Biosynthesis of phosphatidyl glycerophosphate in *Escherichia coli*

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ABSTRACT An enzyme (L-glycerol 3-phosphate: CMP phosphatidyltransferase) catalyzing the synthesis of phosphatidyl glycerophosphate from CDP-diglyceride and L-glycerol 3-phosphate has been rendered soluble by treatment of the particulate, membrane-containing fraction of *E. coli* with Triton X-100 and has been partially purified. The enzyme, devoid of phosphatidyl glycerophosphatase activity, is specific for L-glycerol 3-phosphate and is completely dependent upon added Mg^{++} or Mn^{++} for activity. It has high affinity for CDP-diglyceride and can be used for the assay of this nucleotide.

Other properties of the enzyme are also described.

KEY WORDS phosphatidyl · glycerophosphate · CDP-diglyceride · L-glycerol 3-phosphate · enzymatic · biosynthesis · *E. coli* · particulate fraction · Triton · extraction

PHOSPHATIDYL GLYCEROL appears to be the most widely distributed glycerophosphatide, being an important constituent of the lipids of animals, plants, and microorganisms, including the bacteria. Benson and Maruo (1) have called attention to the rapid metabolism of phosphatidyl glycerol in plants, while Kanfer and Kennedy (2) have reported that the pattern of labeling of phosphatidyl glycerol in rapidly growing cultures of *Escherichia coli* suggests either a rapid turnover of this lipid or its conversion to some end product that is not extractable with chloroform–methanol. In contrast, phosphatidyl ethanolamine was quite stable under these experimental conditions. Macfarlane (3) first demonstrated the occurrence of *O*-amino acid esters of phosphatidyl glycerol in *Clostridium welchii*. More recently, Lennarz (4) discovered an enzyme in extracts of *Staphylococcus aureus* that catalyzes the synthesis of *O*-lysyl-phosphatidyl glycerol from phosphatidyl glycerol and lysyl sRNA.

In the light of these findings, a detailed examination of the pathways for the formation and metabolism of phosphatidyl glycerol in bacterial systems appears to be of special interest. In 1963, Kanfer and Kennedy (5) reported that crude particulate fractions derived from *E. coli* catalyzed the synthesis of phosphatidyl glycerol, apparently by the same enzymatic steps (Fig. 1) previously demonstrated in liver mitochondria (6). In the study of the mitochondrial system (6) some evidence was obtained to indicate that separate enzymes are involved in reactions 1 and 2, but the enzymes were neither extracted from the particulate mitochondrial fraction nor purified.

In this report we describe a study of L-glycerol 3-phosphate:CMP phosphatidyltransferase from *E. coli*. This enzyme, which catalyzes reaction 1, has been rendered soluble by treatment of the particulate, membrane-containing fraction with Triton X-100, and has been partially purified and separated from phosphatidyl glycerophosphatase, which catalyzes reaction 2. Some properties of the enzyme have been determined.

MATERIALS AND METHODS

E. coli B cells (a gift of Dr. Standish C. Hartman) were grown commercially on a large scale. Cells were grown at 37°C in rich medium up to the early stationary phase and were harvested by centrifugation. They were immediately frozen and were stored at −20°C until used. The frozen cells appeared to retain the desired enzyme activities for many months. *E. coli* ML 308 cells were grown at 37°C with vigorous shaking, on medium 63 (7) plus

Abbreviations: CMP, cytidine 5′-phosphate; CDP, cytidine 5′-diphosphate.

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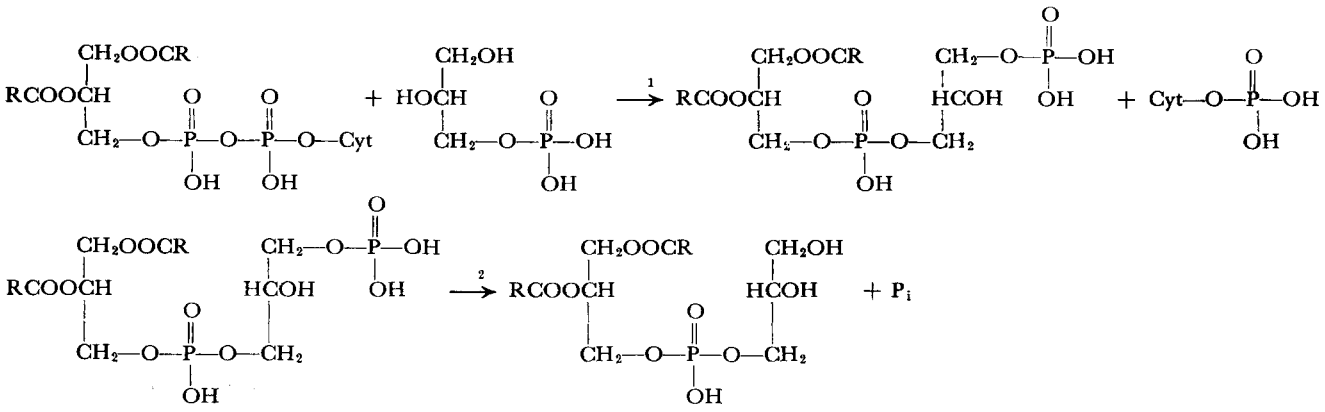

FIG. 1. Enzymatic synthesis of phosphatidyl glycerophosphate and phosphatidyl glycerol (6).

