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Partial Purification and Characterization of Cytidine 5'-Diphosphate-Diglyceride Hydrolase from Membranes of *Escherichia coli*

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Cytidine 5'-diphosphate (CDP)-diglyceride is hydrolyzed to phosphatidic acid and cytidine 5'-monophosphate by a specific membrane-bound enzyme in cell-free extracts of *Escherichia coli*. The hydrolase can be extracted from the particulate fraction with Triton X-100 and purified 1,000-fold in the presence of this detergent. Several nucleoside disphosphate diglycerides were synthesized to determine the substrate specificity of the hydrolase. CDP-diglyceride was hydrolyzed preferentially, although uridine 5'-diphosphate-diglyceride, guanosine 5'-diphosphate-diglyceride, and adenosine 5'-diphosphate (ADP)-diglyceride were also slowly hydrolyzed. Surprisingly, the purified enzyme did not catalyze detectable cleavage of deoxy-CDP (dCDP)-diglyceride. The liponucleotide pool of *E. coli* contains dCDP-diglyceride and CDP-diglyceride in approximately equal amounts (Raetz and Kennedy, 1973). Water-soluble nucleoside pyrophosphates, such as CDP-choline, nicotinamide adenine dinucleotide, or adenosine 5'-triphosphate are not attacked by this specific hydrolase. Hydrolysis of CDP-diglyceride is strongly inhibited by adenosine 5'-monophosphate and by ADP-diglyceride.

Cytidine 5'-diphosphate (CDP)-diglyceride and deoxy-CDP (dCDP)-diglyceride function as donors of phosphatidyl residues for the biogenesis of membrane phospholipids in *Escherichia coli* (3, 10, 19). Their combined levels in vivo do not exceed 0.05% of the total cellular phospholipid (19), and both compounds turn over rapidly. The formation of CDP-diglyceride (and dCDP-diglyceride) from phosphatidic acid may be rate-making for phospholipid biosynthesis in the organism (19).

In 1972, a preliminary communication (17) from this laboratory reported the discovery of a specific hydrolase, localized in the membrane fraction of *E. coli*, that hydrolyzes CDP-diglyceride according to the following equation: CDP-diglyceride + water → cytidine 5'-monophosphate (CMP) + phosphatidic acid.

The present paper describes the further fractionation of this enzyme, which has now been obtained 1,000-fold purified, and other properties of the enzyme. A detailed examination of its specificity reveals the unexpected finding that dCDP-diglyceride is not hydrolyzed by the purified enzyme, a finding consistent with a regulatory function of the enzyme. Uridine diphosphate-diglyceride, adenosine diphosphate (ADP)-diglyceride, and guanosine diphosphate-diglyceride have been synthesized and tested as substrates. Of these, ADP-diglyceride, although itself slowly hydrolyzed, is an especially potent inhibitor of the hydrolase.

(Part of this work was taken from a dissertation submitted by C.R.H.R. to the Faculty of Arts and Sciences of Harvard University in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Cells of *E. coli* B were usually grown in a rotary shaker at 37 C on mineral medium 63 with 1% glycerol as a carbon source (5), unless otherwise indicated. Cell-free extracts were prepared by sonic disruption as described previously (18). For large-scale preparations, frozen cells of *E. coli* B were purchased from the Grain Processing Co., Muscatine, Iowa.

Enzyme assays. Hydrolysis of CDP-diglyceride was measured by the release of water-soluble CMP from CDP-diglyceride tritiated in the cytidine moiety (17, 20). Conditions described earlier (17) were employed, except that bovine serum albumin (0.7 mg/ml) was also added to the assay system. Hydrolysis of other, nonradioactive nucleoside diphosphate diglycerides was measured spectrophotometrically (Table 4).

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The liponucleotides employed in all assays were dipalmitoyl derivatives, synthesized from dipalmitoyl-L-α-glycerophosphate and nucleoside monophosphomorpholidates (19). Other enzymes of phospholipid metabolism were assayed as described previously (18).

Preparations of radioactively labeled CDP-diglyceride. CDP-dipalmitin tritiated in the cytidine moiety was prepared enzymatically by the CMP-CDP-diglyceride exchange reaction (20), associated with the phosphatidylserine synthetase of *E. coli*. CDP-dipalmitin labeled with ^{32}P in the phosphatidyl moiety was synthesized from dipalmitoyl-L-α-[^{32}P]glycerophosphate and CMP-morpholidate as in the case of the nonradioactive liponucleotides (19). Dipalmitoyl-L-α-[^{32}P]glycerophosphate was made enzymatically from adenosine 5'-triphosphate labeled with ^{32}P in the γ position and 1,2-dipalmitin by incubation with the diglyceride kinase of *E. coli* (16, 22). Conditions for this enzymatic reaction have been reported by Schneider and Kennedy (22). CDP-dipalmitin, doubly labeled with ^{32}P in the phosphatidyl residue and tritium in the cytidine moiety, was obtained by mixing solutions of the singly labeled liponucleotides, which were synthesized separately.

