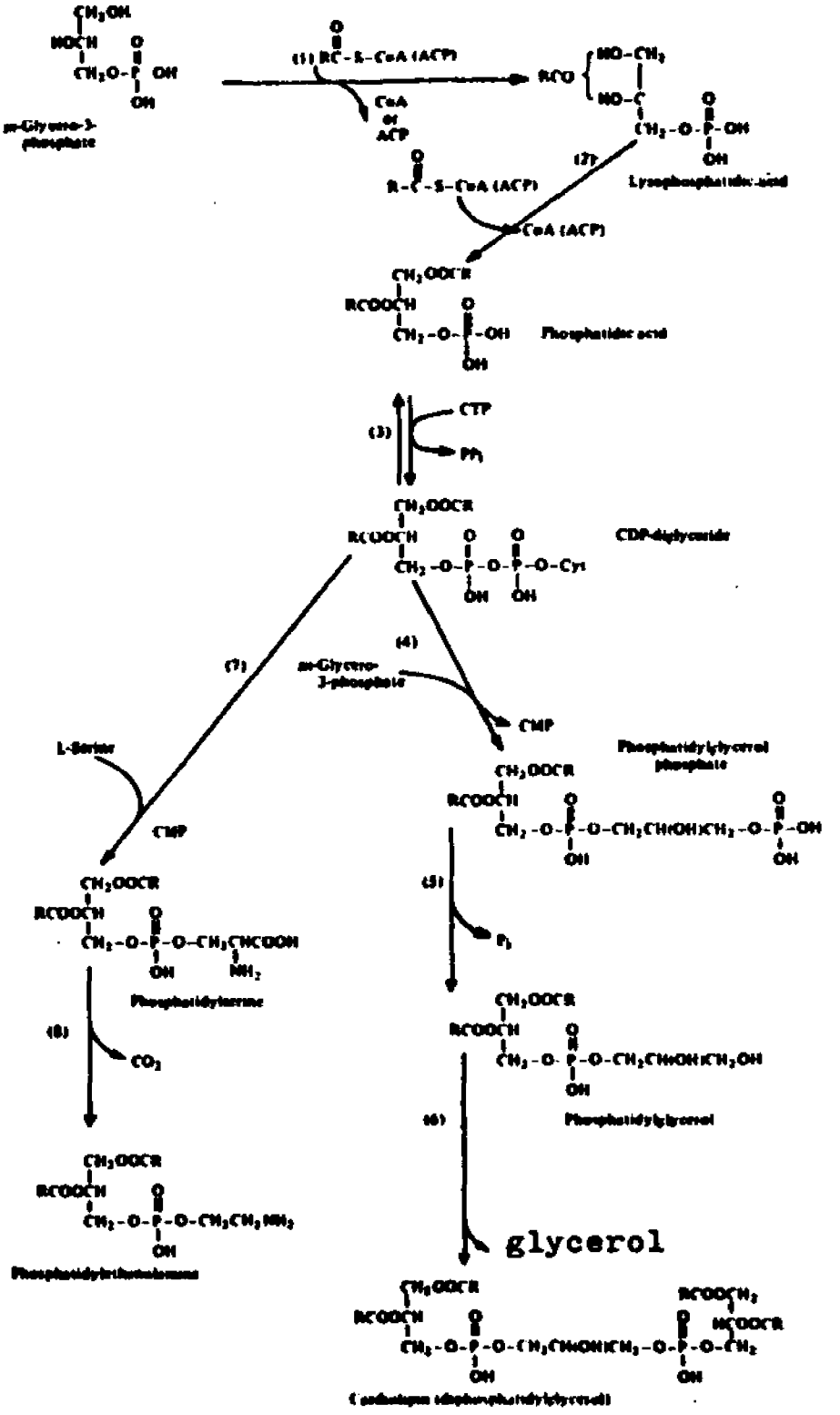
The finding that 3,4-dihydroxybutyl-1-phosphonate is not a substrate for acyl CoA:sn-glycerol 3-phosphate acyl transferase (24) is further supported by the characterization of lipids synthesized from this compound in vivo. This, however, raises the question of what effects could be expected if such a reaction were possible in E. coli. This matter has been partially resolved by studies involving phosphonic acid analogues of CDP-diglyceride, reported in chapter 2. The results indicate that the acylation of 3,4-dihydroxybutyl-1-phosphonate might markedly affect phosphoglyceride metabolism.

The availability of a phospholipid such as the phosphonic acid analogue of phosphatidylglycerophosphate intreduces a new variable into the study of lipids in model systems. The presence of a species bearing such a large negative charge should have interesting effects on the physical properties of lipid bilayers, and the permeability of liposomes. Such studies might also reveal possible effects of this lipid analogue on membrane structure in vivo. There have been some attempts to study the effect of perturbing membrane phospholipid structure on the activity of membrane bound enzymes in E. coli (15). 3,4-Dihydroxybutyl-1-phosphonate, through the formation of the analogue of phosphatidylglycerophosphate, may prove useful in extending these studies. By employing this new lipid in reconstitution experiments, it should be possible to further probe the behavior and lipid specificity of membrane bound enzymes

in vitro.

Figure 1: Pathways for the biosynthesis of phospholipids in E. coli; modified, from Bell et al. (13). The enzymes catalyzing the reactions are as follows: 1 - glycerol 3-phosphate acyl transferase; 2 - acylglycerol 3-phosphate acyl transferase; 3 - CTP:phosphatidic acid cytidyl transferase; 4 - CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase; 5 - phosphatidylglycerophosphate phosphatase; 6 - cardiolipin synthetase; 7 - CDP-diglyceride:L-serine phosphatidyl transferase; 8 - phosphatidylserine decarboxylase.

FIG 1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CH2O-P
OH
sn-Glycerol-3-phosphate
(1) RC-S-CoA (ACP)
CoA or ACP
RCO
HO-CH2
HO-C
CH2-O-P-OH
OH
Lysophosphatidic acid
(2)
R-C-S-CoA (ACP)
CoA (ACP)
CH2OOCR
RCOOCH
CH2-O-P-OH
OH
Phosphatidic acid
(3)
CTP
PPi
CH2OOCR
RCOOCH
CH2-O-P-O-P-O-Cyt
OH OH
CDP-diglyceride
(4)
sn-Glycerol-3-phosphate
CMP
Phosphatidylglycerol phosphate
CH2OOCR
RCOOCH
CH2-O-P-O-CH2CHOHCH2-O-P-OH
OH OH
(5)
Pi
CH2OOCR
RCOOCH
CH2-O-P-O-CH2CHOHCH2OH
OH
Phosphatidylglycerol
(6)
glycerol
(7)
L-Serine
CMP
CH2OOCR
RCOOCH
CH2-O-P-O-CH2CHCOOH
OH NH2
Phosphatidylserine
(8)
CO2
CH2OOCR
RCOOCH
CH2-O-P-O-CH2CH2NH2
OH
Phosphatidylethanolamine
CH2OOCR
RCOOCH
CH2-O-P-O-CH2CHOHCH2-O-P-O-CH2
OH
RCOOCH2
HCOOCR
Cardiolipin (diphosphatidylglycerol)

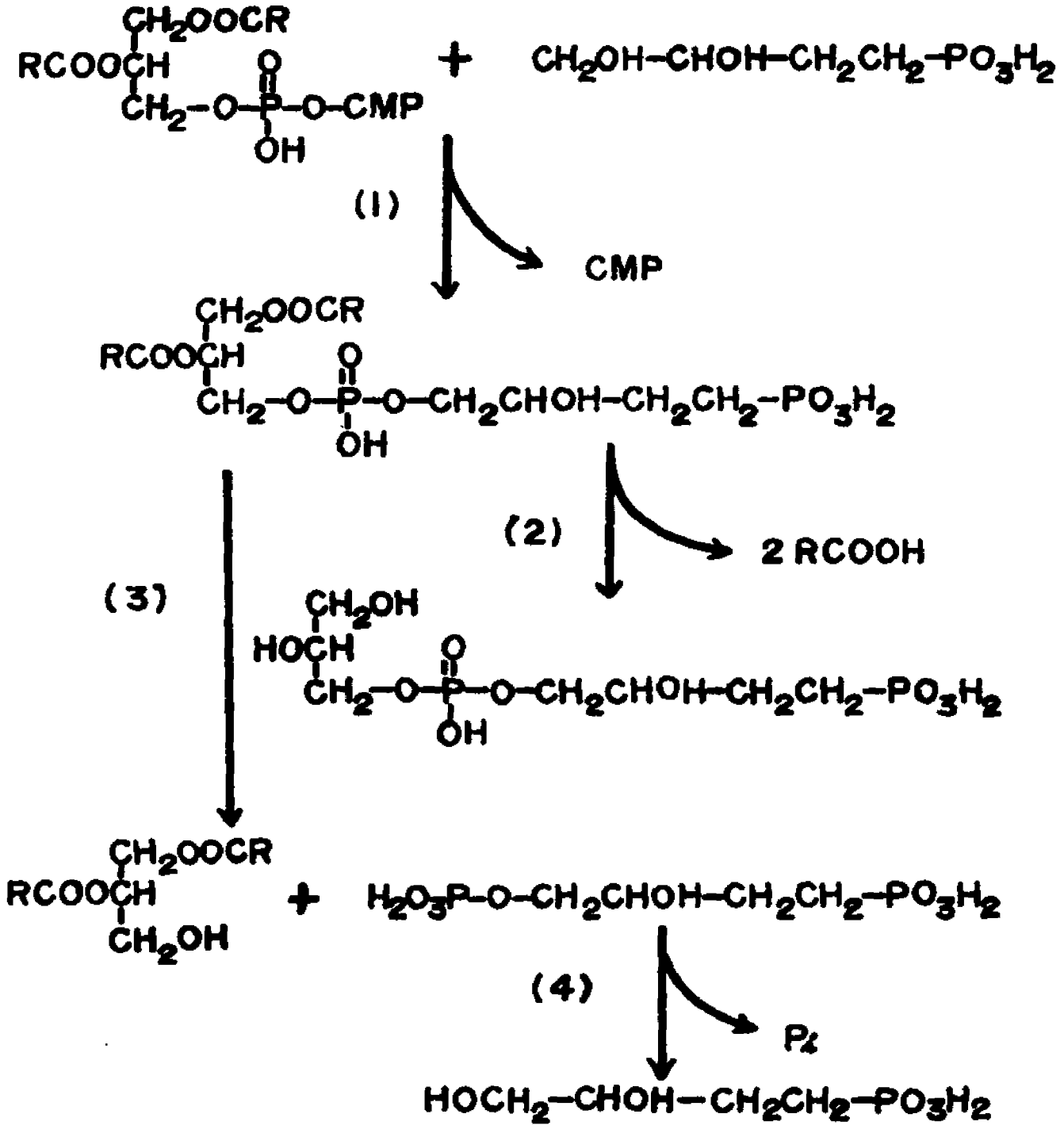
Table 1: Thin layer chromatography of labeled lipids. Lipid material synthesized *in vivo* and *in vitro* from 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate was analyzed by thin layer chromatography as indicated. The chromatograms were divided into 1 cm sections, and counted as previously described (22). The solvent systems used were as follows: 1 - chloroform-methanol-water (65:25:3); 2 - chloroform-methanol-acetic acid (65:25:8); 3 - n-butanol-acetic acid-water (6:2:2); 4 - diisobutylketone-acetic acid-water (40:30:7); 5 - chloroform-methanol-0.3M ammonium acetate, pH 6.9 (2:3:1).

Table 1

Adsorbent	Solvent System	Distribution of ^3H labeled material synthesized *in vivo*	Distribution of ^3H labeled material synthesized *in vitro*
Anasil G	1	95% at or just above origin	100% at or just above origin
"	2	95% at or just above origin	100% at or just above origin
"	3	76% R_f 0.48 24% R_f 0.65	100% R_f 0.48
"	4	78% R_f 0.30 22% R_f 0.53	100% R_f 0.30
DEAE cellulose	5	85% R_f 0.44 15% R_f 0.84	100% R_f 0.44

Figure 2: Formation of the phosphonic acid analogue of phosphatidylglycerophosphate and degradative methods used in its characterization. Reaction 1: Synthesis of the phosphonic acid analogue of phosphatidylglycerophosphate [(1,2-diacyl)-sn-glyceryl D-4'-phosphoryloxy-3'-hydroxybutyl-1'-phosphonate] by CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase in vitro and in vivo. Reaction 2: Mild alkaline hydrolysis of the lipid analogue to remove the fatty acids. Reaction 3: Hydrolysis with B. cereus phospholipase C to produce diglyceride and 4-phospho-3-hydroxybutyl-1-phosphonate. Reaction 4: Treatment of the water soluble phospholipase C hydrolysis product with alkaline phosphatase to produce phosphate and 3,4-dihydroxybutyl-1-phosphonate in a 1:1 molar ratio.

