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Partial Purification and Properties of CTP:Phosphatidic Acid Cytidylyltransferase from Membranes of *Escherichia coli*

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The cytosine liponucleotides CDP-diglyceride and dCDP-diglyceride are key intermediates in phospholipid biosynthesis in *Escherichia coli* (C. R. H. Raetz and E. P. Kennedy, J. Biol. Chem. **248**:1098-1105, 1973). The enzyme responsible for their synthesis, CTP:phosphatidic acid cytidylyltransferase, was solubilized from the cell envelope by a differential extraction procedure involving the detergent digitonin and was purified about 70-fold (relative to cell-free extracts) in the presence of detergent. In studies of the heat stability of the enzyme, activity decayed slowly at 63°C. Initial velocity kinetic experiments suggested a sequential, rather than ping-pong, reaction mechanism; isotopic exchange reaction studies supported this conclusion and indicated that inorganic pyrophosphate is released before CDP-diglyceride in the reaction sequence. The enzyme utilized both CTP and dCTP as nucleotide substrate for the synthesis of CDP-diglyceride and dCDP-diglyceride, respectively. No distinction was observed between CTP and dCTP utilization in any of the purification, heat stability, and reaction mechanism studies. In addition, CTP and dCTP were competitive substrates for the partially purified enzyme. It therefore appears that a single enzyme catalyzes synthesis of both CDP-diglyceride and dCDP-diglyceride in *E. coli*. The enzyme also catalyzes a pyrophosphorolysis of CDP-diglyceride, i.e., the reverse of its physiologically important catalysis.

Cytosine-containing coenzymes play crucial roles in the biosynthesis of phospholipids in eucaryotic and procaryotic cells (31). *Escherichia coli* contains both CDP-diglyceride and dCDP-diglyceride (27). All of the phospholipids of the organism are derived from these cytosine-containing liponucleotides.

The original suggestion that CDP-diglyceride might play a role in phospholipid biosynthesis was made by Agranoff et al. in 1958 (1). Since then, cell-free particulate enzymes catalyzing CDP-diglyceride synthesis from CTP and phosphatidic acid (CTP:phosphatidic acid cytidylyltransferase; EC 2.7.7.41) have been described from a number of sources (5, 6, 15, 19, 23, 24).

Unexpectedly, it has also been found that the cytosine liponucleotides of *E. coli* consist of approximately equal amounts of CDP-diglyceride and dCDP-diglyceride (27). The pool of these coenzymes is very small, and the turnover of both ribonucleotide and deoxyribonucleotide forms of the coenzyme is rapid (27). It is possible that the presence of the two forms is significant in the regulation of lipid synthesis.

A detailed examination of the enzymatic synthesis of CDP-diglyceride and dCDP-diglyceride in *E. coli* in the present study was prompted by the obvious importance of these liponucleotides as precursors of all of the membrane phospholipids of the organism. A more particular aim was to determine whether the syntheses of the two forms of the cytosine liponucleotide are catalyzed by separate enzymes. After solubilization and 70-fold purification (relative to cell-free extracts), no significant separation of activities with CTP or dCTP as substrate was detected. We also found that CTP and dCTP were competitive substrates for the partially purified enzyme. These and other results support the view that a single enzyme catalyzes the synthesis of both CDP-diglyceride and dCDP-diglyceride in *E. coli*.

MATERIALS AND METHODS

Materials. [^{3}H]CTP (1.26 Ci/mmol) was the tetralithium salt from Schwarz/Mann. It was judged 80% radiochemically pure by chromatography in solvent system 3 (Table 1), the major contaminant being CDP. Correction for the impurity was made in calculating activities. [5-^{3}H]dCTP (22.8 Ci/mmol; tetrasodium salt from New England Nuclear Corp.) required purification before use. Unlabeled dCTP (5 mg) was added to about 0.2 mCi of the tritiated material and purified by chromatography on Dowex-1 formate (14).

^{32}P-labeled sodium pyrophosphate (PP_i) (5.8 Ci/mmol) was from New England Nuclear Corp.

TABLE 1. *Thin-layer and paper chromatography systems*

Medium	Solvent system	R_f value			
		Phosphatidic acid	CDP-dipalmitin		dCDP-dipalmitin
Silica gel plates[a]	1. Chloroform-methanol-acetic acid-water (50:28:4:8, vol/vol)	0.89	0.61		0.66
	2. *t*-Butanol-water-ammonia (63:39:9, vol/vol)		0.85		0.85
		CTP	dCTP	PP_i	P_i
Whatman no. 1 paper	3. Isobutyrate-ammonia-water (66:1:33, vol/vol)	0.11	0.16	0.10	0.21
PEI-cellulose plates[b]	4. 2.5 M sodium formate, pH 3.4	0.24	0.25	0.13	0.85
	5. 2.5 M sodium formate, pH 3.4[c]	0.55	0.60	0.22	

[a] Silica Gel 1B, J.T. Baker Chemical Co. The plates were heated at 110°C for 30 to 60 min immediately before use.