Other materials. [G-^{3}H]CMP and adenosine 5'-triphosphate labeled with ^{32}P in the γ position were products of the New England Nuclear Corp., Boston, Mass. Triton X-100 (octylphenoxypolyethoxyethanol) was obtained from Rohm and Haas (Philadelphia, Pa.). Uridine diphosphate-glucose, ADP-glucose, CDP-glycerol, CDP-choline, and all nucleoside monophosphates and monophosphomorpholidates were obtained from the Sigma Chemical Co. (St. Louis, Mo.).

Miscellaneous procedures. Protein concentration was determined by the method of Lowry et al. (11). Sucrose gradients were prepared in 12-ml polyallomer tubes (9.5 cm in length), and an International B-60 preparative ultracentrifuge equipped with an SB283 rotor was employed for high-speed sedimentation. Fractions were collected by piercing the bottom of the tube with a short 22-gauge needle.

RESULTS

Hydrolysis of CDP-diglyceride by cell-free extracts of E. coli. When CDP-dipalmitin, labeled with tritium in the cytidine moiety, was incubated with extracts of freshly grown *E. coli* prepared by sonic disruption or passage through a Mantin-Gaulin press, a rapid release of water-soluble CMP was observed even in the absence of added phosphatidyl acceptors such as L-serine or *sn*-glycero-3-phosphate. The initial rate of this reaction was linear with time, and the reaction was greatly stimulated by Triton X-100 (Fig. 1). The hydrolysis of CDP-diglyceride went to completion with prolonged incubation. The initial rate of CDP-diglyceride hydrolysis was directly proportional to protein concentration up to 100 μg of added protein (data not shown). Although the reaction was normally carried out in 0.1 M phosphate buffer, hydrolysis also took place at a somewhat slower rate in 0.3 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) in the absence of phosphate. The enzyme had no requirement for added metal ions under the standard assay conditions employed.

Effect of growth conditions on the activity of CDP-diglyceride hydrolase. The specific activity of the hydrolase was about 1.5-fold higher in exponentially growing cells than in stationary cultures. Slightly higher specific activities were obtained when glucose was used as the carbon source (Table 1). *E. coli* B and *E. coli* K-12 did not differ significantly in their content of this enzyme.

Localization of the hydrolase in disrupted cell preparations. Sucrose gradient centrifugation of sonically disrupted cell preparations resulted in the pattern of activity shown in Fig. 2. Most of the hydrolytic activity was concentrated at the bottom of the gradient on the 70% sucrose shelf along with the larger membrane fragments. This pattern closely resembles that of the phosphatidylserine decarboxylase (18), an enzyme known to be associated with the inner membrane (1, 21, 25). There does not appear to be a soluble CDP-diglyceride hydrolase since this would have been detected in the top two or three fractions of the sucrose gradient shown in Fig. 2.

Extraction and purification of the membrane-bound hydrolase. The hydrolytic activ-

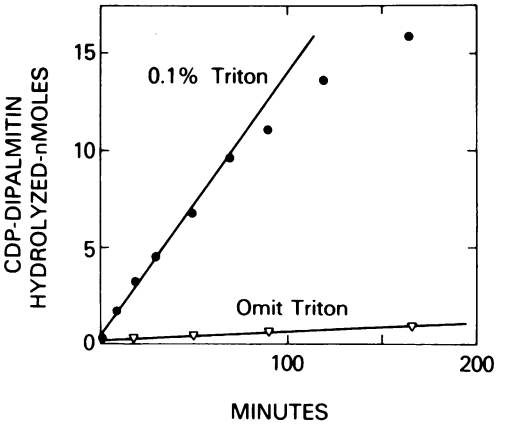

FIG. 1. *Hydrolysis of CDP-dipalmitin, tritiated in the cytidine moiety, by a cell-free extract of E. coli. This shows the time course of CDP-diglyceride hydrolysis catalyzed by 40 μg of crude extract protein (fraction 1, Table 2) in the presence and absence of Triton X-100. The stimulation by Triton X-100 may reflect either the solubilization of the substrate or a direct effect on the enzyme.*