FIG 2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+
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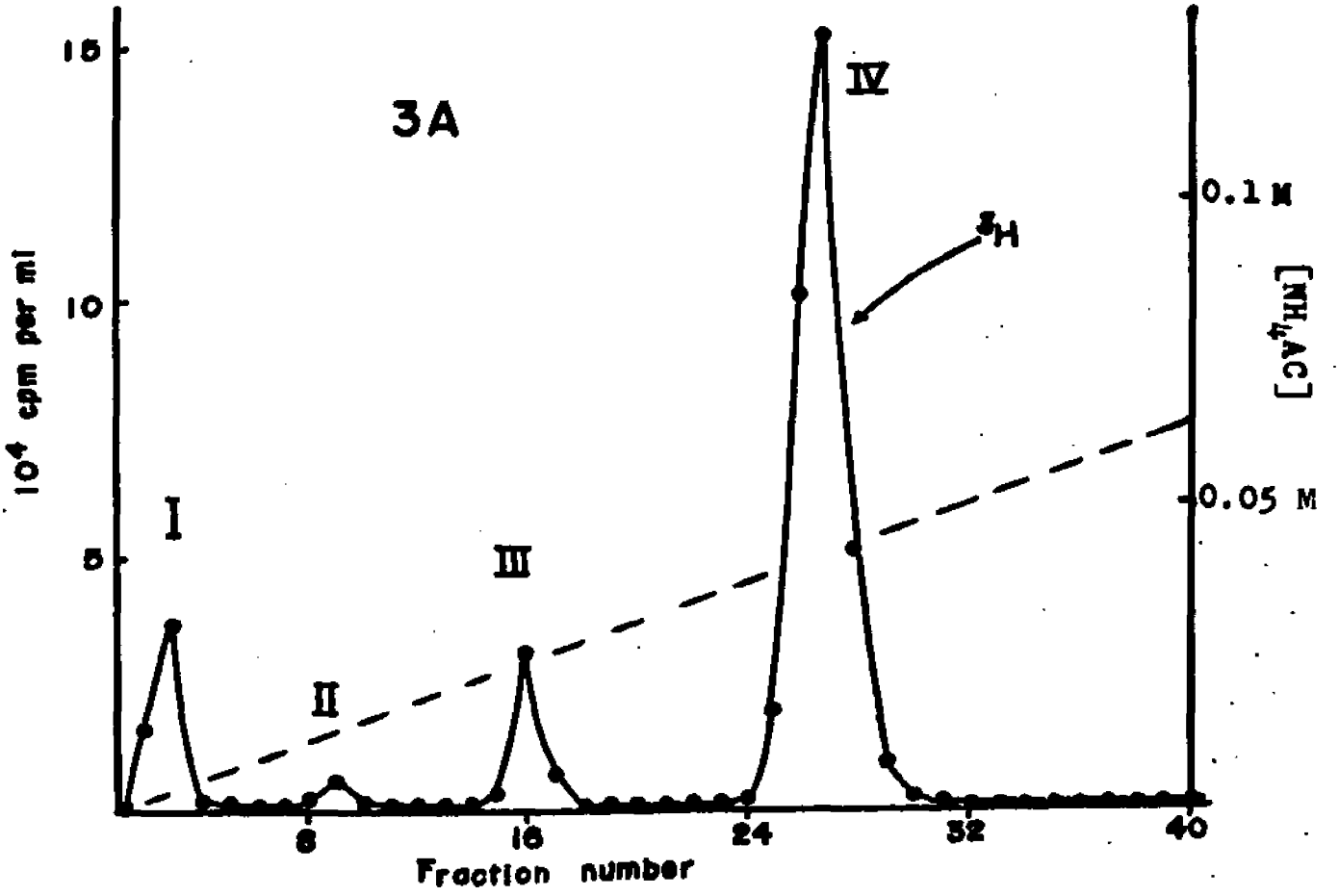
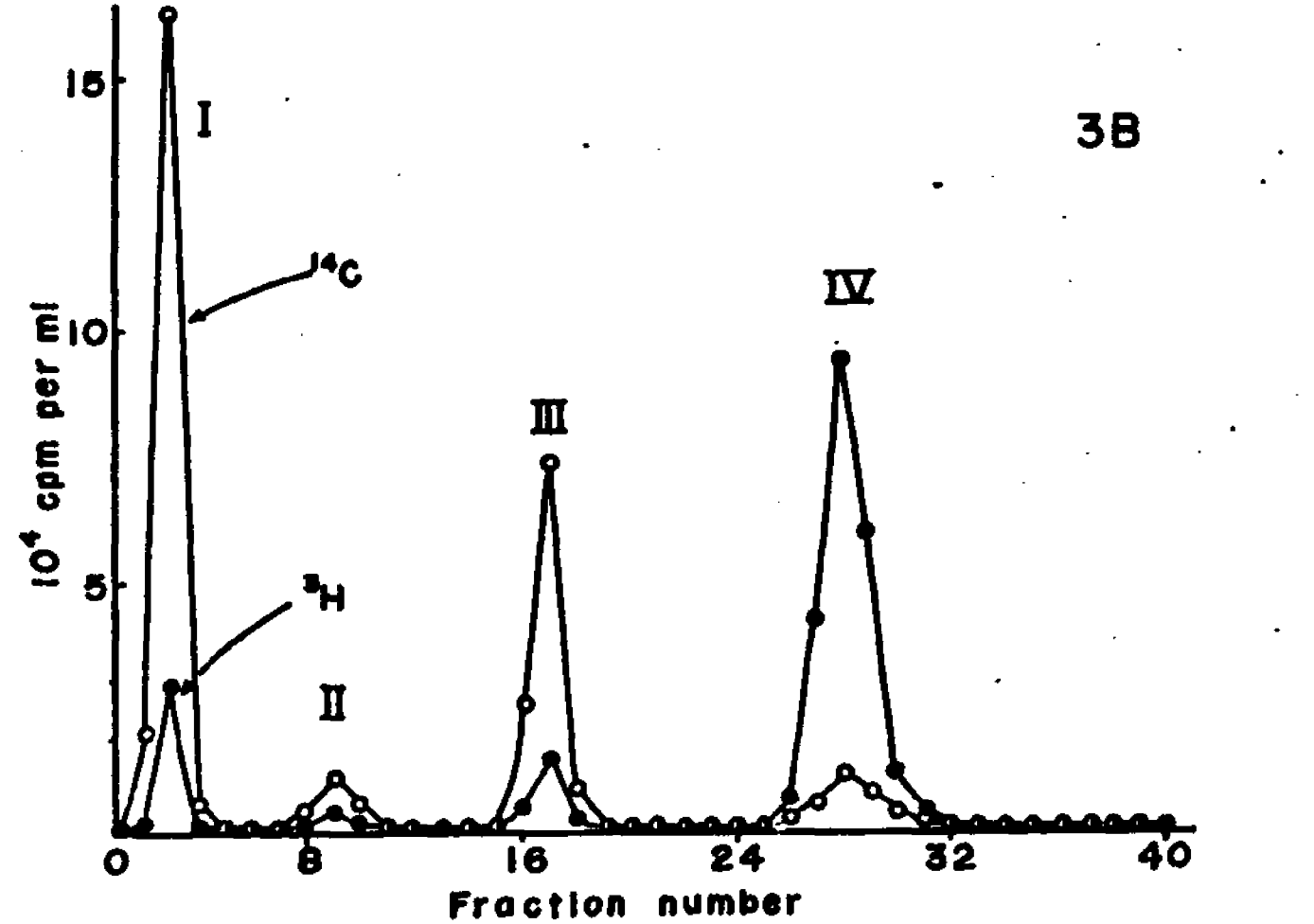
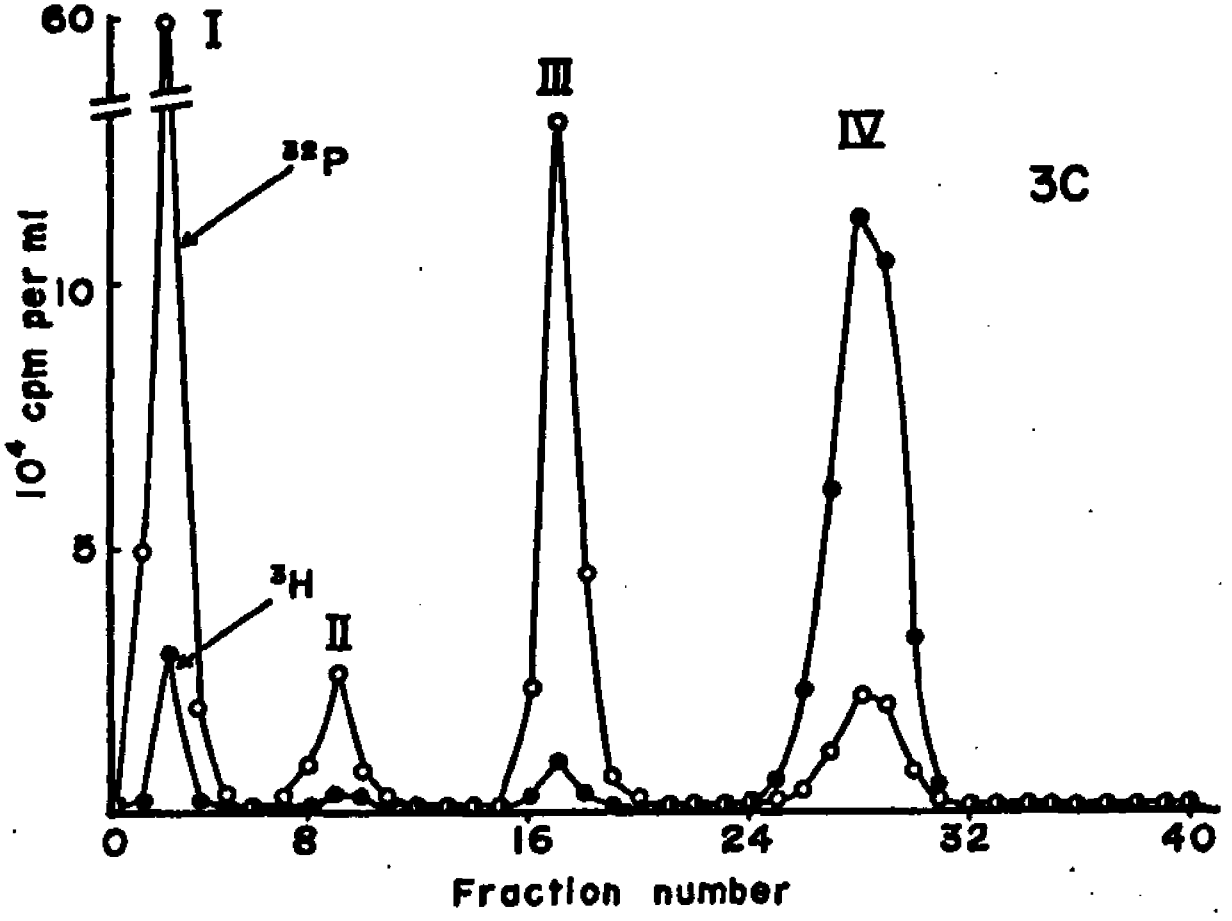
CMP
CH2OOCR
RCOOCH
CH2-O-P-O-CH2CHOH-CH2CH2-PO3H2
OH
(2)
2 RCOOH
(3)
CH2OH
HOCH
CH2-O-P-O-CH2CHOH-CH2CH2-PO3H2
OH
CH2OOCR
RCOOCH
CH2OH
+
H2O3P-O-CH2CHOH-CH2CH2-PO3H2
(4)
Pi
HOCH2-CHOH-CH2CH2-PO3H2

Figure 3 A-D: Fractionation of lipids derived from 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate. Labeled lipids were chromatographed on a 1 x 30 cm column of DEAE cellulose as described in the Materials and Methods section. The isotope content of each fraction in (A), (B), and (D) was determined by counting a 0.1 ml aliquot as previously described (22). Samples of fractions in (C) were counted in 1 ml of water and 10 ml of Patterson-Greene scintillation fluid (39) after evaporation of the solvent. In each case, 97-99% of all the radioactivity applied to the column was recovered. Phosphatidylethanolamine, phosphatidylglycerol, and cardilipin were eluted, respectively, in fractions I, II, and III. (A): Chromatography of lipids isolated from a 40 ml culture of E. coli strain 8, incubated in the presence of 0.03 mM 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (31 mCi/mmole) for 2 hours. ^{3}H —●—. (B): Chromatography of lipids obtained from cells cultured in the presence of labeled phosphonate as in (A), and [1-^{14}C]acetate, as described in the Materials and Methods section. ^{3}H —●—; ^{14}C —○—. (C): Chromatography of lipids isolated from cells cultured in the presence of labeled phosphonate as in (A), and [^{32}P]phosphate (2 μCi/ml). ^{3}H —●—; ^{32}P —○—. (D): Chromatography of lipids synthesized in vitro by CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase (1.8 x 10^5 cpm), from CDP-dipalmitin and 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (480 mCi/mmole). ^{3}H —●—. (NH_4AC = ammonium acetate).