0.015 M potassium succinate as the carbon source. The cells were harvested at log-phase.

L-Glycerol 3-phosphate-2-^{3}H was synthesized enzymatically from glycerol-2-^{3}H by phosphorylation with ATP in the presence of glycerol kinase (EC 2.7.1.30) (8). The incubation mixture contained Tris–HCl buffer of pH 8.0 (0.05 M); glycerol-2-^{3}H (4 mM) purchased from New England Nuclear Corp. with a specific activity of approximately 200 mc/mmole; ATP (20 mM); $MgCl_2$ (20 mM); mercaptoethanol (20 mM); serum albumin (1 mg/ml), and glycerol kinase (0.01 mg/ml); final volume, 5 ml. After incubation at 37°C for 3 hr, the mixture was heated in a boiling water bath for 5 min and centrifuged briefly. The supernatant solution was then passed over a Dowex-1 (formate) column 16 cm in length and 1.3 cm in diameter. The column was first eluted with a few bed volumes of distilled water. Unreacted glycerol-2-^{3}H was not adsorbed on the column and appeared in the first 20 ml of water. Gradient elution was then begun with an arrangement of apparatus similar to that of Hurlbert, Schmitz, Brumm, and Potter (9) with 250 ml of water in the lower mixing chamber, and 4 M formic acid in the upper reservoir. 10-ml fractions were collected, and each fraction was assayed for radioactivity. A single radioactive peak was eluted from the column, usually in fractions 23–28. The peak fractions were pooled and taken to dryness under vacuum in a rotary evaporator at a 10°C bath temperature. The dried material was dissolved in 2–3 ml of distilled water. Aliquots were counted and the content of L-glycerol 3-phosphate was determined by assay with L-glycerol 3-phosphate dehydrogenase (8). The radiochemical purity of the L-glycerol 3-phosphate was checked by paper chromatography in solvent system A (see below), which showed a single radioactive spot with an R_f of 0.27, identical with that of authentic DL-glycerol 3-phosphate.

L-Glycerol 3-phosphate-1,3-^{14}C was prepared, in a similar procedure, from glycerol-1,3-^{14}C.

The following simplified method was used to synthesize L-glycerol 3-phosphate-^{32}P from $^{32}P_i$. An incubation mixture for synthesizing ATP-γ-^{32}P was prepared as described by Glynn and Chappell (10) in a final volume of 10 ml, and incubated for 1 hr at 26°C. At this time, a mixture containing 500 μmoles of unlabeled glycerol, 400 μmoles of mercaptoethanol, 1 mg of serum albumin, and 0.1 mg of glycerol kinase, in a final volume of 1 ml, was added. After incubation for 2 hr longer at 37°C, the solution was directly applied to a Dowex-1 (formate) column (20 cm in length and 1.2 cm in diameter), which was then eluted with a gradient of formic acid as described for the synthesis of L-glycerol 3-phosphate-2-^{3}H. The radioactive glycerophosphate appeared in a peak at fraction 25–30 under these conditions. The yield represented about 80% of the radioactivity added as $^{32}P_i$.

CDP-dipalmitin was prepared from CMP and dipalmitoyl L-glycerol 3-phosphate by the method of Paulus and Kennedy (11).

Glycerol-1,3-diphosphate was synthesized by the method of Kiyasu, Pieringer, Paulus, and Kennedy (6).

Glycerophosphoryl glycerol (GPG) was kindly provided by Dr. J. R. Carter of this laboratory and had been synthesized from glycidol and DL-glycerol 3-phosphate.

Glycerol kinase and L-glycerol 3-phosphate dehydrogenase were obtained from Boehringer and Soehne.

The nonionic detergent Triton X-100 (*t*-octylphenoxypolyoxyethanol) was a product of Rohm & Haas Co.

Superbrite glass beads #100 (average diameter 200 μ) used for enzyme preparation were obtained from the Minnesota Mining & Manufacturing Co. 10 lb. of glass beads was stirred for 3–4 hr with 10 liters of 1.5 N HCl and then washed extensively with tap water until neutral. Finally, the beads were rinsed with distilled water and dried in an oven at 100°C overnight.

The DEAE-cellulose was purchased from Bio-Rad Laboratories and was washed before use with NaOH and HCl according to the method of Peterson and Sober (12). The acetate form of DEAE-cellulose was prepared by the method of Rouser, Kritchevsky, Heller, and Leiber (13).

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (14) and phosphorus by the method of Gomori (15). Fatty acid ester bonds were determined by the method of Stern and Shapiro (16).

The solvent systems for paper chromatography were as follows. (A) (1 M ammonium acetate of pH 7.5)–absolute ethanol 35:65; ascending technique. (B) 1-butanol–acetic acid–water 5:4:1; ascending technique. (C) (Saturated ammonium sulfate)–water–isopropanol 79:19:2; descending technique.

The R_f values of some phosphate esters in each solvent system are shown in Table 1.

Assay of L-Glycerol 3-Phosphate:CMP Phosphatidyltransferase. Two general methods of assay were used in this work. Method I was based on the measurement of the conversion of radioactive L-glycerol 3-phosphate to a lipid, while in method II the release of CMP from CDP-diglyceride in the presence of L-glycerol 3-phosphate and enzyme was measured spectrophotometrically. The incubation mixture, which was the same for both assays, contained Tris–HCl buffer of pH 8.5 (0.25 M), DL-glycerol 3-phosphate (0.8 mM), CDP-dipalmitin Tris salt (0.1 mM), Triton X-100 (2 mg/ml), $MgCl_2$ (10 mM), and mercaptoethanol (5 mM); final volume, 1 ml. When the purified enzyme preparation was used, 1 mg of serum albumin was included in the incubation mixture. The system was incubated at 28°C for 20 min.