ity could be extracted quantitatively from the particulate fraction with 5% Triton X-100. In large-scale preparations, a frozen paste of *E. coli* B (1.1 kg) was suspended in 1.6 liters of 0.1 M potassium phosphate, pH 6.8, containing 5 mM $MgSO_4$ and 10 mM 2-mercaptoethanol. All procedures were carried out between 0 and 4 C unless otherwise indicated. After four passages through a Mantin-Gaulin press at 560 atm, the broken cells (Table 2, fraction 1) were centrifuged at 45,000 × *g* for 5 h, and the supernatant was discarded. The pellet was resuspended in 970 ml of 0.1 M potassium phosphate buffer, pH 7.15, containing 10 mM 2-mercaptoethanol and 5% Triton X-100, with the aid of a Waring blender. Remaining insoluble material was removed by a 90-min centrifugation at 45,000 × *g*. The supernatant (fraction 2) was mixed with 200 ml of glycerol, and the pH was lowered to 4.9 with 0.5 M acetic acid. Acetone (2.4 liters) was rapidly added, and the precipitate was collected by centrifugation. The enzyme was extracted from this pellet by resuspension in 3 liters of 0.01 M sodium acetate, pH 5, containing 1% Triton X-100, 15% glycerol, and 10 mM 2-mercaptoethanol. After 10 min of mixing, the protein that did not redissolve was removed by centrifugation. The supernatant containing the hydrolase, which redissolves selectively under these conditions, was titrated to pH 7.2 with saturated tris(hydroxymethyl)aminomethane-free base solution (fraction 3).

TABLE 1. *Specific activity of CDP-diglyceride hydrolase under various growth conditions*[a]

Growth phase	Carbon source[b]	Hydrolase activity (units/mg of protein)
Mid-log	Casamino Acids	3.50
	Glycerol	2.63
	Glucose	3.84
Stationary	Casamino Acids	1.85
	Glycerol	1.78
	Glucose	3.04

[a] Cells of *E. coli* B were grown at 37 C on mineral medium 63 (5) supplemented with the carbon sources as indicated above. Cell extracts were prepared as described in Materials and Methods. Extracts from commercially available cells (Table 2) contained somewhat higher levels of the enzyme. A unit of activity catalyzes the hydrolysis of 1 nmol of CDP-diglyceride per min at 37 C.

[b] Added to a final concentration of 1% (wt/vol).

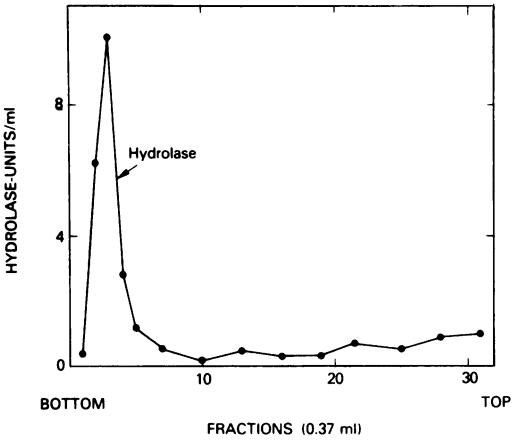

FIG. 2. *Sedimentation profile of CDP-diglyceride hydrolase during centrifugation in a sucrose gradient. Conditions of centrifugation and preparation of extracts were identical to those described previously (18). Cell-free extract (0.5 ml), containing 10 mg of protein per ml, was layered on top of a 5 to 20% sucrose gradient (9 ml) in a polyallomer tube, at the bottom of which was a 70% sucrose shelf (1.5 ml). The gradient was then centrifuged at 200,000 × g_{av} for 1.5 h at 4 C. Membrane fragments and hydrolase activity were concentrated on the 70% sucrose shelf near the bottom of the gradient.*

Chromatography on DEAE-cellulose. The enzyme was then applied to a diethylaminoethyl (DEAE)-cellulose column (8 by 24 cm), Whatman DE-52, at 25 C in 0.01 M potassium phosphate buffer, pH 7.4, 1% Triton X-100, 10 mM 2-mercaptoethanol, and 10% glycerol. After a 1-liter wash with the same buffer, the hydrolase was eluted at room temperature with a linear gradient (0 to 0.4 M NaCl in 12 liters). The enzyme was eluted in a well-defined peak in which the concentration of NaCl at midpoint was about 0.12 M (Fig. 3). Chromatography on DEAE-cellulose also served to separate the hydrolase from the phosphatidylserine decarboxylase (Fig. 3), which was purified with the hydrolase in the preceding fractions.

The hydrolase was stable for a period of weeks when fractions from the column were stored at 0 C. The pooled fractions were concentrated by pressure dialysis to a volume of 90 ml with Amicon XM-50 filters (fraction 4). Since some Triton X-100 was retained, the concentrate was rather viscous; the activity, however, was quantitatively recovered and was stable after concentration.