3A
15
10
5
10^4 cpm per ml
I
II
III
IV
^3H
0.1 M
0.05 M
$[NH_4Ac]$
8
16
24
32
40
Fraction number


3B
15
10
5
10^4 cpm per ml
I
^{14}C
^3H
II
III
IV
0
8
16
24
32
40
Fraction number


60
I
III
IV
^{32}P
3C
10^4 cpm per ml
10
5
^3H
II
0
8
16
24
32
40
Fraction number

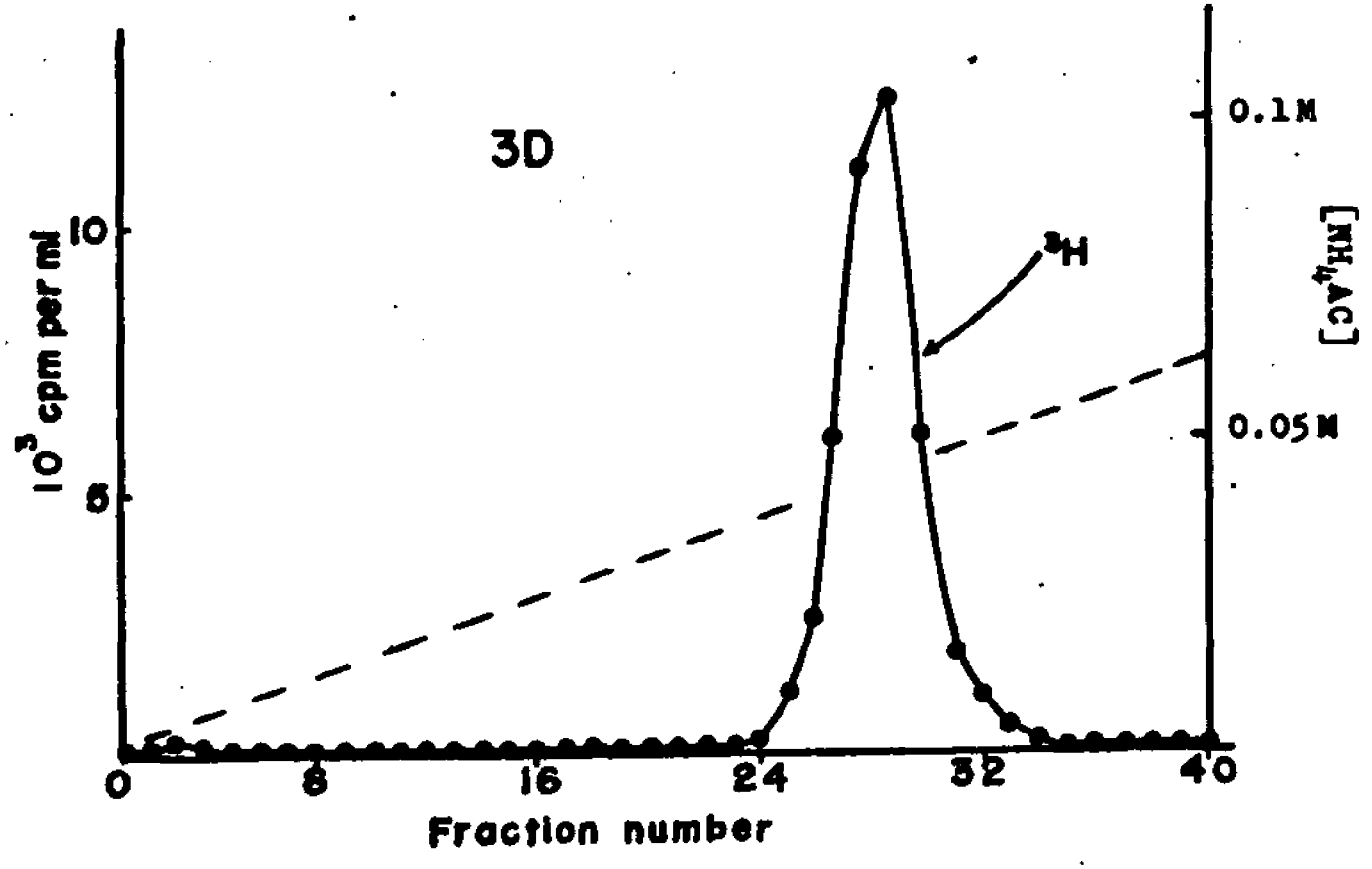

3D
10^3 cpm per ml
10
5
^3H
0.1M
0.05M
[NH$_4$AC]
0
8
16
24
32
40
Fraction number

Table 2: Potential factors influencing the distribution of labeled lipids derived from 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate. Phospholipids were isolated from bacterial cultures and chromatographed as described in Figure 3, after various changes in culture conditions. In experiments 1-5, E. coli strain 8 was cultured on the medium of Garen and Levinthal (30) supplemented with 0.6 mM phosphate and 0.5% potassium succinate. Experiment 1: A typical distribution obtained after incubation with 0.03 mM 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (31 mCi/mmole) for 2 hours. Experiment 2: Distribution after incubation as in experiment 1 for 20 minutes. Experiment 3: Distribution after pulse labeling cells for 5 minutes with 0.03 mM 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (480 mCi/mmole). Experiment 4: Distribution in a culture in which the concentration of labeled analogue was increased to 0.3 mM (12.4 mCi/mmole). Lipids were isolated after 2 hours of incubation. Experiment 5: Distribution immediately after washing cells (A) and 3 hours later (B) in the turnover experiment described in Figure 5. Experiment 6: E. coli strain 8 was cultured on the medium of Garen and Levinthal (30) supplemented with 0.6 mM phosphate, 0.5% glucose, and 0.1% glycerol. Lipids were isolated after 2 hours of incubation in the presence of 0.03 mM 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (310 mCi/mmole). Experiment 7: Distribution of labeled lipids isolated from strain BB20-14, cultured in a manner identical to that of strain 8 in experiment 6.

Table 2

Experiment	% Distribution of ^{3}H label in lipid fractions: I	II	III	IV	nmoles phosphonate incorporated per ml culture
1	8	2	4	86	2.9
2	5	7	8	80	0.19
3	6	9	3	82	0.03
4	3	1	8	88	0.65
5A	6	2	3	89	3.2
5B	10	2	3	85	3.2
6	6	2	3	89	0.77
7	5	3	3	89	0.62

Table 3: Chromatographic comparison of deacylated lipids. Samples of fraction IV (1 x 10^5 cpm ^3H) and material synthesized *in vitro* (1 x 10^5 cpm ^3H) were placed in screw-cap test tubes, and the solvent removed under nitrogen. The lipids were redissolved in 0.5 ml of chloroform-methanol (1:4), and 0.05 ml of 1.2 N NaOH in methanol-water (1:1) were added. The samples were incubated at 37°C for 10 minutes. At the end of this period the samples were neutralized by the addition of 0.075 ml of 1 N acetic acid. This was followed by 1 ml of chloroform-methanol (9:1), 0.5 ml of isobutanol, and 1 ml of water. The aqueous layer was carefully removed, and reduced *in vacuo*. The water soluble products were chromatographed on Whatman No. 1 paper in either methanol-91% formic acid-water, 80:14:6 (solvent system 1), or n-propanol-ammonia-water, 6:3:1 (solvent system 2). After development, each lane was cut into 1 cm sections, and each counted in 1 ml of water and 10 ml of Patterson-Greene scintillation fluid (39). Authentic 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate migrated with an R_f of 0.62 in solvent system 1, and 0.28 in solvent system 2.

Table 3

Lipid Material Deacylated	Distribution of ^3H label in: Solvent System 1	Solvent System 2
Fraction IV	100% R_f 0.31	100% R_f 0.22
In vitro	100% R_f 0.31	100% R_f 0.22

Table 4: Chromatographic characterization of water soluble phospholipase C hydrolysis products. Crude lipids formed *in vivo* (1×10^5 cpm ^3H) and that synthesized *in vitro* (1.2×10^5 cpm ^3H) were treated with *B. cereus* phospholipase C (in 0.1 M tris-HCl buffer, pH 7.2) as described in the Materials and Methods section. The incubation times were as follows: 10 minutes for lipid synthesized *in vitro* and 60 minutes for lipids formed *in vivo*. The water soluble products were isolated by the method of Bligh and Dyer (31). This material was chromatographed on Sil-N-HR before and after treatment with alkaline phosphatase, using methanol-0.01 N HCl as the solvent system. The chromatograms were cut into 1 cm sections, and counted as described in Table 3. Authentic 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (4CP) migrated with an R_f of 0.64 in this system.

Table 4

	% ^{3}H label at origin	% ^{3}H label migrating as 4CP
In Vitro sample treated with Phospholipase C	90	10
Crude *In Vivo* sample treated with Phospholipase C	88	12
In Vitro sample treated with Phospholipase C and Alkaline Phosphatase	1	99
Crude *In Vivo* sample treated with Phospholipase C and Alkaline Phosphatase	4	96

Figure 4: Chromatographic characterization of water soluble phospholipase C hydrolysis products. A sample of fraction IV isolated as in Figure 3C, containing 1 x 10^5 cpm ^3H and 1.8 x 10^4 cpm ^{32}P, was incubated for 60 minutes with B. cereus phospholipase C (in 0.1 M ammonium acetate buffer, pH 6.0) as described in the Materials and Methods section. The water soluble products were isolated by the method of Bligh and Dyer (31). A portion of this material was chromatographed on a CEL 300 PEI thin layer sheet before (A) and after (B) treatment with alkaline phosphatase, using the solvent system 1 M LiCl:1 M formic acid (1:1). After development, each lane was cut into 1 cm sections, and each counted in 1 ml of 0.1 N HCl and 10 ml of Patterson-Greene scintillation fluid (39) to determine the distribution of ^3H (open areas) and ^{32}P (cross-hatched areas). Phosphate (Pi) and 3,4-dihydroxybutyl-1-phosphonate (4CP) standards had R_f values, respectively, of 0.54 and 0.80 in this system. These two compounds were visualized with a spray reagent for phosphate (45).

FIG 4

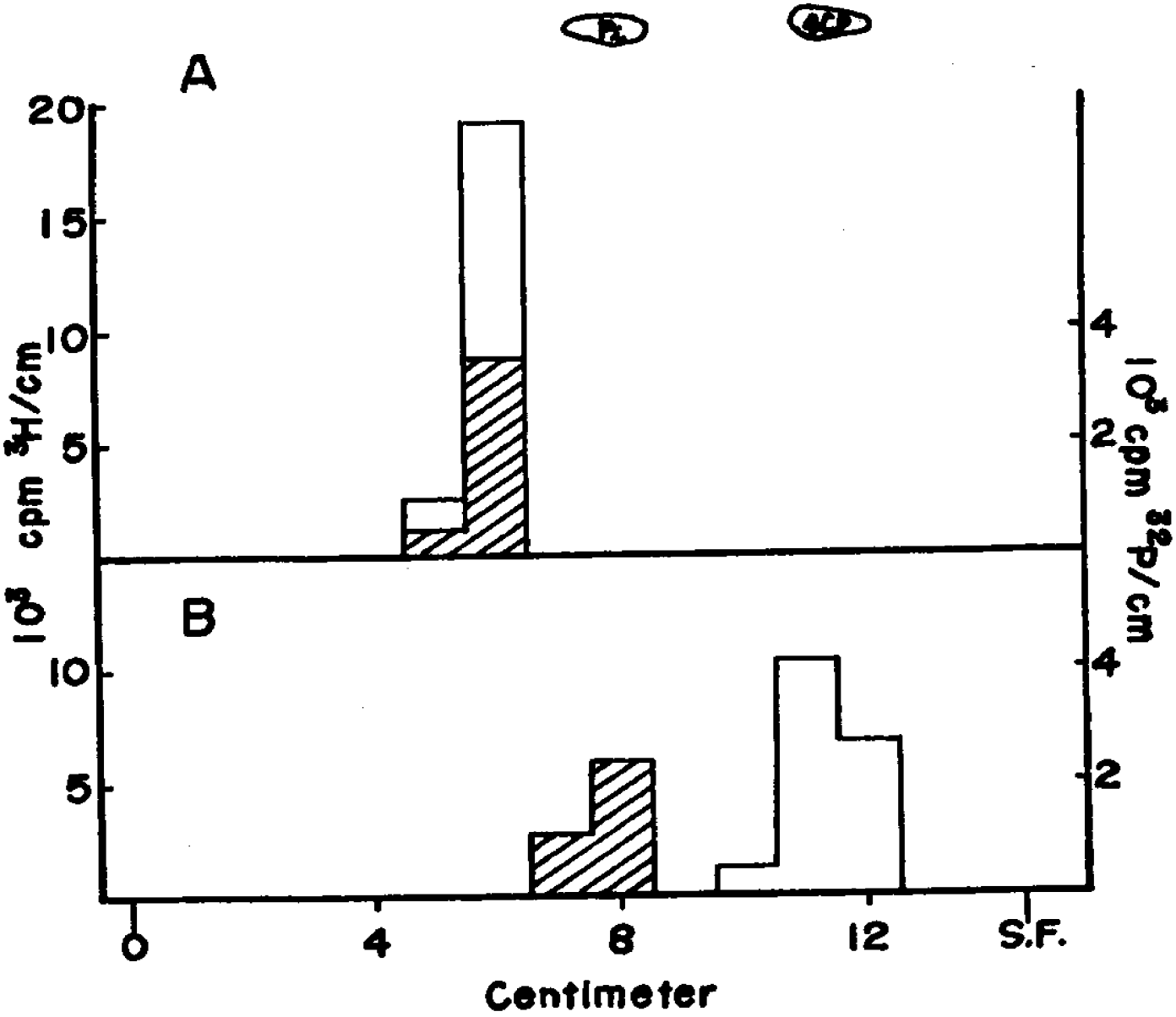

A
B
20
15
10
5
10
5
4
2
4
2
10^3 cpm ^3H/cm
10^3 cpm ^{32}P/cm
0
4
8
12
S.F.
Centimeter

Figure 5: Chromatographic characterization of water soluble phospholipase C hydrolysis products. The experiment was identical to that described in Figure 4, except that the chromatography was performed on Whatman No. 1 paper, using 1 M ammonium acetate (pH 7.1):ethanol (1:1) as the solvent system. Phosphate (Pi) and 3,4-dihydroxybutyl-1-phosphonate (4CP) standards had R_f values, respectively, of 0.46 and 0.70 in this system. The chromatogram was counted as described in Table 3.