When method I was used for assay, radioactive L-glycerol 3-phosphate was used in the incubation mixture. The incubation was carried out in conical-tipped, glass-stoppered vessels of 40 ml capacity. At the end of the incubation period, 5 ml of methanol was added, followed by 10 ml of chloroform. The solution was thoroughly stirred and then filtered through a plug of glass wool into a vessel of the same type. About 20 ml of 2 M $MgCl_2$ was then added and the tightly stoppered vessels were thoroughly shaken. The upper aqueous phase was drawn off and the lower chloroform phase was washed once with 20 ml of water. Aliquots of the washed chloroform extract were taken to dryness in counting vials, to which 10 ml of Buhler's solution (17) was added. The contents were then counted in a liquid scintillation counter. Controls, in which methanol was added immediately after the enzyme, were included in every set of determinations to insure that the removal of labeled L-glycerol 3-phosphate from the washed lipid extract was complete. 1 unit of enzyme activity is that amount which catalyzes the conversion of 1 μmole of L-glycerol 3-phosphate to lipid per min under these conditions.

TABLE 1 R_f VALUES OF PHOSPHATE ESTERS

Compound	Solvent System A	B	C
GP	0.27	0.29	
GPG	0.64	0.24	
GPGP	0.20	0.12	
GPGPG	0.50		
PGP	0.05		
CMP			0.73
Cytidine			0.60

GP, DL-glycerol 3-phosphate; GPG, 3-(DL-3-glycerophosphoryl)-DL-glycerol; GPGP, 1-(L-3-glycerophosphoryl)-L-glycerol 3-phosphate; GPGPG, 1,3-di(DL-3-glycerophosphoryl) glycerol; PGP, glycerol-1,3-diphosphate.

In method II, the enzyme incubation mixture was the same as described above, except that nonradioactive DL-glycerol 3-phosphate was used. At the end of the incubation, 1 ml of 7% (v/v) perchloric acid was added, followed by 0.1 ml of 5% (w/v) serum albumin. The tubes were chilled in ice for 10 min, and the precipitate was removed by centrifugation. The cloudy perchloric acid supernatant solution was then extracted twice with 3 ml of chloroform in order to remove Triton X-100. The treatment reduced the absorbance at 280 mμ of the control samples, to which perchloric acid had been added immediately after the enzyme, to a very low value, while CMP released during the course of the enzymatic reaction was recovered quantitatively. The CMP released was calculated from the difference in absorbance at 280 mμ between the complete system and the controls, a value of 13.6 being used as the mM extinction coefficient. 1 unit of enzyme is that amount which catalyzes the release of 1 μmole of CMP per min under these conditions, and is thus identical with 1 unit of enzyme in assay method I.

RESULTS

Some Properties of the Particulate Enzyme Preparation

In confirmation of the observations of Kanfer and Kennedy (5), the enzyme that catalyzes the synthesis of phosphatidyl glycerophosphate was found to be localized in the particulate fraction sedimented by centrifugation at 100,000 *g* for 1 hr, whereas little activity was observed in the supernatant fraction.

The incorporation of radioactive L-glycerol 3-phosphate into lipids catalyzed by *E. coli* particulate fractions required added CDP-diglyceride and divalent cation. A detergent, Triton X-100, was also required for maximal activity. The reaction product was a mixture of phosphatidyl glycerophosphate and phosphatidyl glycerol.

The effect of Triton X-100 is shown in Fig. 2. The formation of lipid product by the particulate fraction increased until a Triton concentration of 5 mg/ml was reached. Higher concentrations of Triton were inhibitory. In later experiments with a partially purified, soluble enzyme, this inhibitory effect of higher Triton concentrations was not observed.

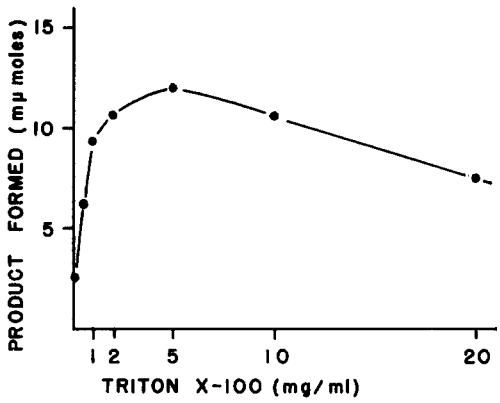

FIG. 2. Effect of Triton X-100. The incubation system (final volume, 1 ml) contained Tris–phosphate buffer of pH 8.0 (250 mM), CDP-dipalmitin Tris salt (0.1 mM), L-glycerol 3-phosphate-1,3-^{14}C (0.2 mM, 2.54 × 10^6 cpm/μmole), $MnCl_2$ (1 mM), mercaptoethanol (5 mM) and the 100,000 *g* particulate fraction derived from sonically disrupted cells (1 mg of protein). The concentration of Triton X-100 was varied as shown. The system was incubated for 20 min at 27°C. The amount of radioactive lipid formed was determined by method I as described in the text.

The requirement for cation is shown in Table 2. Manganese was more effective than magnesium at low concentrations, but higher concentrations were somewhat inhibitory and led to the formation of precipitates. For this reason, magnesium was routinely used in later studies. Ca^{++} and Zn^{++} produced little activation.

Extraction and Purification of Enzyme

All steps were carried out at 0°C unless otherwise noted.

Preparation of Blendor Extract. Partially thawed cells of *E. coli* B (1 kg) were suspended in 660 ml of ice-cold buffer A (0.1 M Tris of pH 8.0, 0.01 M in mercaptoethanol) in a large Waring Blendor (model CB 4). Stirring was begun at low speed and 3 Kg of washed glass beads was added gradually. When the mixture was homogeneous, the speed of the blendor was increased. Stirring was then continued for 30 min. The operation was done in a cold room and the temperature of the blendor extract was maintained below 10°C by means of cold tap water running through the blendor jacket. At the end of this period, the speed of the blendor was reduced and 1340 ml of ice-cold buffer A were added gradually. Stirring was continued at moderate speed for another 10 min. After the beads had been allowed to settle for 10 min, the supernatant solution was decanted. To the sediment, another 500 ml of buffer A was added, and the mixture was stirred for 10 min. After the beads had settled, this supernatant solution was decanted and combined with the first to give the total blendor extract.