Only 75% of the hydrolase activity applied to the DEAE-cellulose column in Fig. 3 was recovered in the peak emerging at a sodium chloride concentration of 0.12 M. The remaining hydrolase activity was not retained by the column at all and emerged in the run-through. When the run-through fractions were exposed to fresh DEAE-cellulose again, none of the activity was adsorbed. That portion of the hydrolase activity not bound to DEAE-cellulose may

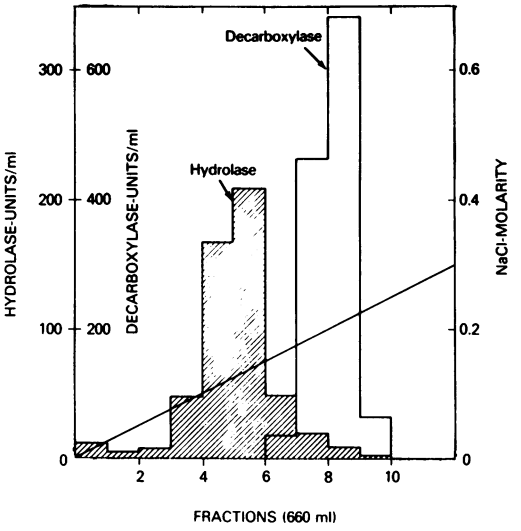

FIG. 3. *Separation of CDP-diglyceride hydrolase and phosphatidylserine decarboxylase on a column of DEAE-cellulose. Conditions are described in the text. A unit of decarboxylase activity is the same as a unit of hydrolase (Table 1).*

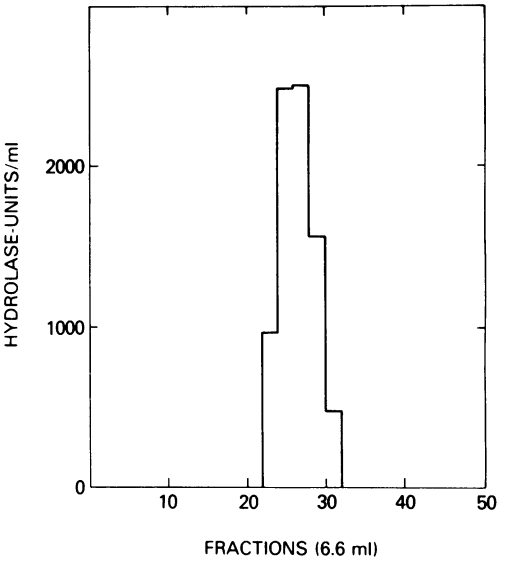

FIG. 4. *Chromatography of CDP-diglyceride hydrolase on a column of Sephadex G-150. The enzyme emerged in a narrow peak near the excluded volume. Conditions are described in the text.*

represent a different form of the CDP-diglyceride hydrolase or may be an entirely different pyrophosphatase. The present work was carried out with the fractions that were bound to DEAE-cellulose.

Ethanol precipitation. Fraction 4 was titrated to pH 4.9 with 0.5 M acetic acid. After the addition of ethanol to a final concentration of 30% (vol/vol), the preparation was held on ice for 1 h, after which the precipitate was removed by a 20-min centrifugation at 10,000 × *g*. To the supernatant, which contained most of the activity, ethanol was added to give a final concentration of 80% (vol/vol). After standing for 2 h at 0 C, the protein was centrifuged as described above, and the supernatant was discarded. The precipitate was redissolved in 25 ml of buffer (fraction 5), containing 0.01 M potassium phosphate (pH 7.4), 50 mM NaCl, 10% glycerol, 10 mM 2-mercaptoethanol, and 0.1% Triton X-100. Ethanol precipitation removed the concentrated Triton X-100 from fraction 4.

Chromatography on Sephadex G-150. Fraction 5 was applied to a Sephadex G-150 column (2.5 by 100 cm) at 25 C, equilibrated with the same buffer used to redissolve the ethanol precipitate, and eluted with this buffer at a flow rate of 30 ml/h. Fractions containing hydrolase, which emerged in a sharp peak near the excluded volume (Fig. 4), were pooled and concentrated by pressure dialysis to a final volume of 2.1 ml (fraction 6). A small amount of precipitate, which formed during concentration, was removed by centrifugation. Fraction 6 was stable for several weeks at 0 C. Analysis of this fraction by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed considerable heterogeneity (at least five major bands), despite the fact that the enzyme was purified more than 1,000-fold relative to crude extracts (Table 2).

TABLE 2. *Purification of the membrane-bound hydrolase*

Fraction	Total vol (ml)	Total protein (g)	Sp act (units/mg of protein)	Yield (%)
1. Broken cells	2,500	128.0	7.3	100
2. Membrane extract[a]	960	16.6	27	48
3. Acetone treatment	2,900	4.5	72	34
4. DEAE-cellulose	90	0.27	790	23
5. Ethanol fractionation	25	0.05	2,150	11
6. Sephadex G-150	2.1	0.013	7,560	10

[a] The remaining activity could be solubilized quantitatively with a second extraction. This was not done in routine preparations. A unit of activity is defined in Table 1.