[b] Polygram Cel 300 PEI, Brinkmann Instruments, Inc.

[c] Plates were developed, dried, and then developed again in the same dimension.

Crude phospholipid from *E. coli* K-12 cells and enzymatically synthesized *sn*-[1,(3)-^{14}C]phosphatidic acid (dipalmitoyl; 29) were gifts from M. Snider. Dipalmitoyl-L-α-glycerophosphate was synthesized chemically by a method based on that of Baer (3). CDP-dipalmitin and dCDP-dipalmitin were prepared by the procedure of Raetz and Kennedy (27), which was adapted from Agranoff and Suomi (2). The synthesis of [^{3}H]CDP-dipalmitin has also been described previously (26). The latter three compounds were prepared by C. R. H. Raetz. Lipids were dispersed in water or in detergent solutions by brief sonic treatment.

Detergents of the Triton series are polyoxyethylene derivatives of octylphenol; these were obtained from Rohm and Haas. Detergents of the Brij series are polyoxyethylene derivatives of long-chain alkanes (Atlas Co.). Digitonin was purchased from Fisher Scientific Co. Tween 80 (polyoxyethylene sorbitan monooleate) was obtained from the Nutritional Biochemicals Corp.

Bacteria. *E. coli* K-12 strain A324, wild type with respect to lipid metabolism, was used as source of CTP:phosphatidic acid cytidylyltransferase activity. Frozen *E. coli* K-12 cells harvested near the end of the log phase of growth and washed by centrifugation were obtained from Grain Processing Co., Muscatine, Iowa. Yields of enzyme from the frozen cells were 10 to 50% of those from the fresh cells, but properties of the enzyme from the two sources appeared to be identical.

Cell growth and isolation of cell envelope. Cells were grown at 37°C in medium 63 (9) supplemented with 1% (wt/vol) glycerol, 0.1% (wt/vol) casein hydrolysate (Difco Laboratories), 20 μg of proline per ml, and 2 μg of thiamine per ml to a cell density of 2×10^8/ml, and were harvested by centrifugation. For large-scale preparations, cells were grown in a fermentor and harvested in a Sharples centrifuge. Cells were washed with 0.1 M potassium phosphate, pH 7.0, containing 5 mM $MgSO_4$ and 10 mM 2-mercaptoethanol (buffer A), resuspended in the same buffer, and broken with an MSE (Measuring & Scientific Equipment, Ltd.) 100-W sonic oscillator, with the temperature below 15°C at all times. Subsequent operations (including purification steps) were carried out at 4°C unless otherwise indicated. After sonic treatment, unbroken cells were removed by centrifugation at 3,000 × *g* for 10 min. The cell envelope fraction was then isolated by centrifugation for 1 h at 100,000 × *g*, or for 5 h at 54,000 × *g* for large volumes. The enzyme activity of the envelope fraction was stable for months when stored at −70°C. Stability was enhanced by the presence of Mg in the buffer.

Enzyme assays. CTP:phosphatidic acid cytidylyltransferase was assayed by a modification of the procedure of Carter (6). Each tube contained the following components at the indicated final concentrations: potassium phosphate, pH 7 (0.05 M); AMP (2 mM); Triton X-100 (0.5%, wt/vol); tritiated CTP or dCTP, specific activity about 90,000 cpm/μmol (2 mM); dipalmitoyl phosphatidic acid (2 mM); and $MgCl_2$ (2.5 mM), in a total volume of 0.1 ml. AMP was included to inhibit CDP-diglyceride hydrolase activity (26). The $MgCl_2$ was always added last, thereby avoiding formation of insoluble Mg phosphatidate as discussed previously (5, 6). After incubation at 37°C, usually for 40 min, the reaction was terminated by addition of 1 ml of 0.25% (wt/vol) bovine serum albumin followed by 0.2 ml of 50% (wt/vol) trichloroacetic acid to precipitate phospholipid and protein. The precipitate was collected by centrifugation, dissolved in 1 ml of 0.2 M Tris base, and reprecipitated with 0.2 ml of 50% (wt/vol) trichloroacetic acid. After centrifugation, the residue was dissolved in 1.5 ml of 0.5 M tris(hydroxymethyl)aminomethane (Tris) base, and 1-ml portions were counted in 10 ml of scintillation fluid (22).