Differential Centrifugation and Extraction of Enzyme. The scheme for fractionation and extraction of the enzyme is shown in Fig. 3.

The total blendor extract was first centrifuged at 3,000 *g* for 15 min and the supernatant solution (supernatant 1) was decanted. The sediment (residue 1), which contained some unsettled beads and whole cells, was discarded. Supernatant 1 was centrifuged at 40,000 *g* for 1 hr and the supernatant solution was discarded. The pellet (residue 2) was suspended in buffer A and stored frozen at −20°C. The frozen residue was thawed and washed twice with buffer A before extraction by centrifugation at 40,000 *g*.

TABLE 2 REQUIREMENT FOR DIVALENT CATIONS

Cations Added	Concn	Lipid Formed
	mM	*mμmoles*
None		0.03
$MnCl_2$	1	22.7
	2	24.0
	5	22.8
$MgCl_2$	1	3.65
	2	9.30
	5	17.5
	10	29.5
$CaCl_2$	1	0.06
$ZnSO_4$	1	0.40

The experimental conditions were similar to those of Fig. 2, except that the concentration of Triton X-100 was kept constant at 2 mg/ml, while the concentration of divalent cations was varied as shown. Tris–HCl buffer of pH 8.0 (250 mM) was used instead of Tris–phosphate buffer in the incubation system.

Extraction with Triton X-100. Preliminary experiments showed that the enzyme could be extracted by buffer A that contained Triton X-100. The specific activity of the enzyme in the extract varied with the concentration of Triton X-100 in buffer A, as shown in Fig. 4. In the procedure finally adopted, the washed residue and buffer A containing 1% Triton were stirred in a Waring Blendor at 0°C for 10 min, at reduced speed to avoid foaming. The mixture was then centrifuged at 40,000 *g* for 1 hr.

Treatment with $MnCl_2$. To 100 ml of the combined Triton extracts with a protein concentration of 6.8 mg/ml, 1 M $MnCl_2$ was added to a final concentration of 0.05 M for the removal of nucleic acids. After the mixture had been stirred for several minutes, it was centrifuged at 10,000 *g* for 15 min at 0°C. Essentially all the activity was retained in the supernatant solution and the precipitate was discarded. Mn^{++} was removed from the treated supernatant by dialysis in buffer A (1 mM in EDTA) at 4°C overnight.

Ethanol Fractionation. Ice-cold absolute ethanol was added dropwise with continued stirring to the $MnCl_2$-treated, dialyzed solution which was set in an ice–salt bath at −8°C. The precipitate at 30% ethanol contained little enzymatic activity and was discarded. The fraction which precipitated at 30–50% ethanol contained most of the enzymatic activity with a slightly lower specific activity than that of the original solution. This was suspended in 20 ml of 0.01 M Tris buffer (pH 8.0), containing