Hydrodynamic properties of the CDP-diglyceride hydrolase after solubilization from the membrane. In the course of purifying the hydrolase it was found that its apparent size relative to globular protein standards was three to four times larger when measured by gel filtration chromatography on Sephadex G-200 columns than by sedimentation in sucrose gra-

dients according to the procedure of Martin and Ames (13). Figure 5A shows that the hydrolase sedimented just ahead of hemoglobin (molecular weight, 64,000) in a sucrose gradient, whereas it chromatographed close to pyruvate kinase (molecular weight, 236,000) on a column of Sephadex G-200 (Fig. 5B). The amounts of Triton X-100 included in the buffers in this experiment did not significantly influence the migration of the standards employed. Levels of detergent greater than 0.1% were necessary, however, to prevent aggregation of the hydrolase.

Identification of products. CDP-diglyceride, double labeled with tritium in the cytidine moiety and ^{32}P in the phosphatidyl moiety, was incubated with the partially purified hydrolase (Fig. 6). Treatment with the enzyme resulted in the conversion of most of the tritium to water-soluble material, whereas all of the ^{32}P remained chloroform soluble. CMP was identified as the only water-soluble, tritiated product by its chromatography on paper with an authentic

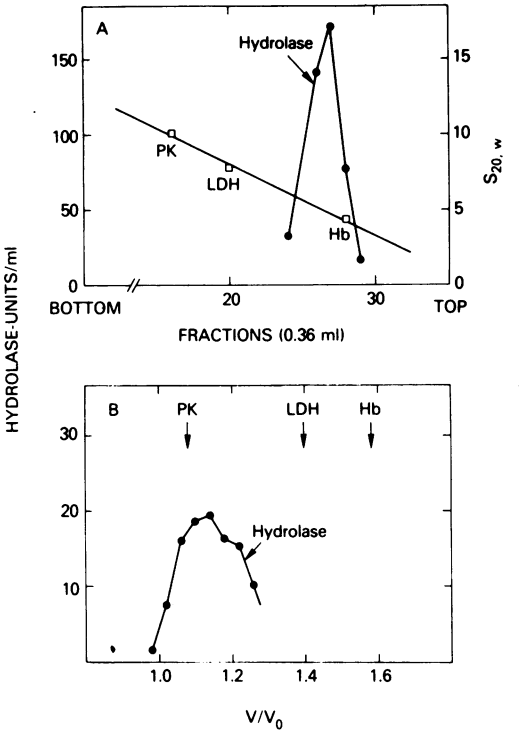

FIG. 5. *Hydrodynamic properties of the solubilized, partially purified hydrolase. (A) The hydrolase sediments just ahead of hemoglobin on a 5 to 20% sucrose density gradient, which was prepared in polyallomer tubes as described in Materials and Methods. Centrifugation was performed for 16 h at 4 C and 200,000 × g_{av}. The gradient contained 0.1% Triton X-100, 10 mM 2-mercaptoethanol, and 10 mM potassium phosphate, pH 7.4, throughout. The sample (0.4 ml) was prepared in the same buffer with 0.5% Triton X-100 and contained 300 units of hydrolase (fraction 6) and 2 mg of bovine hemoglobin, as well as pyruvate kinase and lactate dehydrogenase sufficient for detection by enzymic assay. (B) Results of chromatographing a similar enzyme sample at 4 C on a column (1.4 by 40 cm) of Sephadex G-200, equilibrated with buffer containing 10 mM potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 10% glycerol, and 1% Triton X-100. The arrows indicate the positions of the peaks of the marker proteins.*