FIG 5

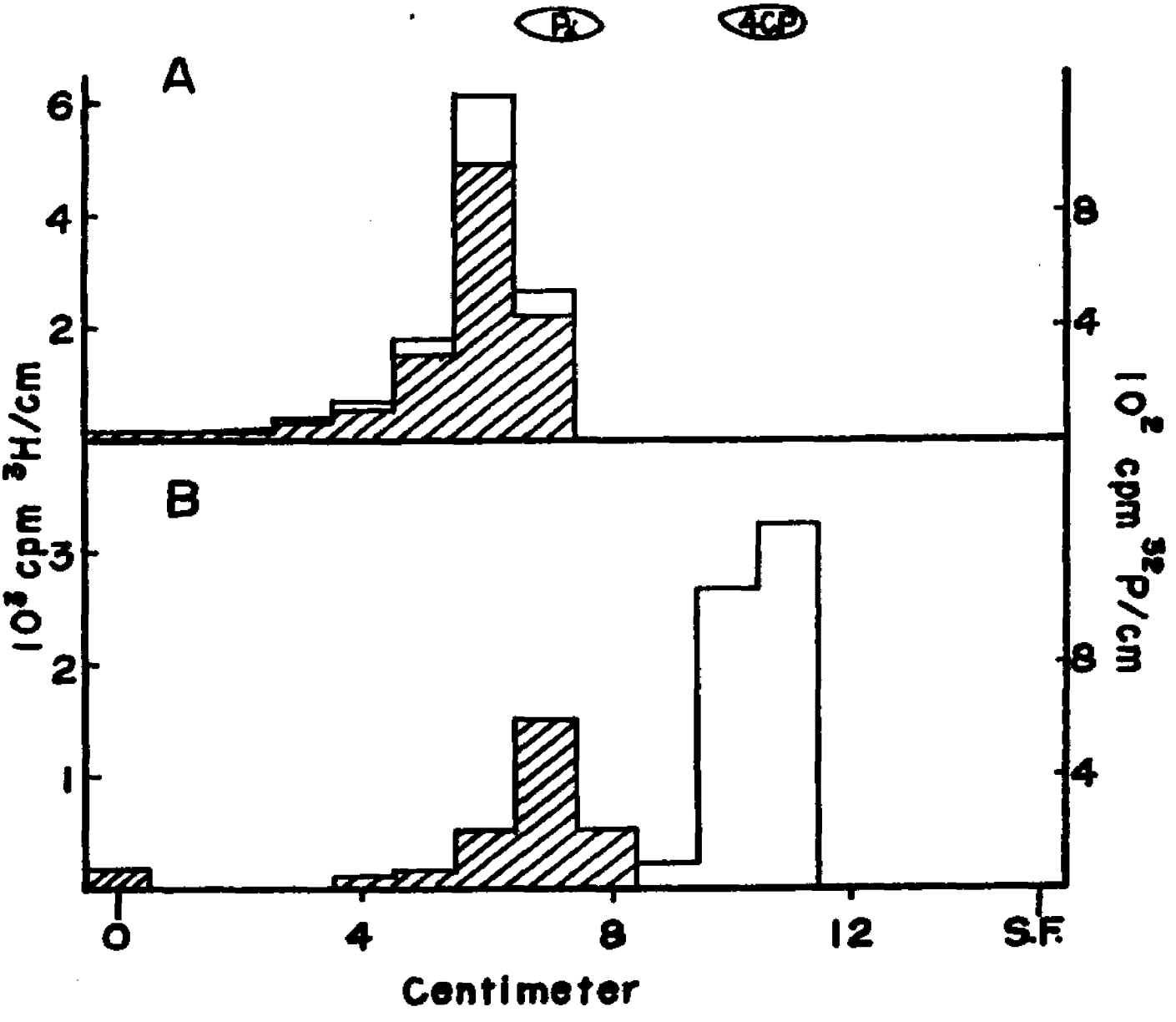

Pc
4CP
A
B
6
4
2
3
2
1
8
4
8
4
10^3 cpm ^{3}H/cm
10^2 cpm ^{32}P/cm
0
4
8
12
S.F.
Centimeter

Figure 6: Enzymatic determination of D-3,4-dihydroxy-[3-^{3}H]butyl-1-phosphonate. The assay mixture contained (0.5 ml total volume): glycine-hydrazine buffer (0.5 M glycine, 0.2 M hydrazine, 2.5 mM EDTA), pH 9.5; 2.5 mM NAD; and the indicated concentration of substrate (rac-3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate, 31 mCi/mmole). The reaction was initiated by the addition of 0.05 mg of rabbit muscle L-glycerol 3-phosphate dehydrogenase (145 U/mg), and the extent of oxidation of rac-3,4-dihydroxy[3-^{3}H]-butyl-1-phosphonate determined as described in the Materials and Methods section.

FIG 6

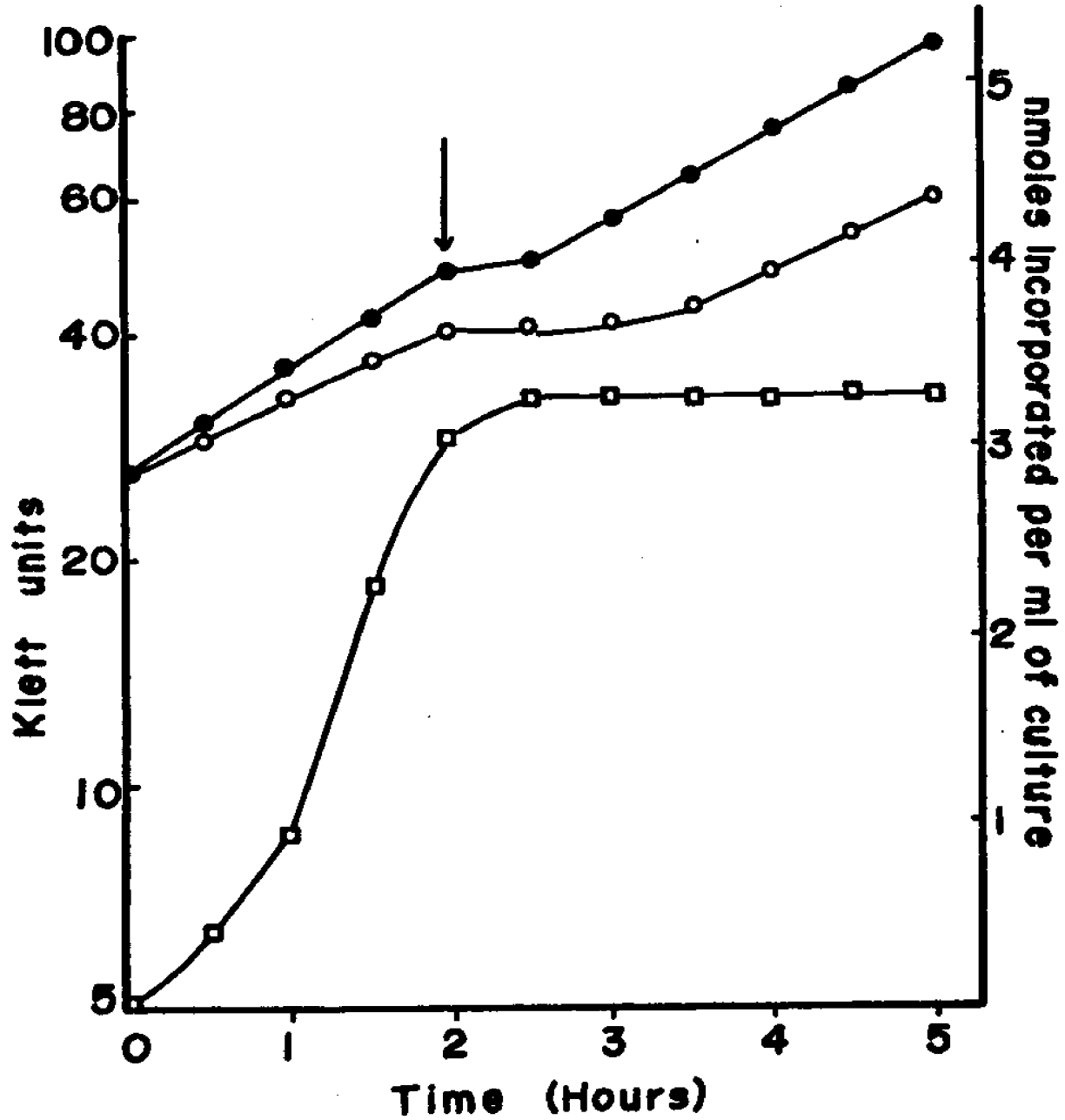
Table 5: Chromatographic analysis of chloroform soluble phospholipase C hydrolysis products. A sample of fraction IV isolated as in Figure 3B, containing 1 x 10^5 cpm ^3H and 1 x 10^4 cpm ^{14}C, was hydrolyzed overnight with B. cereus phospholipase C (in 0.1 M tris-HCl buffer, pH 7.2) as described in the Materials and Methods section. The chloroform soluble products were isolated by the method of Bligh and Dyer (31) and applied to an Anasil G thin layer plate. Dipalmitin, monopalmitin, and palmitic acid were also applied as chromatographic standards, and the chromatogram was developed in either hexane-diethyl ether-acetic acid, 30:70:1 (solvent system 1) or diethyl ether-benzene-ethanol-acetic acid, 40:50:2:0.2 (solvent system 2). After development, 1 cm sections of each lane were scraped into scintillation vials, and counted as previously described (22). Dipalmitin, monopalmitin, and palmitic acid migrated with R_f values, respectively, of 0.50, 0.13, and 0.66 in solvent system 1, and 0.58, 0.20, and 0.50 in solvent system 2. These compounds were visualized with Rhodamine B.

Table 5

Solvent System	% ^{14}C label migrating as: diglyceride	monoglyceride	fatty acid
1	90	5	5
2	90	5	5

Figure 7: Turnover of lipids derived from 3,4-dihydroxy-[3-^{3}H]butyl-1-phosphonate. Forty ml cultures of E. coli strain 8 were incubated with 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (31 mCi/mmole), at a concentration of 0.03 mM, as described in the Materials and Methods section. At 30 minute intervals up to 2 hours, 1 ml samples of culture were removed, and the lipids isolated by the Ames (32) procedure. At the time indicated by the arrow, the cultures were washed on a Millipore filter, and resuspended in fresh media lacking phosphonate. For three additional hours, lipids were extracted from 1 ml culture samples as above. Lipid extracts were counted as described in the Materials and Methods section to determine the incorporation of labeled phosphonate —□— . The growth of treated —○— and untreated —●— cultures was also monitored during the experiment.

FIG 7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40
20
10
5
Klett units
0
1
2
3
4
5
Time (Hours)
5
4
3
2
1
nmoles incorporated per ml of culture

Table 6: Chromatographic characterization of other labeled lipids. Samples of fractions I, II, and III, labeled with ^{3}H, were chromatographed on Anasil G, using the following solvent systems: 1 - chloroform-methanol-water (65:25:3); 2 - chloroform-methanol-acetic acid (65:25:8). The chromatograms were counted as described in Table 1. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) standards migrated with R_f values, respectively, of 0.35, 0.21, and 0.60 in solvent system 1, and 0.32, 0.42, and 0.74 in solvent system 2. The chromatographic behavior of phosphatidylglycerol as two components has been described by other workers, and appears to be an artifact caused by salt effects (32).

Table 6

Distribution of ^{3}H Label

Lipid Fraction	Solvent System 1		Solvent System 2	
	% label at origin:	% label migrating as:	% label at origin:	% label migrating as:
I	1	PE = 99	0	PE = 100
II	36	PG = 64	18	PG = 82
III	5	CL = 95	7	CL = 93

Table 7: Chromatographic analysis of chloroform soluble phospholipase D hydrolysis products. A sample of fraction I (8×10^4 cpm ^3H) was treated with phospholipase D in a biphasic system containing (1.0 ml, total aqueous volume): 0.08 M sodium acetate buffer, pH 5.6; 0.04 M $CaCl_2$; 1 mg of phospholipase D (22 U/mg); and 1 ml of diethyl ether. The sample was shaken on a vortex mixer overnight at 25°C. After evaporation of the ether, the chloroform soluble products were isolated by the procedure of Bligh and Dyer (31). This material was analyzed, along with an untreated sample of Fraction I, by chromatography on Sil-N-HR in the following solvent systems: 1 - chloroform-ethanol-91% formic acid-water (200:20:16:1); 2 - chloroform-pyridine-91% formic acid (50:30:7). The chromatograms were counted as described in Table 1. Phosphatidylethanolamine (PE) and 3,4-dihexadecanoylbutyl-1-phosphonic acid (phosphotidic acid) migrated with R_f values, respectively, of 0.12 and 0.53 in solvent system 1, and 0.22 and 0.76 in solvent system 2. These chromatographic standards were visualized as described in Table 5.

Table 7

Lipid Sample	Solvent System 1		Solvent System 2	
	% ^{3}H label migrating as:		% ^{3}H label migrating as:	
	PE	phosphotidic acid	PE	phosphotidic acid
Fraction I (Untreated)	100	0	100	0
Fraction I treated with Phospholipase D	2	91	1	95

Figure 8: Degradation scheme used to identify the labeled moiety in fraction I (phosphatidylethanolamine). Reaction 1: Hydrolysis of a sample of fraction I (8×10^4 cpm ^3H) with phospholipase D. Reaction 2: Treatment of the chloroform soluble phospholipase D hydrolysis product with mild base to remove the fatty acids. Reaction 3: Treatment of the water soluble deacylation product with alkaline phosphatase. The per cent of the total label in the various products is indicated. The labeled product of reaction 3 was identified a glycerol by chromatography on Whatman No. 1 paper using either n-propanol-ammonia-water (6:3:1) or ethyl acetate-formamide-pyridine (1:2:1) as the solvent system. 3,4-Dihydroxybutyl-1-phosphonate and glycerol had R_f values, respectively, of 0.28 and 0.72 in the former system, and 0.41 and 0.95 in the latter. Chromatograms were counted as described in Figure 5.