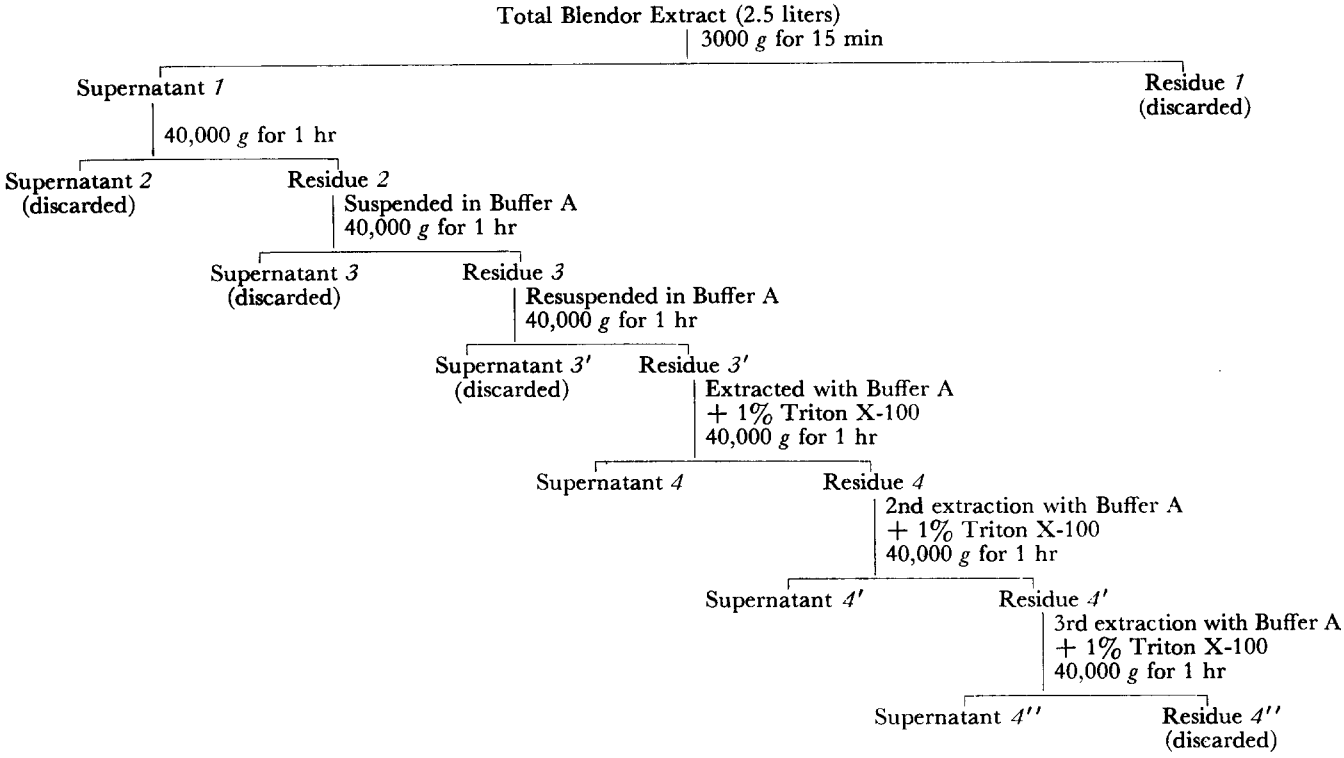

FIG. 3. Differential centrifugation and extraction of enzyme.

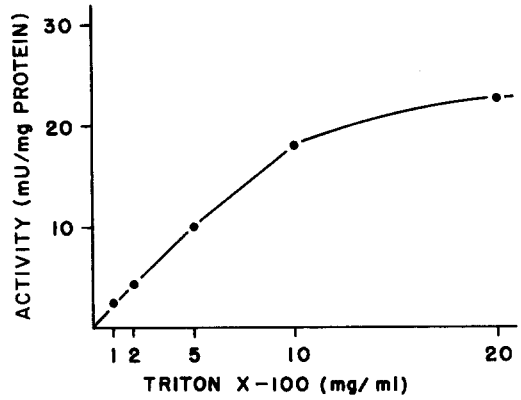

FIG. 4. Extraction of enzyme from particulate fractions with Triton X-100. Equal amounts of residue 2 (about 30 mg of protein) were extracted with 2 ml of buffer A containing Triton X-100 as indicated. Each extract was centrifuged at 40,000 *g* for 1 hr and the supernatant solution was assayed for enzymatic activity by method I.

0.01 M mercaptoethanol and 1% Triton X-100 (buffer B).

The purpose of ethanol fractionation is to facilitate the next step, DEAE-cellulose column fractionation, since the enzyme is not retained on a DEAE-cellulose column at high salt concentration. Dialysis in 0.01 M Tris buffer (pH 8.0) containing 0.01 M mercaptoethanol overnight at 4°C results in the loss of 40% of the total activity. Although ethanol fractionation itself did not give any purification, it reduced the ionic strength and concentrated the enzyme preparation without serious loss of activity.

Fractionation on DEAE-Cellulose. DEAE-Cellulose was suspended in cold buffer B and packed into a column 30 cm in length and 2 cm in diameter.

The 30–50% ethanol fraction, dissolved in buffer B, was passed over the column which was then eluted with the same buffer at a flow rate of 36 ml/hr. Fractions of 10 ml each were collected. The enzyme was loosely bound to the DEAE-Cellulose and could be eluted with buffer B without added salt. Enzyme of the highest specific activity was recovered in tubes 31–50. These were combined and NaCl was added to a final concentration of 0.1 M in order to preserve activity. Fractions so obtained were purified about 30-fold and were free from phosphatidyl glycerophosphate phosphatase activity. It was later found that fractionation of sonically disrupted cells, rather than extracts of cells disrupted with beads, by slightly improved chromatography on DEAE, yielded a product with a specific activity about 4-fold higher.

The purification procedure is summarized in Table 3.

Stability of Enzyme

The enzyme was stable if stored at moderate salt concentrations. The 40,000 *g* particulate fraction (residue 2) suspended in buffer A could be stored at −20°C for months without loss of activity. The 1% Triton extract could be stored at 0°C without loss of activity for at least 2–3 months. However, the $MnCl_2$-treated supernatant lost about 40% of its original activity upon dialysis in 0.01 M Tris–0.01 M mercaptoethanol buffer (pH 8.0) overnight. Therefore, after fractionation on DEAE-cel-

TABLE 3 PURIFICATION OF L-GLYCEROL 3-PHOSPHATE: CMP PHOSPHATIDYLTRANSFERASE

Preparation	Total Activity Recovered	Specific Activity
	%	*mU/mg protein*
Total blendor extract	100	1.0
40,000 *g* Residue	72	2.6
1% Triton extract	40	8.2
$MnCl_2$-treated supernatant	30	8.5
30–50% Ethanol fraction	15	6.4
DEAE-cellulose, fractions 31–50	5.5	30

lulose the enzyme was stored in buffer B containing 0.1 M NaCl, under which conditions it was stable at 0°C for several weeks.

Characteristics of Enzyme

The purified enzyme had a pH optimum of approximately 8.0 in Tris–HCl buffer (Fig. 5). This was slightly different from that of the particulate enzyme preparation, which had a pH optimum of 8.5.

When the concentration of L-glycerol 3-phosphate was fixed at a saturating level and the concentration of CDP-dipalmitin was varied, the K_m for CDP-dipalmitin was 2.2×10^{-5} M (Fig. 6). When the concentration of CDP-dipalmitin was maintained constant and the concentration of L-glycerol 3-phosphate was varied, the K_m for L-glycerol 3-phosphate was 2.5×10^{-4} M (Fig. 7). At concentrations of CDP-dipalmitin above 4×10^{-4} M, a precipitate formed and inhibition was observed.

The rate of the reaction was linear with the enzyme concentration under the conditions of the experiment of Fig. 6 until at least 15 mμmoles of product had been formed. The rate of the reaction catalyzed by the purified enzyme preparation was linear for at least 1 hr.