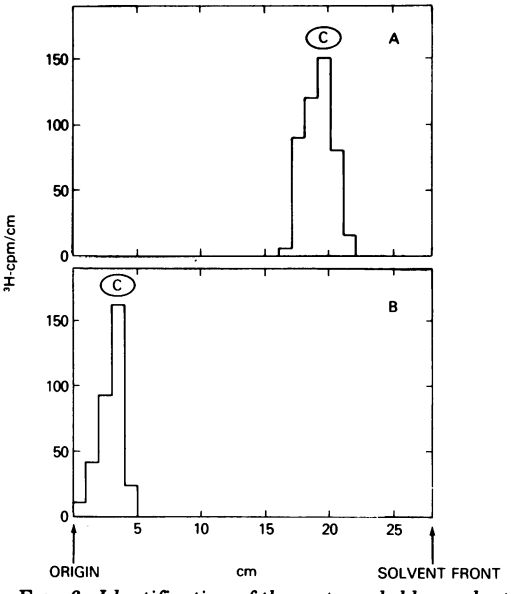

FIG. 6. *Identification of the water-soluble product of the hydrolase reaction. Twenty nanomoles of CDP-dipalmitin, double labeled with ^3H in the cytidine moiety (150 counts/min per nmol) and ^{32}P in the phosphatidyl moiety (450 counts/min per nmol), was incubated for 2 h with 1 μg of the DEAE-cellulose-purified enzyme (fraction 4), under conditions otherwise identical with those employed for the assay. The reaction was stopped by the addition of 0.45 ml of chloroform-methanol (2:1, vol/vol) containing 0.01 M HCl, followed by 0.24 ml of water. The products were partitioned between the aqueous methanol and chloroform phases. Aliquots of the aqueous phase (which contained the tritium released from CDP-diglyceride by the enzyme) were mixed with 0.1 μmol of CMP carrier and spotted on Whatman no. 1 paper. Two solvent systems were employed for the identification of CMP. (A) Radioactivity profile observed with saturated $(NH_4)_2SO_4$ in 0.1 M potassium phosphate (pH 6.8)-propanol (100:2, vol/vol). (B) Results obtained with ethanol-1 M ammonium acetate (pH 7.4) (7:3, vol/vol). Circles indicate schematically the position of carrier CMP, located by its absorbance under ultraviolet light. Radioactivity of the chromatograms was measured by liquid scintillation counting after cutting the paper into 1-cm strips, 3 cm in width.*

standard in two solvent systems (Fig. 6A and B).

The material that remained chloroform-soluble after enzymatic treatment contained 10% of the total tritium, presumably residual CDP-diglyceride, and all of the ^{32}P, 95% of which migrated with phosphatidic acid in the thin-layer chromatogram shown in Fig. 7. Further proof that phosphatidic acid was the major lipid product was obtained by paper chromatographic identification of α-glycerophosphate as the principal water-soluble product of mild alkaline hydrolysis (2).

As reported previously (17), phosphatidic acid and CMP are formed in approximately equal amounts. Since no further degradation of [^{32}P]-phosphatidic acid to $^{32}P_i$ was observed, it is unlikely that our enzyme preparations contain phosphatidic acid phosphatase (24). Likewise [^3H]CMP was not degraded to [^3H]cytidine, indicating the absence of significant 5'-nucleotidase activity.

As noted above, a small fraction of the CDP-diglyceride hydrolase activity extracted from the membranes was not bound by columns of DEAE-cellulose. This fraction appears to be a similar pyrophosphatase, however, since it also splits CDP-diglyceride, tritiated in the cytidine moiety, to generate [^3H]CMP.

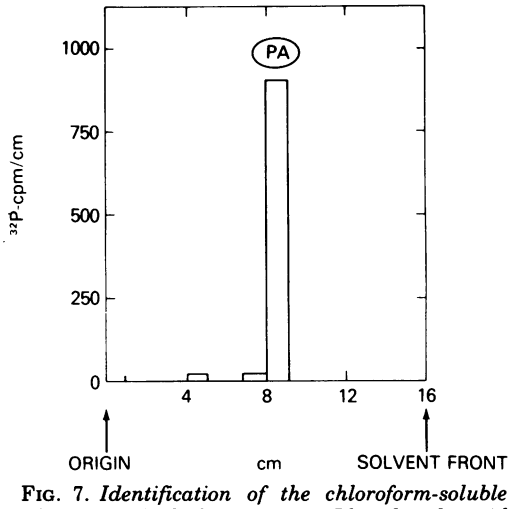

FIG. 7. *Identification of the chloroform-soluble product of the hydrolase reaction. Phosphatidic acid was identified as the major product in the chloroform layer after the incubation described in Fig. 6 by thin-layer chromatography on silica gel plates in the solvent system chloroform-pyridine-formic acid (50:30:7, vol/vol/vol). The chloroform-soluble material recovered after prolonged incubation of CDP-diglyceride (labeled with ^{32}P in the phosphatidyl moiety) with the enzyme migrated with an R_f of 0.5, identical with that of a dipalmitoyl phosphatidic acid standard, which was located with a phosphate spray reagent (7) as indicated by the circle. All chromatograms were developed at room temperature.*

Substrate specificity. Water-soluble pyrophosphates, such as nicotinamide adenine dinucleotide, CDP-choline, or ADP-glucose, are not attacked by the CDP-diglyceride hydrolase. The three major membrane lipids of *E. coli* are not altered by the purified enzyme. The hydrolase does not catalyze the exchange of free CMP with the CMP moiety of CDP-diglyceride.