FIG 8

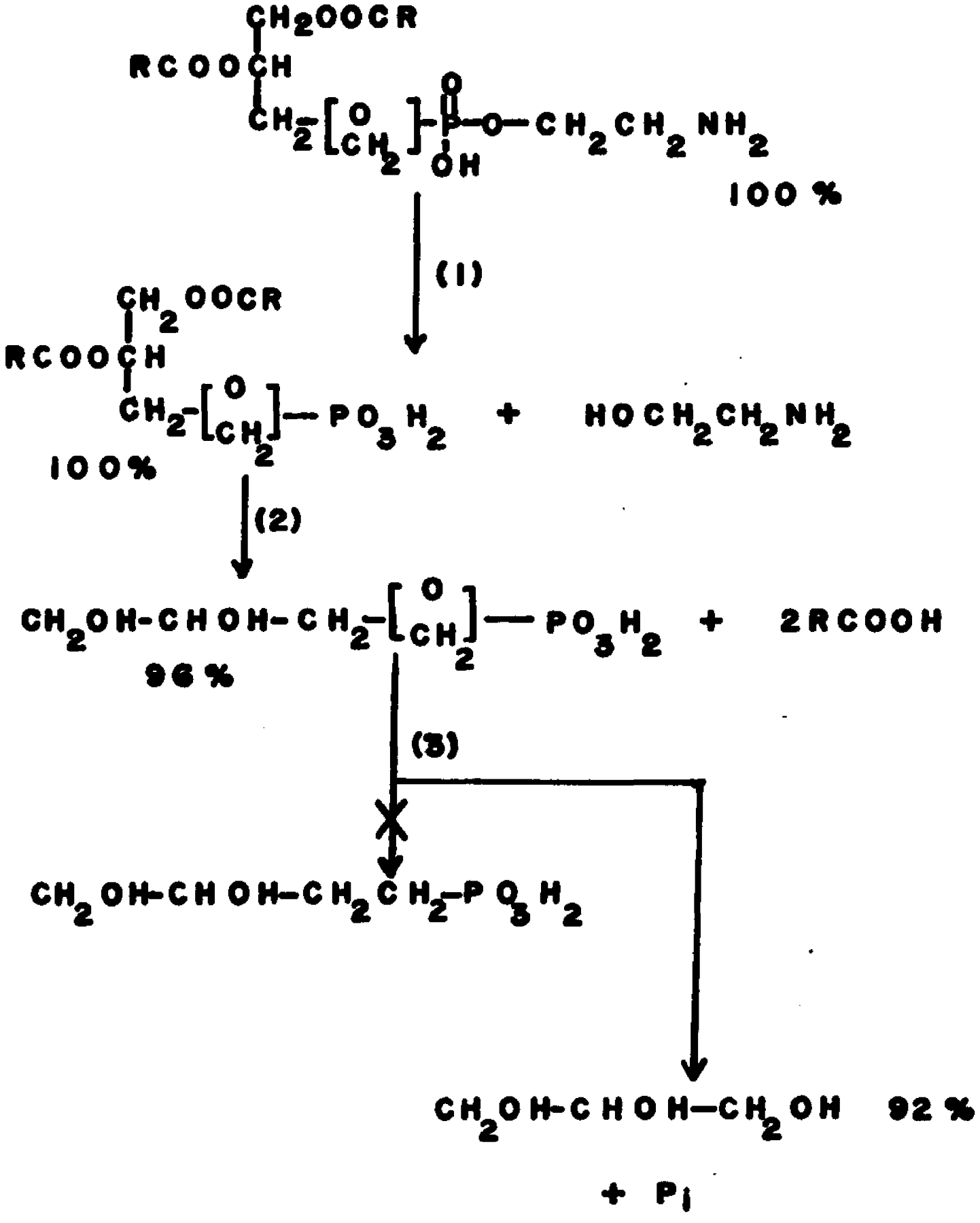

CH2OOCR
RCOOCH
CH2-[O, CH2]-P(=O)(OH)-O-CH2CH2NH2
100 %
(1)
CH2OOCR
RCOOCH
CH2-[O, CH2]-PO3H2 + HOCH2CH2NH2
100 %
(2)
CH2OH-CHOH-CH2-[O, CH2]-PO3H2 + 2RCOOH
96 %
(3)
CH2OH-CHOH-CH2CH2-PO3H2
CH2OH-CHOH-CH2OH 92 %
+ Pi

Figure 9: Degradation scheme used to identify the labeled moiety in fraction II (phosphatidylglycerol). Reaction 1: Hydrolysis of a sample of fraction II (1×10^4 cpm ^3H) with phospholipase C. Reaction 2: Treatment of the chloroform soluble phospholipase C hydrolysis product with mild base to remove the fatty acids. Reaction 3: Treatment of the water soluble phospholipase C hydrolysis product with alkaline phosphatase. The per cent of the total tritium in the products is indicated. The labeled product of reactions 2 and 3 was identified as glycerol as described in Figure 8.

FIG 9

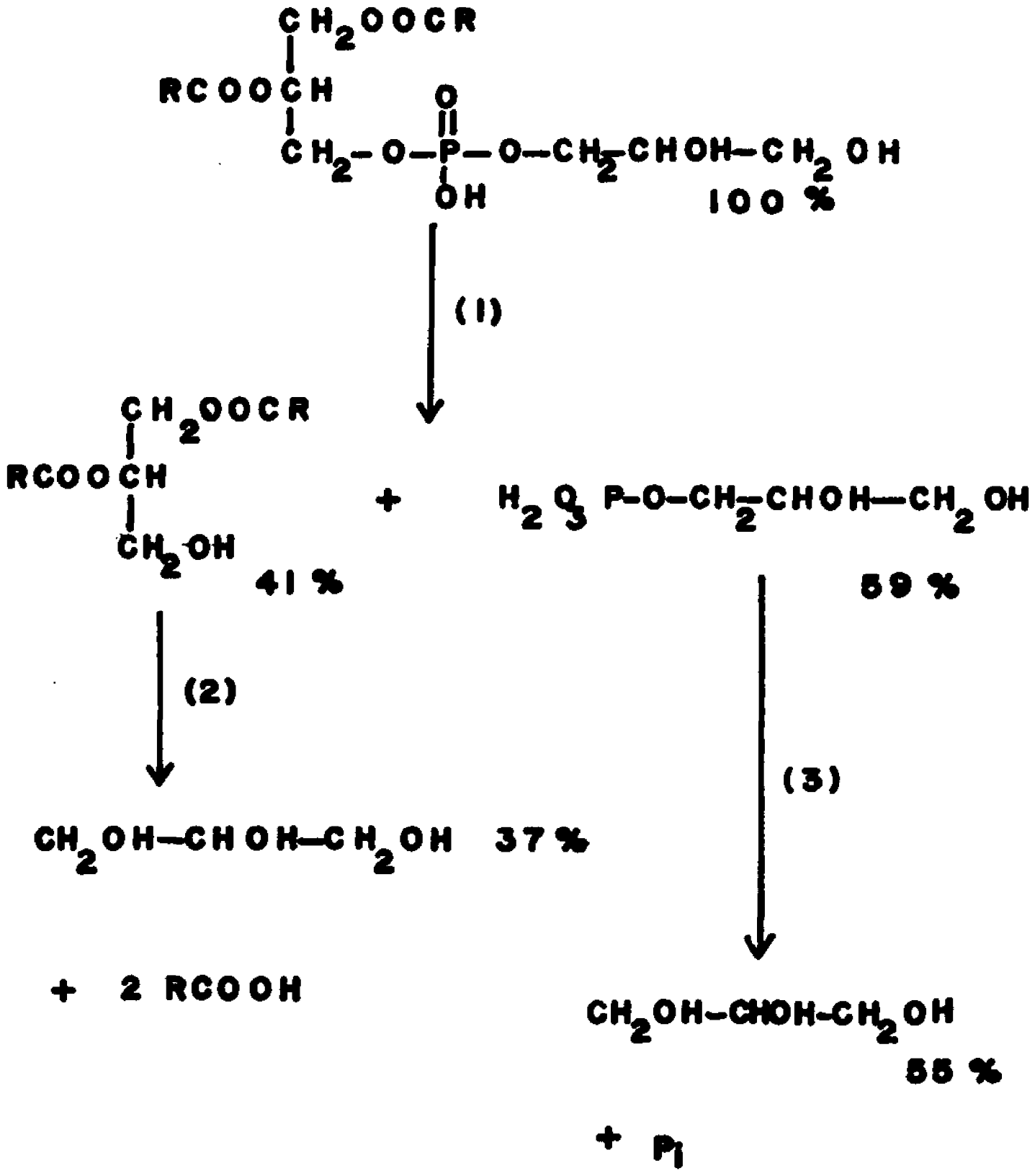
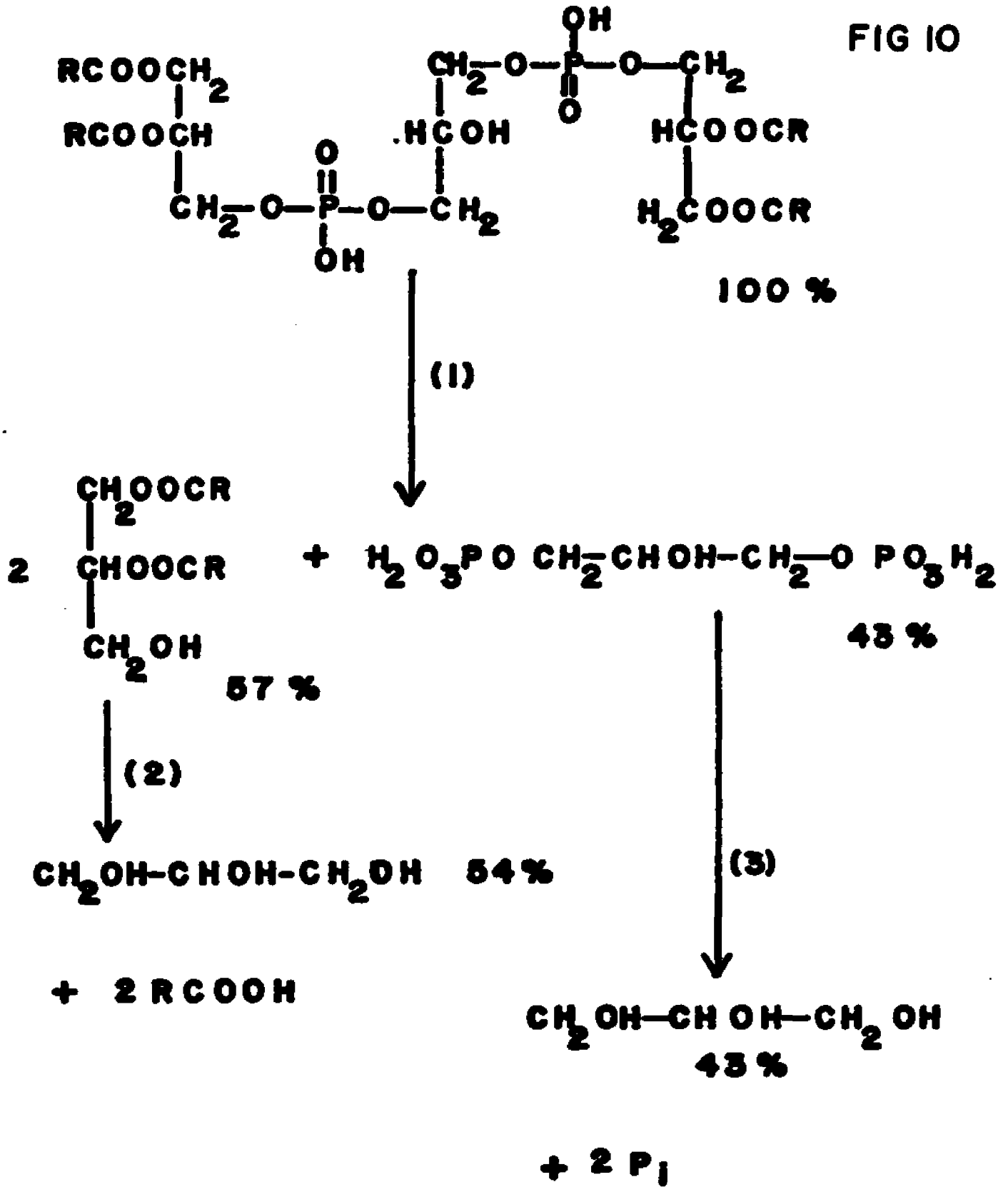

CH2OOCR
RCOOCH
CH2-O-P-O-CH2-CHOH-CH2OH
OH
100 %
(1)
CH2OOCR
RCOOCH
CH2OH
41 %
+
H2O3P-O-CH2-CHOH-CH2OH
59 %
(2)
(3)
CH2OH-CHOH-CH2OH 37 %
+ 2 RCOOH
CH2OH-CHOH-CH2OH
55 %
+ Pi

Figure 10: Degradation scheme used to identify the labeled moiety in fraction III (cardiolipin). A sample of fraction III (1×10^5 cpm ^3H) was treated in a manner identical to that described for fraction II in Figure 9. The per cent of the total label in the various products is indicated. The labeled product of reactions 2 and 3 was identified chromatographically as glycerol as described in Figure 8.

FIG 10



CHAPTER 2

SUBSTRATE ACTIVITY OF PHOSPHONIC ACID ANALOGUES OF CDP-DIGLYCERIDE IN THE SYNTHESIS OF PHOSPHOGLYCERIDES IN *ESCHERICHIA COLI*[1]

Introduction

The availability of phosphonic acid analogues of phosphoglycerides (46-49) and glycerol 3-phosphate (25,37,46, 50) has helped to elucidate the substrate specificities of several enzymes involved in phospholipid and glycerol 3-phosphate metabolism (24,47-49). This chapter reports the synthesis of two new phosphonic acid analogues of cytidine diphosphate-diglyceride (CDP-diglyceride), DL-2-hexadecoxy-3-octadecoxypropylphosphonyl-O-(cytidine 5'-phosphate) and DL-3,4-dioctadecoxybutylphosphonyl-O-(cytidine 5'-phosphate) (I and II respectively in Figure 1). These analogues have been used to examine aspects of the specificity of CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase and CDP-diglyceride:L-serine phosphatidyl transferase obtained from *Escherichia coli*. The analogues are able to serve as substrates for both enzymes. A thin layer chromatographic analysis of the products formed suggests some interesting characteristics of phosphatidylglycerophosphate phosphatase and phosphatidylserine decar-

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1. The studies reported in this chapter have been published: *Biochim. Biophys. Acta* 388, 29-37 (1975)

boxylase.