The affinity of the purified enzyme for magnesium ion is shown in Fig. 8. Half the maximal activity was observed at about 6–8 mM Mg^{++}. The activity of the purified enzyme preparation was not affected by sulfhydryl reagents such as $HgCl_2$ and *N*-ethylmaleimide. Fluoride (5 mM) inhibited activity about 60%.

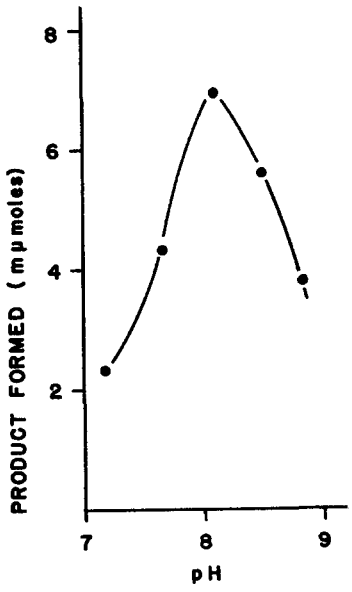

FIG. 5. Effect of pH on the activity of L-glycerol 3-phosphate: CMP phosphatidyltransferase. The experimental conditions were the same as those of assay method I, except that the pH of the system was varied with Tris–HCl buffer (250 mM) of various pH values. The pH of the incubation mixture was measured with a glass electrode. The concentration of Triton X-100 was 5 mg/ml. The enzyme preparation used was DEAE-cellulose combined fractions 31–50 (0.01 mg of protein).

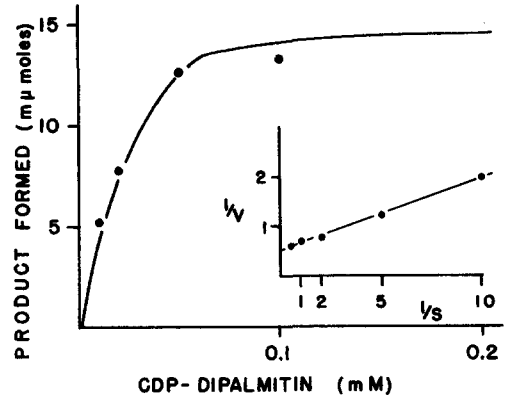

FIG. 6. Affinity of L-glycerol 3-phosphate: CMP phosphatidyltransferase for CDP-dipalmitin. The incubation mixture (final volume, 1 ml) contained Tris–HCl buffer of pH 8.0 (250 mM), mercaptoethanol (5 mM), Triton X-100 (5 mg/ml), $MgCl_2$ (20 mM), L-glycerol 3-phosphate-2-^{3}H (1 mM, 1.45×10^6 cpm/μmole), serum albumin (1 mg/ml) and DEAE-cellulose fractions 31–50 (0.01 mg of protein). The concentration of CDP-dipalmitin was varied as shown. The system was incubated for 20 min at 28°C. The product formed was measured by method I.

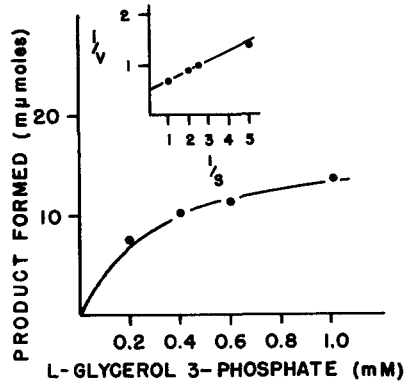

FIG. 7. Affinity of enzyme for L-glycerol 3-phosphate. The experimental conditions were the same as those described in Fig. 6, except that the concentration of CDP-dipalmitin was held constant at 0.1 mM, while the concentration of L-glycerol 3-phosphate was varied as shown.

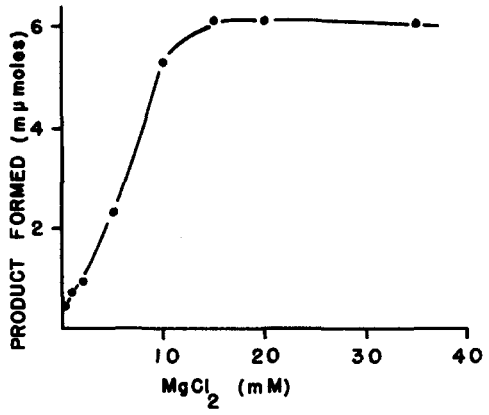

FIG. 8. Affinity of enzyme for Mg^{++}. The experimental conditions were the same as those described in Fig. 5, except that the pH of the incubation mixture was kept constant (pH 8.5), while the concentration of $MgCl_2$ was varied as shown.

Specificity of Enzyme

The enzyme is highly specific for L-glycerol 3-phosphate. When the release of CMP was measured under conditions in which the amount of L-glycerol 3-phosphate was limiting (experiment I of Table 4), the activity of DL-glycerol 3-phosphate was no greater than its content of L-isomer. In confirmation of this conclusion, the conversion of radioactive L-glycerol 3-phosphate to lipid was not affected by the presence of the D-enantiomorph (experiment II of Table 4).

A number of other substrates were tested for activity, as measured by the release of CMP in assay method II. No activity could be detected with glycerol, glycerol-2-phosphate, *myo*-inositol, and L-serine.

Stoichiometry of Reaction

Table 5 shows the results of an experiment in which the disappearance of L-glycerol 3-phosphate from the water-soluble fraction and the release of CMP are compared with the formation of phosphatidyl glycerophosphate in pairs of identical tubes. Within the limits of experimental error, 1 mole of glycerophosphate disappeared and 1 mole of CMP was released for each mole of phosphatidyl glycerophosphate formed in this system.