A series of nucleoside diphosphate diglycerides was synthesized chemically (19), and the relative rates of hydrolysis were determined (Table 4). CDP-diglyceride was hydrolyzed preferentially. A comparable degree of selectivity has been shown to exist with phosphatidyl transferases, which utilize CDP-diglyceride in the biosynthesis of phosphatidylserine and phosphatidylglycerophosphate (19). ADP-diglyceride was hydrolyzed relatively slowly, even though its affinity for the hydrolase is much greater (Table 3).

A surprising result of the present study is the observation (Table 4) that dCDP-diglyceride is not hydrolyzed at an appreciable rate (less than 1%) by the partially purified enzyme (Table 2, fraction 4, and subsequent, more highly purified fractions). The preparation of dCDP-diglyceride used in these experiments did not contain an inhibitor of CDP-diglyceride hydrolysis (Table 3), and it served as a substrate for the enzymatic synthesis of phosphatidylserine and phosphatidylglycerophosphate (19).

TABLE 3. *Inhibition of CDP-diglyceride hydrolase by adenine-containing nucleotides*[a]

Addition	Relative initial rate of hydrolysis of CDP-dipalmitin
None	100
AMP	7
ADP	19
ATP	64
dAMP	100
3'-AMP	103
ADP-diglyceride (0.33 mM)	5
ADP-diglyceride (0.033 mM)	50
dCDP-diglyceride (0.20 mM)	108

[a] The initial rate of hydrolysis of CDP-dipalmitin (0.33 mM) was measured in the presence of the indicated nucleotide at a concentration of 3.3 mM, unless otherwise indicated, and is expressed as the percentage of the rate observed for the reaction in the absence of added inhibitor. Approximately 6 out of 20 nmol of CDP-diglyceride was hydrolyzed in this control, expressed as 100% activity. Incubations were performed for 20 min (with about 1 μg of fraction 4, Table 2) as described in the text for the standard assay. ATP, Adenosine 5'-triphosphate.

TABLE 4. *Nucleoside specificity of the CDP-diglyceride hydrolase*[a]

Substrate	Relative rates of hydrolysis (%)
CDP-diglyceride	100
UDP-diglyceride	39
GDP-diglyceride	17
ADP-diglyceride	6
dCDP-diglyceride	<1

[a] Incubations were performed under standard assay conditions (17), except that the final reaction volume was 0.3 ml and nonradioactive liponucleotides were used. Approximately 5 μg of fraction 4 was added to start the reaction, and 30% of the CDP-diglycerice present initially was hydrolyzed in 15 min. This rate is defined as 100%. To determine the extent of hydrolysis, the reaction was stopped with 2 ml of chloroform-methanol (2:1, vol/vol) and 0.5 ml of 0.1 M HCl. After mixing and separation of the phases, the optical density at 260 nm of the aqueous-methanol layer was determined. As with the regular assay (17), plastic tubes were employed for this incubation. UDP, Uridine 5'-diphosphate; GDP, guanosine 5'-diphosphate.

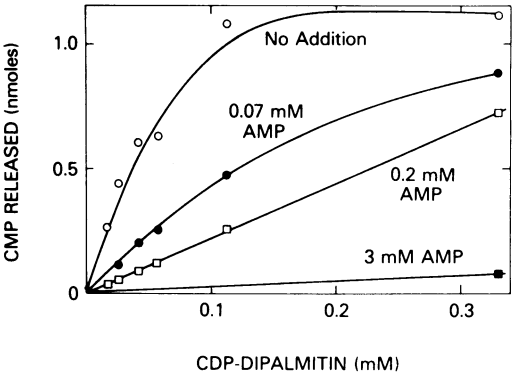

FIG. 8. *Inhibition of hydrolysis of CDP-diglyceride by AMP. The release of CMP was measured as described in Materials and Methods, except that the CDP-dipalmitin concentration was varied at the levels of AMP shown in the figure. Incubations were performed for 5 min with 0.4 μg of DEAE-cellulose-purified enzyme (fraction 4). The initial rate of CMP release was linear for about 10 min over the entire range of substrate concentrations examined.*

In crude extracts, in contrast to the purified fractions, substantial degradation of dCDP-diglyceride was observed. Most of this activity, however, was not associated with the membrane fraction and has not been characterized. The fraction of the CDP-diglyceride hydrolase activity not bound by DEAE-cellulose also did not hydrolyze dCDP-diglyceride.

Inhibition by adenine nucleotides. In contrast to CMP, adenosine 5'-monophosphate (AMP) and other adenosine-containing nucleotides are potent inhibitors of CDP-diglyceride hydrolase (17; C. R. H. Raetz, C. B. Hirschberg, W. Dowhan, W. T. Wickner, and E. P. Kennedy, Fed. Proc. 31:896, 1972). Inhibition by AMP appears to follow simple, competitive inhibition kinetics (Fig. 8). An analysis of these results in terms of Michaelis-Menten kinetics may be an oversimplification, however, since the rate measurements were performed in the presence of detergent. Nevertheless, the inhibition by AMP shows a high degree of chemical specificity, since neither 3'-AMP nor deoxy-AMP exerts comparable effects (Table 3). AMP itself is not a substrate for the enzyme.