These *in vitro* experiments were also performed to examine the possible consequences of the acylation of 3,4-dihydroxybutyl-1-phosphonate in *E. coli*, a question which was posed in chapter 1. Such a reaction might allow the synthesis of a compound similar to analogue II *in vivo*. The results indicate that this could cause a further inhibition of phospholipid synthesis.

Materials and Methods

Chemicals: Cytidine diphosphate-DL-dipalmitin (CDP-dipalmitin) was purchased from Serdary Research Laboratories, London, Ontario, Canada. Bacillus cereus phospholipase C, bovine serum albumin, the non-ionic detergent Triton X-100 (octylphenoxypolyethoxyethanol), tris(hydroxymethyl)aminomethane (tris), rac-glycerol 3-phosphate (disodium salt, grade X), DL-serine, and cytidine monophosphate-morpholidate (CMP-morpholidate), were obtained from Sigma Chemical Co., St. Louis, Mo. The "chromatographically pure" bacterial phospholipids, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, were products of Supelco, Inc., Bellefonte, Pa. Bovine phosphatidylserine was obtained from the same source. Phosphatidylglycerophosphate was prepared by treating bacterial cardiolipin with B. cereus phospholipase C as described by DeHaas et al. (34).

Sil-N-HR thin layer plates were purchased from Brinkmann Instruments Inc., Westbury, N.Y. Anasil G thin layer plates were obtained from Analabs, New Haven, Conn. sn-[^{14}C]Glycerol 3-phosphate was a product of New England Nuclear Corp., Boston, Mass. DL-[3-^{14}C]Serine was purchased from ICN Corp., Irvine Calif. All other chemicals were of reagent grade.

Bacteria: E. coli K-12, cultured on enriched media, and

harvested in the stationary phase of growth, were obtained from the Grain Processing Corp., Muscatine, Iowa.

Synthesis of DL-2-hexadecoxy-3-octadecoxypropylphosphonyl-O-(cytidine 5'-phosphate) (I) and DL-3,4-dioctadecoxybutylphosphonyl-O-(cytidine 5'-phosphate) (II) as tris salts[2]: The synthesis of both compounds was identical, employing the respective phosphonic acids, whose preparation has been previously described (51,52). The method used was a modification of the procedure used by Raetz and Kennedy (8) for the synthesis of liponucleotides of natural structure, and is described in detail for analogue II.

A mixture of 3,4-dioctadecoxybutylphosphonic acid (103 μmoles) and CMP-morpholidate (159 μmoles) in anhydrous pyridine (6.0 ml) containing several pellets of molecular sieve (Linde type 3A) was held at 37°C for 72 hours, and then at room temperature for an additional 48 hours. The solvent was removed in vacuo, and the residue partitioned between 25 ml of chloroform-methanol-water (2:3:1) and 40 ml of 0.05 N HCl. The chloroform phase was washed twice with 40 ml of distilled water, and 15 ml of methanol were added, followed by tris base (1 M) to bring the pH to approximately 7. The resulting solution was applied to a 1 x 50 cm column of DEAE cellulose (Whatman DE-52, acetate form). The column was eluted with a linear gradient of

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2. The synthesis was performed by Dr. Arthur Rosenthal of the Department of Laboratories, The Long Island Jewish Medical Center, New Hyde Park, N.Y.

ammonium acetate (0 - 0.2 M) in chloroform-methanol-water (2:3:1), and fractions of 6.0 ml were collected. Fractions 22-39 contained unreacted phosphonic acid. The product emerged in fractions 49-57. These were pooled, washed with HCl, distilled water, and neutralized as above. The solvent was evaporated *in vacuo*. This was followed by repeated dehydration involving re-evaporation after the addition of isopropanol. This yielded 78 mg of product, which was dispersed in water and dialyzed against distilled water at 3°C for 72 hours. The residual material, dehydrated as above, was dissolved in chloroform-methanol (1:1) and centrifuged to remove a small amount of insoluble matter. After the supernatant was evaporated, the product retained a small amount of a red non-polar impurity which was removed by precipitation of the product from chloroform first with acetonitrile, and then with acetone. The final yield of product was 22 mg (64 μmoles; 20 per cent based upon phosphonic acid).

The synthetic analogues of CDP-diglyceride were tested for purity by thin layer chromatography on Anasil G, using the following solvent systems: (A) - chloroform-methanol-water (65:25:3); (B) - chloroform-methanol-concentrated ammonia (60:35:5); and (C) - chloroform-methanol-water-acetic acid (25:15:4:2). In all three systems, both analogues migrated as single spots having identical R_f values (R_f in solvent system A = 0.36; B = 0.18; C = 0.58), as judged by ultraviolet absorption, rhodamine B, and spray

reagents for phosphate (53). Charring with sulfuric acid revealed a barely detectable impurity in the analogue preparations not observed by any of the other methods. Since there was some uncertainty concerning the degree of hydration of the analogues, and the exact quantity of counterions, concentrations were determined spectrophotometrically. Samples were hydrolyzed with 70 per cent perchloric acid for ten minutes in a boiling water bath. After cooling, the samples were diluted and brought to pH 1 by the addition of a calculated amount of KOH. After removing the precipitate by centrifugation, the absorbance of the resulting supernatant was determined at 276 nm. The molar extinction coefficient of cytosine (10.0×10^3 $M^{-1}cm^{-1}$ at 276 nm and pH 1) was used to calculate concentrations of CDP-diglyceride and its analogues. The ultraviolet spectrum of the analogues was as expected for a liponucleotide containing cytosine. Solutions of CDP-dipalmitin and its analogues, stored at -15°C, appeared to be stable for several months.

Preparation of CDP-diglyceride:sn-Glycerol 3-phosphate Phosphatidyl Transferase: Frozen cells of E. coli K-12 (2 g) were washed and suspended in 40 ml of 0.1 M tris-HCl buffer, pH 8.0, containing 10 mM β-mercaptoethanol. The cell suspension was treated as described in chapter 1. The 40,000 x g pellet, containing the particulate enzyme, was resuspended at a concentration of 0.9 mg protein per

ml. A similar preparation, containing 3.2 mg protein per ml, was used as the source of phosphatidylserine decarboxylase.

Preparation of CDP-diglyceride:L-Serine Phosphatidyl Transferase: Frozen cells of E. coli K-12 (5 g) were washed and suspended in 20 ml of 0.01 M tris-HCl buffer, pH 8.0, containing 1 mM $MgCl_2$. The cell suspension was treated as described for CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase. The 40,000 x g supernatant was centrifuged at 100,000 x g for 5 hours to sediment the ribosome-bound CDP-diglyceride:L-serine phosphatidyl transferase described by Raetz and Kennedy (10). The pellet was resuspended in 0.01 M tris-HCl buffer, pH 8.0, containing 1 mM $MgCl_2$, at a concentration of 2.0 mg protein per ml.

All manipulations performed during the preparation of enzymes were at 0-4°C. Protein concentrations were determined by the method of Lowry et al. (33), using bovine serum albumin as the standard.

Assay for CDP-diglyceride:sn-Glycerol 3-phosphate Phosphatidyl Transferase: The assay for CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase, based upon the conversion of sn-[^{14}C]glycerol 3-phosphate into chloroform soluble material, was a modification of that described by Chang and Kennedy (4). The assay mixture con-

tained: 0.25 M tris-HCl buffer, pH 8.5; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol; 2 mg per ml Triton X-100; 0.8 mM sn-[^{14}C]glycerol 3-phosphate (2.6 μCi/umole); 45 μg of particulate enzyme protein; and the indicated concentration of either CDP-dipalmitin or one of its analogues; in a final volume of 0.25 ml. Assays were performed at 37°C, and initiated by the addition of enzyme. At 0, 10, 20, and 30 minutes, a 50 μl sample was removed from the incubation mixture, and placed in a screw-cap test tube containing 0.35 ml of distilled water, and 1.5 ml of chloroform-methanol (1:2). After mixing on a vortex mixer, the resulting monophasic system was rendered biphasic according to the procedure of Bligh and Dyer (31). The aqueous layer was carefully removed, and the chloroform layer washed once with 1 ml of 2 M KCl, and twice with 1 ml of distilled water. A 0.7 ml sample of the chloroform layer was pipetted into a scintillation vial, and the chloroform removed under a stream of warm air. The sample was counted as previously described (22).

Chromatographic Characterization of Reaction Products of CDP-diglyceride:sn-Glycerol 3-phosphate Phosphatidyl Transferase: Incubations were performed as described above in a final volume of 0.25ml. The liponucleotide concentration was fixed at 0.07 mM. After 30 minutes, 0.15 ml of distilled water, and 1.5 ml of chloroform-methanol (1:2) were added to the incubation mixture, and the chloroform soluble

material isolated as above. The chloroform extracts were reduced to a small volume under a stream of nitrogen, and applied to a Sil-N-HR thin layer sheet. Authentic phosphatidylglycerophosphate and phosphatidylglycerol were also applied, and the chromatogram was developed in chloroform-methanol-water (65:25:3). After air drying, each lane was cut into 1 cm sections which were placed in scintillation vials and counted as previously described (22).

Only two products were formed when CDP-dipalmitin was used as substrate. One of the products migrated with an R_f of 0.16, identical to that of phosphatidylglycerophosphate. The other product migrated with an R_f of 0.40, identical to that of authentic phosphatidylglycerol. When the analogues were used as substrates, two products were also formed, which had R_f values similar to those formed with CDP-dipalmitin.

Assay of CDP-diglyceride:L-Serine Phosphatidyl Transferase: CDP-diglyceride:L-serine phosphatidyl transferase activity was determined by measuring the conversion of DL-[3-^{14}C]serine into chloroform soluble material in a manner similar to that described by Kanfer and Kennedy (9). The incubation mixture contained: 0.04 M tris-HCl buffer, pH 8.0; 0.1 M sodium sulfate; 2 mg per ml Triton X-100; 2 mM DL-[3-^{14}C]serine (0.5 μCi/μmole); 32 μg of ribosomal enzyme protein; and the indicated concentration of CDP-dipalmitin or one of its analogues; in a final volume of 0.17

ml. Incubations were performed at 37°C, and the assays were initiated by the addition of enzyme. At various times up to 20 minutes, 50 µl samples were withdrawn from the reaction mixture, and treated as described for CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase.

Chromatographic Characterization of Reaction Products of CDP-diglyceride:L-Serine Phosphatidyl Transferase: Incubations were performed in screw-cap test tubes essentially as described above, in a final volume of 0.17 ml. The liponucleotide concentration was fixed at 0.6 mM, and the reaction mixture contained 96 µg of ribosomal enzyme protein. Incubations were performed with and without an additional 100 µg of a particulate preparation enriched for phosphatidylserine decarboxylase activity. After 20 minutes of incubation, 0.23 ml of distilled water and 1.5 ml of chloroform-methanol (1:2) were added to the reaction mixture. The chloroform extractable material was isolated and treated as described for CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase and applied to an Anasil G thin layer plate. Phosphatidylserine and phosphatidylethanolamine standards were also applied, and the chromatogram developed in chloroform-methanol-concentrated ammonia (60:35:5). The chromatogram was air dried, and 1 cm sections of each lane were scraped into scintillation vials. The samples were counted as previously described (22).

Only two products were observed when CDP-dipalmitin was the substrate. One product had an R_f of 0.05, while the other had an R_f of 0.53. These R_f values were identical to those found for phosphatidylserine and phosphatidylethanolamine, respectively. When the analogues were substituted for CDP-dipalmitin in the reaction mixture, two products were also formed. The R_f values of these products were very similar to those of the products formed with CDP-dipalmitin.

In the assays described above, no activity was obtained for either enzyme unless CDP-dipalmitin or its analogues were included in the incubation mixtures. Reaction rates varied linearly with enzyme concentration and time within the reported ranges. Although nonsaturating concentrations of sn-glycerol 3-phosphate and DL-serine were used, at the end of the incubation periods, less than ten per cent of the initial concentrations of these substrates had been converted into products. All K_m values are apparent values, and are reported as concentrations of racemic mixtures.

Results

Preliminary experiments indicated that both analogues of CDP-diglyceride could serve as substrates for CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase and CDP-diglyceride:L-serine phosphatidyl transferase. Figure 2 is a Lineweaver-Burk plot (54) for CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase. The K_m obtained for CDP-dipalmitin was 0.044 mM. This value is in agreement with that reported by Chang and Kennedy (4). Similar K_m values were obtained for the analogues; analogue II had a K_m of 0.060 mM, and analogue I, a K_m of 0.080 mM. The V_{max} for all three substrates was identical.

Phosphatidylglycerophosphate phosphatase is found in particulate preparations of CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase (5). It was therefore not surprising to find labeled materials which exhibited chromatographic behavior identical to that of phosphatidylglycerophosphate and phosphatidylglycerol, when CDP-dipalmitin was the substrate (Figure 3). When the analogues replaced CDP-dipalmitin, two products (presumably the corresponding analogues of phosphatidylglycerophosphate and phosphatidylglycerol) were also found. However, the ratios of the more polar to the less polar product changed. The major product formed with the analogues of CDP-diglyceride migrated as might be expected for the analogues of phosphatidylglycerophosphate. Figure 3 suggests that the

phosphonic acid analogues of phosphatidylglycerophosphate are less acceptable substrates of the phosphatase than is the natural substrate.