In similar experiments, in which 2 M KCl was substituted for 2 M $MgCl_2$ in the washing procedures of assay method I, recoveries of phosphatidyl glycerophosphate were only about 60% of the theory based on CMP release. Evidently the extraction of the magnesium salt of phosphatidyl glycerophosphate into chloroform is more efficient than the extraction of the potassium salt.

Reversibility of Reaction

When limiting amounts of CDP-diglyceride were used in incubations with large amounts of enzyme, the conversion of CDP-diglyceride to phosphatidyl glycerophosphate was almost complete. The yield of product was not affected by the addition of CMP in concentrations of 1–5 mM, which suggests that the equilibrium greatly favors synthesis. Nevertheless, the reversibility of the reaction could be demonstrated when enzymatically syn-

TABLE 4 SPECIFICITY OF THE ENZYME FOR L-GLYCEROL 3-PHOSPHATE

Experiment I Addition		CMP Released
	μmoles	*mμmoles*
L-Glycerol 3-phosphate	0.1	14.2
	0.2	20.0
	0.4	24.2
DL-Glycerol 3-phosphate	0.2	13.9
	0.4	18.3
	0.8	23.8

Experiment II Addition	Radioactive Lipid Formed
	total cpm
L-Glycerol 3-phosphate-2-^{3}H (0.01 μmole) plus L-glycerol 3-phosphate (1 μmole)	12,550
L-Glycerol 3-phosphate-2-^{3}H (0.01 μmole) plus DL-glycerol 3-phosphate (2 μmoles)	13,000

The incubation mixtures in both experiments were the same as those described in Fig. 6, except that the concentration of CDP-dipalmitin was kept constant at 0.1 mM, while the glycerophosphate was varied as shown. The purified enzyme preparation with a specific activity of 120 mU/mg of protein was used (6.4 μg of protein in experiment I, 3.2 μg of protein in experiment II). The specific activity of L-glycerol 3-phosphate-2-^{3}H was 8.4×10^7 cpm/μmole. In experiment I, CMP release was measured by assay method II, while in experiment II, the radioactivity of lipid formed was measured by method I.

TABLE 5 STOICHIOMETRY OF REACTION

Tube	L-Glycerol 3-Phosphate Disappeared	CMP Released	Phosphatidyl Glycerophosphate Formed
	mμmoles		
1	92	95	—
2	104	96	—
3	—	—	95
4	—	—	108

The incubation mixture in all tubes (final volume, 1 ml) contained Tris–HCl buffer of pH 8.0 (250 mM), mercaptoethanol (5 mM), Triton X-100 (5 mg/ml), $MgCl_2$ (20 mM), CDP-dipalmitin (0.2 mM), L-glycerol 3-phosphate-2-^{3}H (0.2 mM, 1.31×10^5 cpm/μmole), serum albumin (1 mg/ml) and DEAE-cellulose combined fractions 31–50 (0.04 mg of protein). All tubes were incubated for 1 hr at 37 °C. In tubes 1 and 2, the release of CMP was measured by assay method II. After the CMP was measured, the amounts of L-glycerol 3-phosphate-2-^{3}H remaining in the perchloric acid supernatant of tubes 1 and 2 were also determined. The amount of L-glycerol 3-phosphate disappearing from the acid supernatant was the difference between the amount of L-glycerol 3-phosphate-2-^{3}H recovered in the zero time control tube and in tube 1 or 2. In tubes 3 and 4, the formation of phosphatidyl glycerophosphate was measured by assay method I.

thesized phosphatidyl glycerophosphate-2′-^{3}H was incubated with CMP in the presence of a pool of unlabeled glycerophosphate (Table 6). When the pool of unlabeled glycerophosphate was omitted, no release of radioactive glycerophosphate could be detected (experiment not shown), which further supports the view that the equilibrium constant favors synthesis of phosphatidyl glycerophosphate.

The synthesis of phosphatidyl glycerophosphate in reaction 1 should also lead to the production of one equivalent of hydrogen ion. It was, therefore, of interest to examine the pH optimum for the reversal of synthesis. The pH optimum for reversal was found to be about pH 7.0 (Table 6), which is distinctly lower than for the forward reaction.

Identification of Lipid Product

An experiment was carried out under conditions essentially similar to those described for assay method I, except on a scale 50 times larger, with L-glycerol 3-phosphate-2-^{3}H as tracer. The lipid product, isolated by chloroform extraction, was converted to the sodium salt by passage over Chelex ion-exchange resin (18), and concentrated to dryness in a rotary evaporator. It was then dissolved in 2–3 ml of chloroform–methanol–water 20:9:1 and chromatographed on DEAE-cellulose in the acetate form, suspended in the same solvent system, as described by Henrickson and Ballou (19). A single radioactive peak was eluted from the column immediately after the fractions containing unreacted CDP-dipalmitin (detected by absorbancy at 280 mμ), and incompletely separated from CDP-dipalmitin. Fractions containing radioactivity but no appreciable absorbance at 280 mμ were pooled and analyzed. The molar ratio of P/ester was 1.05:1.00, close to the theory for phosphatidyl glycerophosphate.

When the product was subjected to deacylation by mild alkaline hydrolysis (20), a single radioactive water-soluble derivative was detected, with an R_f of 0.20 in solvent system A, and 0.12 in solvent system B. Upon hydrolysis in 90% acetic acid for 20 min in a boiling water bath, a single radioactive compound was detected in the water-soluble fraction, with an R_f of 0.05 in solvent system A, identical with that of authentic glycerol-1,3-diphosphate. The yield of water-soluble derivative under these relatively mild conditions was low (about 30%).

If the product of the enzymatic reaction is indeed phosphatidyl glycerophosphate, the extent of labeling from glycerophosphate-^{32}P and from glycerophosphate-2-^{3}H should be identical. Accordingly, an experiment was carried out with doubly-labeled glycerophosphate. The ratio of ^{32}P/^{3}H in the lipid product was unaltered in comparison with that of the precursor glycerophosphate.