The most potent inhibitor as yet identified for the hydrolase is ADP-diglyceride, although this compound is also split at a slow rate. The results of Table 3 demonstrate that the affinity of the enzyme for ADP-diglyceride exceeds that for AMP (and CDP-diglyceride) by an order of magnitude.

DISCUSSION

Enzymes of phospholipid metabolism are generally bound to membranous structures in bacteria, as well as in mammalian cells. The recent development of techniques for the fractionation of membrane proteins has made possible the purification of several of these enzymes.

In our experience, classical methods of protein fractionation such as ion exchange chromatography, sucrose gradient centrifugation, and gel filtration can be applied to many membrane proteins and enzymes "solubilized" with the nonionic detergent Triton X-100. In the present work, two solubilized enzymes of phospholipid metabolism, the CDP-diglyceride hydrolase and the phosphatidylserine decarboxylase, were separated from each other almost quantitatively on a column of DEAE-cellulose (Fig. 3) in the presence of the detergent. In the solubilized form these enzymes behaved as discrete molecular species during most fractionation procedures.

Several other enzymes of phospholipid metabolism in *E. coli* have been resolved from each other and from the bulk of the membrane proteins using this approach. These are the phosphatidylglycerophosphate synthetase (3), the phosphatidylglycerophosphate phosphatase (4), diglyceride kinase (22), and phosphatidylserine decarboxylase (8). In general, attempts to remove the detergent result in aggregation and loss of activity. It is not known whether mild proteolysis removes these enzymes from the membrane, as is the case with nitrate reductase (12) and cytochrome b_5 (23).

The apparent size of the hydrolase is almost four times larger when measured by gel filtration than by sedimentation in sucrose gradients (Fig. 5). In these experiments globular proteins employed as standards were not significantly influenced in their migration by the amounts of Triton X-100 included in the buffers. Meunier and co-workers have recently described a similar phenomenon with the solubilized receptor for acetyl choline (15). In this case the size discrepancy results from the binding of relatively large amounts of Triton X-100 to the solubilized receptor (14), subsequently giving it a lower buoyant density than globular protein standards, which bind relatively little detergent (9, 14). Thus, the solubilized receptor sediments less rapidly than would be expected from its apparent Stokes radius, determined by gel filtration. Although binding of Triton X-100 to the CDP-diglyceride hydrolase was not measured in the present work, the hydrodynamic properties of the hydrolase could also be explained in this manner. Two other membrane-bound enzymes of phospholipid metabolism, the phosphatidylserine decarboxylase and the diglyceride kinase, have analogous hydrodynamic properties when solubilized with Triton X-100 (8).

An unexpected property of the hydrolase is its striking specificity for CDP-diglyceride, in contrast to dCDP-diglyceride, which is not cleaved at an appreciable rate. Since both CDP-diglyceride and dCDP-diglyceride are found in vivo, the hydrolase might function in the regulation of the ribonucleotide to deoxyribonucleotide ratio, although genetic studies will be needed to test this hypothesis.

The data presented in Table 4 reveal that ADP-diglyceride is hydrolyzed relatively slowly when compared to CDP-diglyceride. Under the assay conditions employed these relative rates represent maximal velocities. When ADP-diglyceride was tested as an inhibitor of CDP-diglyceride hydrolysis, however, it was found to have a 10-fold greater affinity for the enzyme than did CDP-diglyceride. AMP and other AMP-containing substances are also potent inhibitors of the hydrolase (Table 3; Fig. 8).

These results at least raise the possibility that ADP-diglyceride, or a related metabolite, might serve as an alternative physiological substrate for the hydrolase. Although its presence cannot be excluded completely, ADP-diglyceride has not been detected in living cells of *E. coli* (19).

In the course of the present studies no phosphatidyl acceptor other than water was found for this enzyme. (L-Serine, glycerol, L-α-glycerol-3-phosphate, ethanolamine, and 5'-CMP [all 1 to 10 mM] were tested under standard assay conditions.) The hydrolase furthermore does not catalyze exchange of CMP with CDP-diglyceride. However, these findings do not altogether exclude a biosynthetic function for the enzyme. Indeed, the phosphatidylserine synthetase of *E. coli* has a CDP-diglyceride hydrolase activity associated with it, although the hydrolytic activity is about two orders of magnitude lower than that of the hydrolase described here (20).

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