When the initial velocity of the reaction catalyzed by CDP-diglyceride:L-serine phosphatidyl transferase was studied as a function of the CDP-dipalmitin concentration, inhibition of the enzyme was observed at concentrations above 1.2 mM (Figure 4). These results are similar to those reported by Kanfer and Kennedy (9). A K_m equal to 1.4 mM was extrapolated from the Lineweaver-Burk plot (54). Figure 4 also illustrates the results obtained when the concentrations of the analogues were varied in a similar manner. The analogues had lower K_m values than CDP-dipalmitin; analogue II had a K_m of 0.40 mM, and analogue I, a K_m of 0.80 mM. The V_{max} for both analogues was identical, but approximately ten-fold lower than that observed for the natural substrate. These results indicate that the enzyme has a greater affinity for the analogues than CDP-dipalmitin.

The presence of two products in the incubation mixtures containing the ribosomal CDP-diglyceride:L-serine phosphatidyl transferase was also expected, since enzyme prepared in this manner may also contain membrane fragments (10). As shown in Table 1, sufficient phosphatidylserine decarboxylase was present in the enzyme preparation to convert a majority of the phosphatidylserine produced to phosphatidylethanolamine. Increasing the amount of decar-

boxylase made this conversion almost quantitative. However, when the same quantity of decarboxylase was added to incubation mixtures containing the analogues, the major product was the corresponding analogue of phosphatidylserine. The results presented in Table 1 are similar to those obtained with phosphatidylglycerophosphate phosphatase, and suggest that the presumed analogues of phosphatidylserine are poor substrates for the decarboxylase.

Discussion

When the structures of the analogues of CDP-diglyceride are compared to that of the natural compound, three important differences are evident. The major difference is that the glycerol 3-phosphate backbone has been replaced by a phosphonic acid moiety. The chain length of this phosphonic acid backbone has also been varied from three to four carbon atoms. Moreover, the analogues are ethers rather than esters. In view of these differences in structure, the CDP-diglyceride analogues should prove to be interesting tools for probing the substrate specificities of the enzymes involved in phosphoglyceride metabolism in *E. coli*.

It is remarkable that both analogues can function as well as CDP-dipalmitin as substrates for CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyl transferase. The specificity of this enzyme is such that all three changes in structure are readily tolerated. In contrast to this, CDP-diglyceride:L-serine phosphatidyl transferase appears to have a stricter substrate structure requirement, as both analogues had lower maximum velocities than the natural substrate. The fact that both analogues had the same V_{max} suggests that either the phosphonic acid group, or the ethers, is responsible for the low activity of these compounds. It is not immediately apparent why the analogues should have a greater affinity for the enzyme than the

natural substrate, although the increased hydrophobicity of the analogue may be an important factor.

Although these results were obtained with crude enzyme preparations, similar results would be expected with purified enzymes. Raetz and Kennedy (11) have reported that the catalytic characteristics of CDP-diglyceride:L-serine phosphatidyl transferase do not change greatly upon purification. Furthermore, the K_m obtained for CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase is in agreement with that reported by Chang and Kennedy (4) using a partially purified preparation.

Thin layer chromatographic analyses of the products of the enzymatic reactions described above suggest that phosphatidylglycerophosphate phosphatase and phosphatidylserine decarboxylase possess a certain degree of substrate specificity. This possibility certainly warrants a closer study with purified enzymes and substrates, since the specificity of the last step in the synthesis of phosphatidylglycerol and phosphatidylethanolamine may play an important physiological role in E. coli.

The substrate specificities of CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase and CDP-diglyceride:L-serine phosphatidyl transferase have also been investigated by varying other aspects of substrate structure. Phosphonic acid analogues of glycerol 3-phosphate have been examined as substrates for CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase.

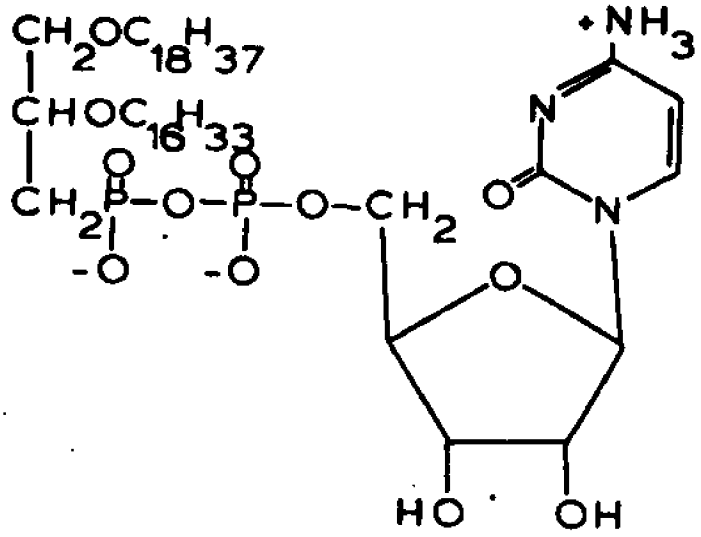
3,4-Dihydroxybutyl-1-phosphonate was found to be both a substrate and a competitive inhibitor with respect to glycerol 3-phosphate (24). 2,3-Dihydroxypropyl-1-phosphonate was a much poorer inhibitor than the four carbon analogue. Raetz and Kennedy (8) have investigated the effect of varying the nucleotide composition of CDP-diglyceride. Liponucleotides that did not contain cytosine were found to be poor substrates for both CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase and CDP-diglyceride:L-serine phosphatidyl transferase. When the substrate activities of dCDP-diglyceride and CDP-diglyceride were compared, dCDP-diglyceride was found to be a better substrate for both enzymes at concentrations below 0.1 mM.

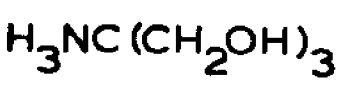
The inability of 3,4-dihydroxybutyl-1-phosphonate to be acylated *in vitro* (24) and *in vivo* (chapter 1) precludes the formation of a compound similar to II in *E. coli*. Even if such an acylation reaction were possible, it is not known whether the products would serve as substrates in the synthesis of CDP-diglyceride. However, the results presented here indicate that an analogue of CDP-diglyceride derived from 3,4-dihydroxybutyl-1-phosphonate would probably inhibit phospholipid synthesis, with a marked effect on phosphatidylethanolamine. Therefore, the acylation, if it were to occur, could possibly permit further perturbations of phosphoglyceride metabolism by 3,4-dihydroxybutyl-1-phosphonate. It may be possible to isolate mutants

of *E. coli* in which this compound is a substrate for acyl CoA:*sn*-glycerol 3-phosphate acyl transferase. Studies involving the alteration of phospholipid synthesis in these strains by 3,4-dihydroxybutyl-1-phosphonate may lead to a greater understanding of the role of specific phospholipids in membrane function.

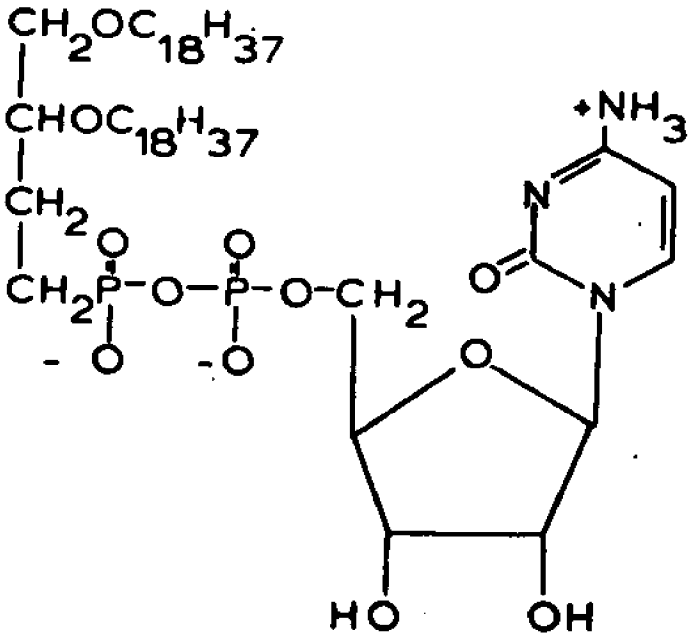
Figure 1: Structures of DL-2-hexadecoxy-3-octadecoxypropylphosphonyl-O-(cytidine 5'-phosphate) (I), and DL-3,4-dioctadecoxybutylphosphonyl-O-(cytidine 5'-phosphate) (II). The compounds were isolated as the tris salts.

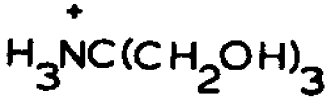
FIG 1





(I)





(II)

Figure 2: Double reciprocal plot for the conversion of sn-[^{14}C]glycerol 3-phosphate into chloroform extractable material by CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase. The assay mixture (0.25 ml, final volume) contained: 0.25 M tris-HCl buffer, pH 8.5; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol; 2 mg per ml Triton X-100; 0.8 mM sn-[^{14}C]glycerol 3-phosphate (2.6 μCi/μmole); 45 μg of particulate enzyme protein; and the indicated concentrations of either CDP-dipalmitin ——●——, analogue II ——○——, or analogue I ——△——. The reaction was initiated by the addition of enzyme. The initial velocity (v), in μmoles per minute, was calculated from the incorporation of labeled sn-glycerol 3-phosphate during a 30 minute period as described in the Materials and Methods section.

FIG 2

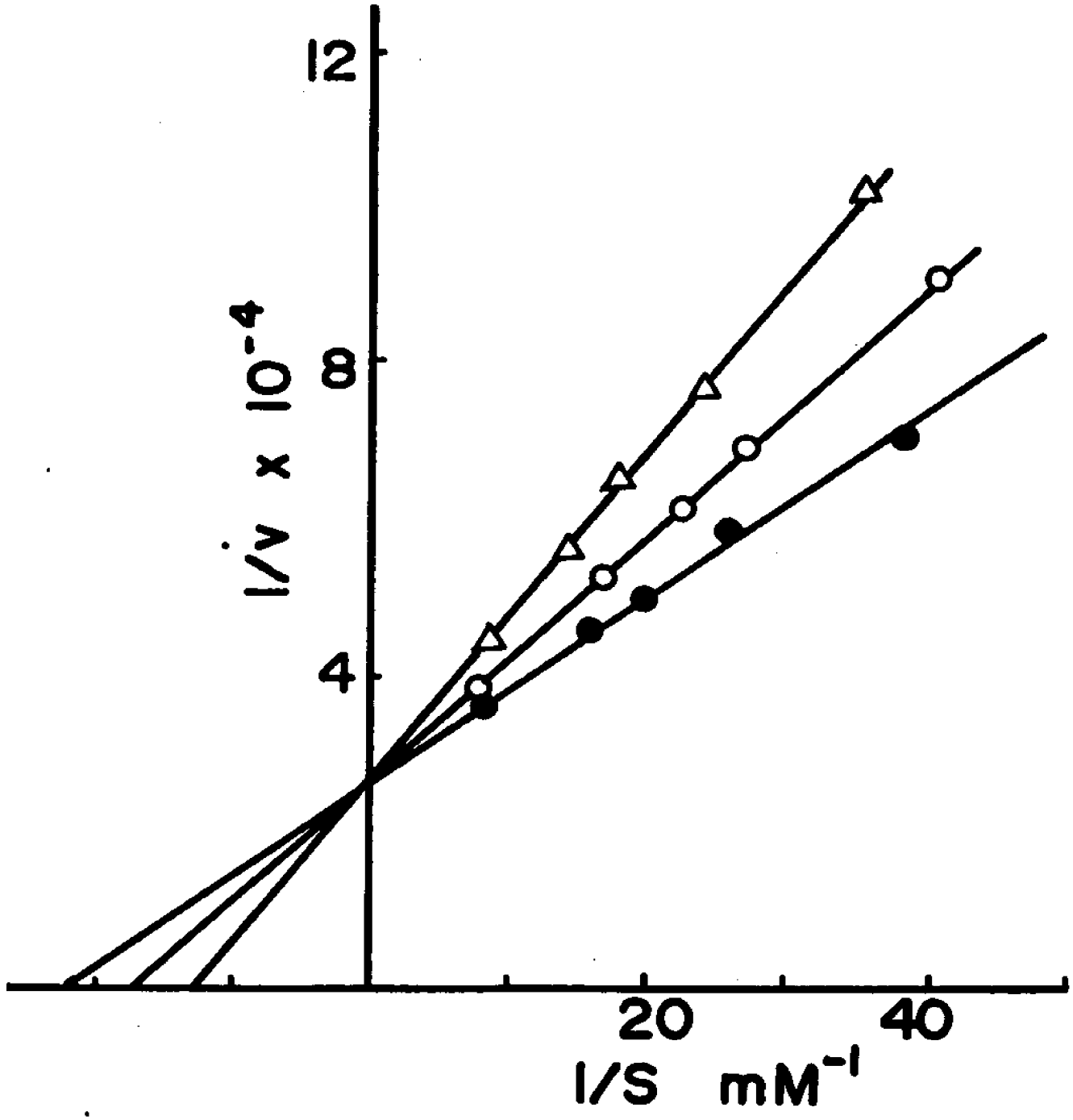


Figure 3: Chromatographic characterization of the products of CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyl transferase. Incubations were performed as in Figure 2, with the concentrations of CDP-dipalmitin (A), analogue II (B), and analogue I (C), fixed at 0.07 mM. After an incubation period of 30 minutes, the chloroform soluble material was isolated as described in the Materials and Methods section. The chloroform extracts, phosphatidylglycerophosphate (PGP), and phosphatidylglycerol (PG), were applied to a Sil-N-HR thin layer sheet, and the chromatogram was developed in solvent system A. Each lane was cut into 1 cm sections, and the relative amounts of labeled products determined as described in the Materials and Methods section. The phospholipids used as standards were visualized by exposure to iodine vapors. PGP and PG had R_f values, respectively, of 0.16 and 0.40 in this solvent system. The phosphatidylglycerophosphate used as a chromatographic standard was prepared by incubating 1 mg of bacterial cardiolipin with 5 units of B. cereus phospholipase C for 2 hours in a biphasic system consisting of 1 ml of diethyl ether and 0.5 ml of 0.1 M tris-HCl buffer, pH 7.2. After evaporation of the ether, the lipid products were isolated by the method of Bligh and Dyer (31).