In other experiments, it was found that the labeled lipid product was converted to phosphatidyl glycerol and orthophosphate as the result of the action of a specific enzyme from *E. coli* (accompanying paper, 21).

TABLE 6 REVERSAL OF THE REACTION CATALYZED BY L-GLYCEROL 3-PHOSPHATE: CMP PHOSPHATIDYLTRANSFERASE

Experiment I

Concentration of CMP	L-Glycerol 3-Phosphate Formed
mM	*mμmoles*
0	0.32
1	2.60
2	3.46
5	5.30
10	5.18

Experiment II

pH	L-Glycerol 3-Phosphate Formed
	mμmoles
8.0	5.39
7.5	6.80
7.0	8.80
6.5	5.20

In experiment I, the incubation mixtures (final volume, 0.2 ml) contained Tris–HCl buffer of pH 7.4 (250 mM), mercaptoethanol (5 mM), Triton X-100 (5 mg/ml), $MgCl_2$ (20 mM), phosphatidyl glycerophosphate-2′-^{3}H (0.1 mM, 1 × 10^6 cpm/μmole), DL-glycerol 3-phosphate (2 mM), serum albumin (1 mg/ml) and an enzyme preparation with a specific activity of 120 mU/mg of protein (0.016 mg of protein). The concentration of CMP was varied as shown. After incubation for 20 min at 28 °C, 0.5 ml of 10% trichloroacetic acid was added followed by 0.1 ml of 4 mM DL-glycerol 3-phosphate and 0.2 ml of 1% (w/v) serum albumin. The tubes were chilled for 5 min and then centrifuged. After the acid supernatant had been extracted twice with 1.5 ml of chloroform, aliquots were counted in a liquid scintillation counter. In experiment II, the incubation mixtures were the same as those described in experiment I, except that the concentration of CMP was held constant at 10 mM, while the pH of the system was varied with Tris–imidazole buffer (250 mM) of various pH values. The assay was the same as that of experiment I.

Identification of CMP as Product of the Reaction

The release of CMP as a water-soluble product of the reaction can be followed spectrophotometrically (Table 5). Evidence that the increase in absorbance at 280 mμ is in fact due to release of CMP was obtained in an experiment similar to that in Table 5, except that the scale was five times larger. The perchloric acid supernatant solution was freed from chloroform by a jet of air, since chloroform interferes with the adsorption of CMP on charcoal. Norit A (15 mg) was then added, the suspension was stirred at room temperature for 15 min, and the charcoal was separated by centrifugation and washed twice with water. The charcoal was then extracted twice with 5 ml of 50% (v/v) aqueous ethanol containing 1% (v/v) concentrated aqueous ammonia. The ammoniacal ethanol extracts were concentrated to dryness and the residue was taken up in a small amount of water and applied to Whatman 3 MM filter paper. Chromatography in solvent system C revealed a spot detectable

under UV light with an R_f of 0.73, identical with that of authentic CMP.

DISCUSSION

Although the L-glycerol 3-phosphate:CMP phosphatidyltransferase from *E. coli* has not been extensively purified, the procedures described here are sufficient to free it almost completely from the specific phosphatase catalyzing its hydrolysis, as shown by the fact that the ratio of $^{32}P/^{3}H$ in the lipid product is the same as that of the doubly-labeled glycerophosphate used as precursor. The satisfactory stoichiometry observed in the experiment in Table 5 also indicates that most other interfering activities are greatly reduced in the purified enzyme fraction.

Although many of the properties of the bacterial enzyme are similar to those observed in the particulate mitochondrial fraction previously studied (6), one striking difference is the complete dependence of the *E. coli* enzyme on added Mg^{++} or Mn^{++}. Such a requirement was not detected in the mitochondrial enzyme.

Since purified enzyme fractions are entirely dependent upon added CDP-diglyceride for activity, and since the enzyme has a high affinity for CDP-diglyceride, the enzyme-catalyzed synthesis of phosphatidyl glycerophosphate can be used as a highly sensitive and convenient assay for CDP-diglyceride. This method has already found application in studying the net enzymic synthesis of CDP-diglyceride (22) and could readily be applied to measurement of CDP-diglyceride levels in tissues.

Phosphatidyl glycerophosphate does not accumulate in cells of *E. coli* nor does it constitute a significant fraction of the total lipids of most other organisms. Recently Wells and Dittmer (23) identified 1-(L-3-glycerophosphoryl)-L-glycerol 3-phosphate as the alkaline deacylation product of a newly discovered brain lipid, presumably phosphatidyl glycerophosphate. In an interesting finding, Kates, Yengoyan, and Sastry (24) showed that a diether analogue of phosphatidyl glycerophosphate is the principal phospholipid in the extreme halophilic bacterium *Halobacterium cutirubrum*. In this lipid, however, the fatty acyl residues are replaced by dihydrophytyl ether residues. The configuration of the phosphatidyl moiety is the opposite of that usually found in nature; the two ether bonds are at the 2- and 3-positions of L-glycerol 1-phosphate. Elucidation of the biosynthesis of this remarkable lipid thus offers a challenging problem.

The phosphodiester bonds of phospholipids appear to be "energy-rich" linkages. Reversal of the enzymatic synthesis of lecithin leads to the formation of CDP-choline and α,β-diglyceride (25). The reversibility of the synthesis of phosphatidyl glycerophosphate described here affords another example of the reactivity of the phosphodiester bond. Although it was not possible to estimate the equilibrium constant from these experiments, the available evidence suggests that the equilibrium must greatly favor synthesis rather than "cytidylolysis."

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