FIG 3

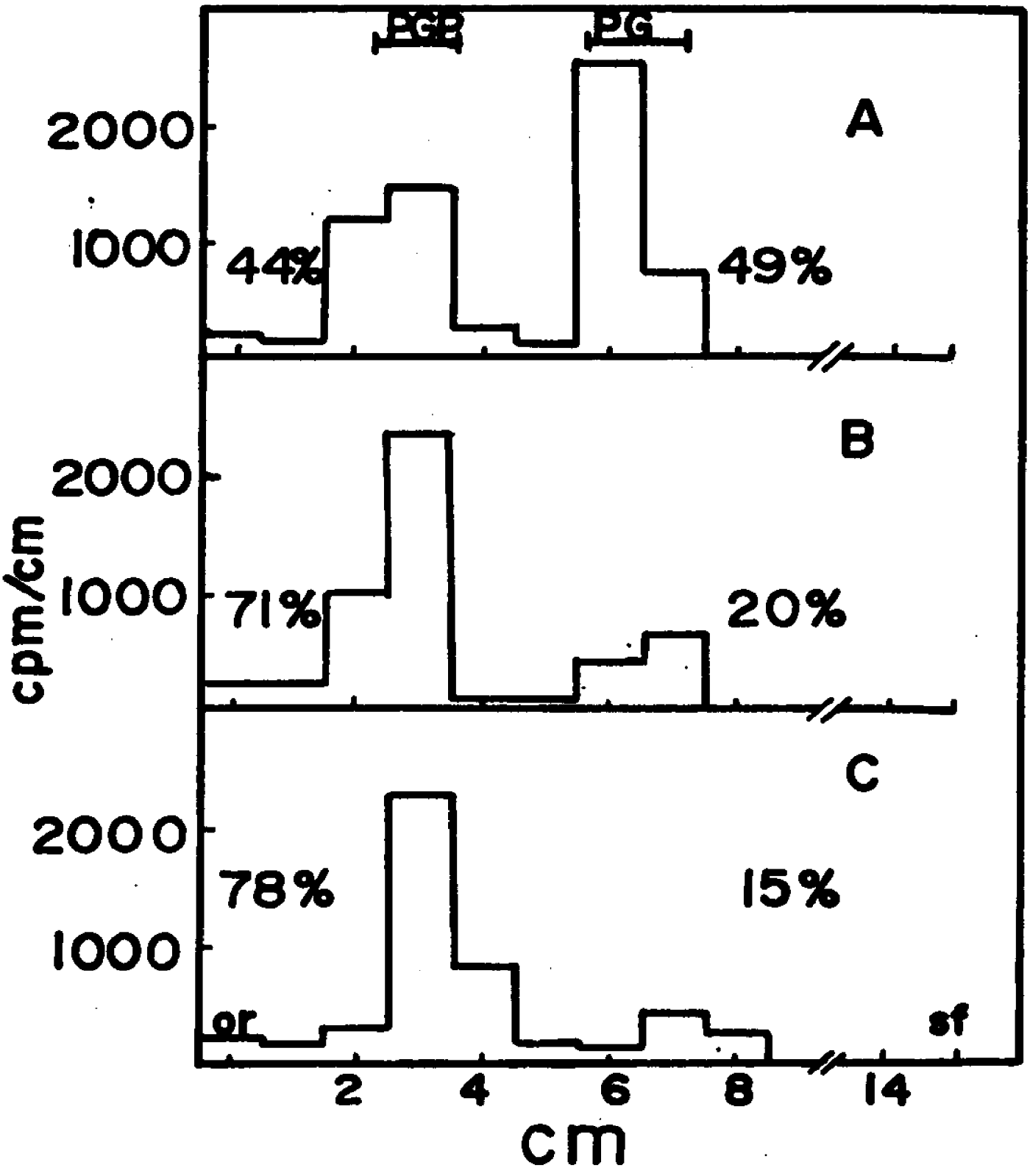

PGB
PG
A
2000
1000
44%
49%
B
2000
1000
71%
20%
cpm/cm
C
2000
1000
78%
15%
or
sf
2
4
6
8
14
cm

Figure 4: Double reciprocal plot for the conversion of DL-[3-^{14}C]serine into lipid material by CDP-diglyceride:L-serine phosphatidyl transferase. The assay mixture (0.17 ml, final volume) contained: 0.04 M tris-HCl buffer, pH 8.0; 0.1 M sodium sulfate; 2 mg per ml Triton X-100; 2 mM DL-[3-^{14}C]-serine (0.5 μCi/μmole); 32 μg of enzyme protein; and the indicated concentrations of CDP-dipalmitin ——●——, analogue II ——○——, or analogue I ——△——. The reaction was initiated by the addition of enzyme. The initial velocity, expressed in μmoles per minute, was calculated from the incorporation of labeled DL-serine during a 20 minute period as described in Figure 2.

FIG 4

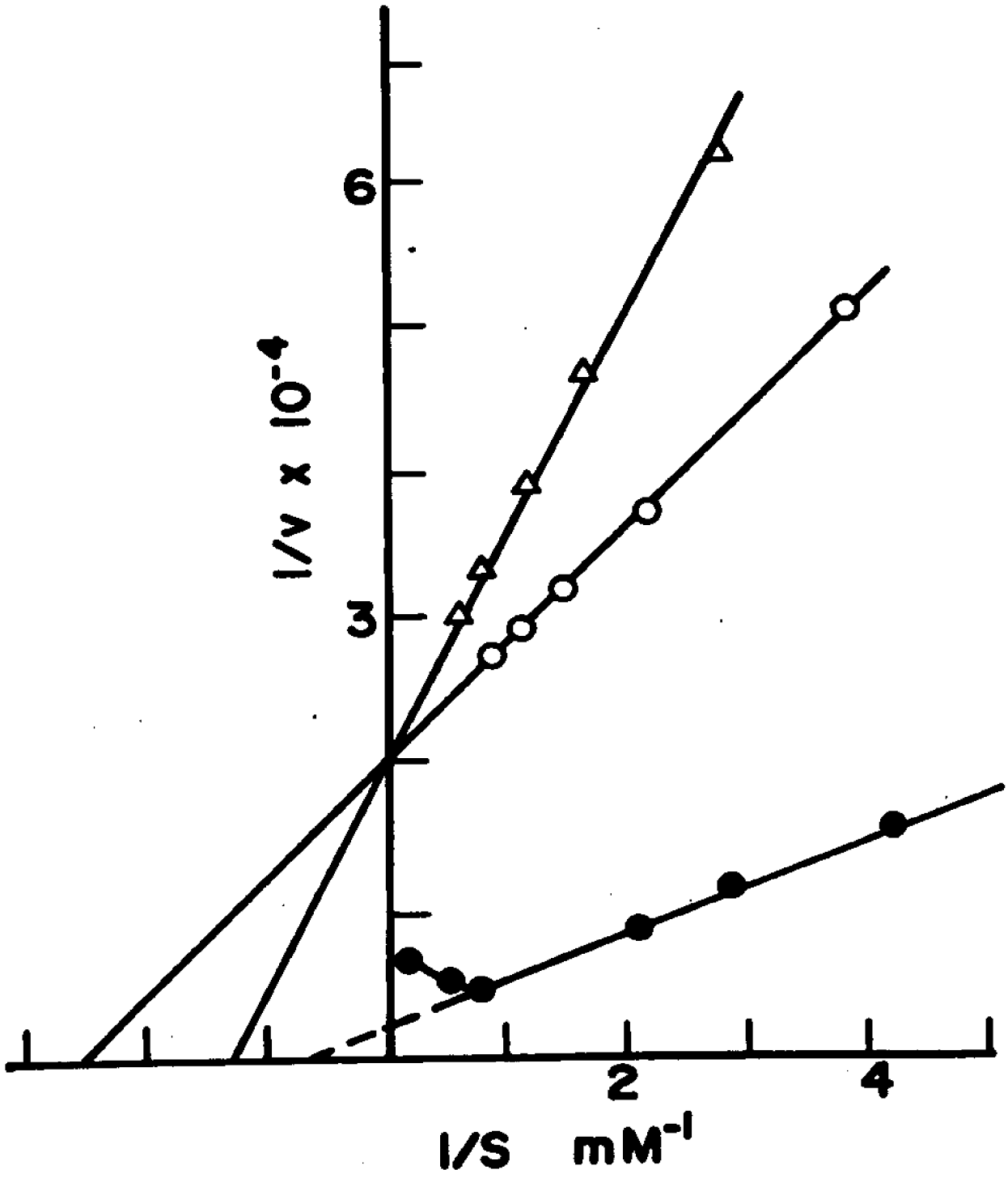


Table 1: Chromatographic characterization of the products of CDP-diglyceride:L-serine phosphatidyl transferase. Incubations contained 96 μg of ribosomal enzyme protein, and were performed as in Figure 4, with the concentrations of CDP-dipalmitin and its analogues fixed at 0.6 mM. Some incubation mixtures contained, in addition, 100 μg of a particulate preparation enriched for phosphatidylserine decarboxylase activity. Chloroform extractable material was isolated as in Figure 3 after a 20 minute period, and applied to an Anasil G thin layer plate, with phosphatidylserine and phosphatidylethanolamine as standards. After developing in solvent system B, the relative amounts of labeled products were determined, and the phospholipid standards visualized, as in Figure 3. Authentic phosphatidylserine (PS) and phosphatidylethanolamine (PE) had R_f values of 0.05 and 0.53, respectively, in this solvent system.

Table 1

INCUBATION CONDITIONS

	- additional decarboxylase		+ additional decarboxylase	
	% cpm migrating as or similar to:		% cpm migrating as or similar to:	
	PS	PE	PS	PE
SUBSTRATE				
CDP-dipalmitin	23	77	7	93
Analogue II	85	15	74	26
Analogue I	89	11	85	15

APPENDIX A

SCINTILLATION COUNTING IN DOUBLE LABEL EXPERIMENTS

In experiments involving ^{14}C and ^{3}H, the standard non-adjustable channels provided with the Beckman LS-200 scintillation counter for counting ^{14}C and ^{14}C above ^{3}H were used. The ^{14}C channel covered the energy spectrum of both isotopes, while only ^{14}C cpm were recorded in the ^{14}C over ^{3}H channel. Lipid samples were counted in toluene based scintillation fluid (22). By counting such samples, labeled only with ^{14}C, it was found that 72-75% of the ^{14}C cpm were recorded in the ^{14}C over ^{3}H channel (Table 1). This was used to calculate the total ^{14}C content of doubly labeled samples. Since the ^{14}C channel recorded the combined total of ^{3}H and ^{14}C cpm, the ^{3}H cpm were determined as the difference between the total ^{14}C cpm (as calculated from the ^{14}C over ^{3}H channel) and the number of cpm recorded in the ^{14}C channel.

The efficiency of counting ^{14}C and ^{3}H under these conditions was 83% and 54%, respectively, based on standards supplied by Beckman Instruments. The amount of quenching in the standards was very similar to that found in the samples containing labeled lipids.

In experiments in which double labeling was performed with ^{32}P and ^{3}H, samples were counted in 1 ml of water and 10 ml of Patterson-Greene scintillation fluid (39). This change in scintillation fluid was made so that aqueous

samples could be counted and compared to 3,4-dihydroxy-[3-^{3}H]butyl-1-phosphonate and [^{32}P]phosphate standards under the same quenching conditions. The standard ^{32}P channel, encompassing the entire energy spectrum, was employed to determine the combined total of ^{3}H and ^{32}P cpm. However, quenching caused the ^{32}P energy distribution to shift, so that the fixed channel supplied for counting ^{32}P over ^{3}H could not be used. With the aid of a series of samples containing only [^{32}P]phosphate, the variable discriminator on the counter was adjusted in order to define a new ^{32}P over ^{3}H channel. When the lower limit was set at 90 discriminator units, and the upper at infinity, 98-99% of the total ^{32}P cpm were recorded (Table 2), while all ^{3}H cpm were rejected. Since the fixed ^{32}P channel registered the combined total of ^{32}P and ^{3}H cpm, the ^{3}H cpm were calculated as the difference between the cpm in the ^{32}P channel, and the total ^{32}P cpm determined from the new ^{32}P over ^{3}H channel.

Under these conditions, ^{3}H was counted with an efficiency of 32%.

Table 1

cpm ^{14}C in ^{14}C channel	cpm ^{14}C in ^{14}C over ^{3}H channel	% cpm ^{14}C in ^{14}C over ^{3}H channel
174	125	72
858	625	73
1132	825	73
4443	3230	73
9904	7421	75
50903	37973	75

Lipid samples containing only ^{14}C were counted as described in the text to determine the per cent of the total ^{14}C cpm appearing in the ^{14}C over ^{3}H channel.

Table 2

cpm ^{32}P in ^{32}P channel	cpm ^{32}P in new ^{32}P over ^{3}H channel	% cpm ^{32}P in new ^{32}P over ^{3}H channel
332	324	98
670	655	98
1350	1335	99
2790	2741	98
4384	4318	99
9643	9518	99
19542	19246	99
48376	47677	99

Samples containing [^{32}P]phosphate were counted as described in the text to determine the per cent of the total ^{32}P cpm appearing in the newly defined ^{32}P over ^{3}H channel.

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