The system for the assay of the reverse reaction contained: PP_i (8 mM); either tritiated CDP-dipalmitin (2 mM) or dCDP-dipalmitin (2 mM), specific activity 100,000 cpm/μmol; and $MgCl_2$ (10 mM). Other additions (potassium phosphate, AMP, and Triton X-100) and conditions were the same as for the assay in the forward direction described above. The reaction

was terminated by addition of 1.6 ml of chloroform-methanol (2:1, vol/vol, containing 0.01 N HCl) followed by 1 ml of water. After centrifugation, the aqueous phase was removed and a sample was counted. For both the forward and reverse reactions, 1 U is defined as the amount of enzyme catalyzing production of 1 nmol of product per h. Both assays were linear over the range of enzyme concentrations used. For incubation times greater than 40 min, the rate slowly declined; this was at least partially caused by slow inactivation of the enzyme at 37°C in the presence of Triton X-100 (10 to 15% loss after 30 min at 37°C). The precision of both assays was ±5%.

Assays for phosphatidylserine decarboxylase (11), phosphatidylglycerophosphate synthetase (13), and CDP-diglyceride hydrolase (26) have been previously described.

Assays for isotope exchange. Enzyme-catalyzed isotope exchange tests were performed by including appropriate radiolabeled compounds in the reaction mixtures. All incubations were carried out in a total volume of 0.1 ml. Concentrations of potassium phosphate (pH 7), Triton X-100, AMP, and $MgCl_2$ were the same as described for the cytidylyltransferase assay. Concentrations of CTP, dCTP, PP_i, phosphatidic acid, and CDP-dipalmitin were as indicated in Table 4 for the individual tests.

For the test of exchange between CTP and PP_i, the reaction mixture included [^{3}H]CTP (86 cpm/nmol) and [^{32}P]PP_i (102 cpm/nmol). Incorporation of ^{32}P into CTP was determined in the following way: CTP was isolated by adsorption to charcoal (Norit A), eluted, and counted for ^{3}H and ^{32}P. Apparent exchange is expressed as the percentage of CTP which contains ^{32}P, calculated on a molar basis from the known specific radioactivities of the input reactants. Exchange between dCTP and PP_i was measured in the same way, except that [^{3}H]dCTP (101 cpm/nmol) was present instead of [^{3}H]CTP.

Exchange between CTP and CDP-dipalmitin was tested with [^{3}H]CDP-dipalmitin (100 cpm/nmol) included in the reaction mixture. ^{3}H in the aqueous phase was measured after partitioning of the reaction mixture between organic and aqueous phases as described above for the assay of the reverse of the cytidylyltransferase reaction. Apparent exchange represents ^{3}H rendered water soluble, expressed as percentage of total ^{3}H.

Exchange between phosphatidic acid and CDP-dipalmitin was tested with [^{14}C]phosphatidic acid (dipalmitoyl; 89 cpm/nmol) present in the reaction mixture. The reaction mixture was extracted by the chloroform-methanol-water procedure described above for the reverse of the cytidylyltransferase assay. The chloroform phase was brought to dryness under a stream of N_2, taken up in 30 μl of chloroform-methanol (2:1, vol/vol), and chromatographed on silica gel plates (system 1, Table 1). ^{14}C present in phosphatidic acid and CDP-dipalmitin was determined. Apparent exchange represents ^{14}C present in CDP-dipalmitin, expressed as percentage of total ^{14}C (in phosphatidic acid and CDP-dipalmitin).

Thin-layer and paper chromatography. Systems used for chromatography of phospholipids, nucleotides, and PP_i are summarized in Table 1, with R_f values for compounds of interest. Phospholipids on silica gel thin-layer plates were detected by spraying with a solution of rhodamine 6G (0.05%, wt/vol) in 95% ethanol or with the molybdenum reagent of Dittmer and Lester (10). Nonradioactive nucleotides were located on polyethyleneimine-impregnated cellulose (PEI-cellulose) plates by illumination with a UV light source. Nucleotides were eluted from PEI-cellulose plates (when necessary) by shaking cut strips in 0.7 M $MgCl_2$–2 M Tris-hydrochloride, pH 7.4 (100:1, vol/vol), for 1 h at 23°C (28) and from paper chromatograms by shaking strips in 0.1 M potassium phosphate, pH 7. Usually radioactivity on silica gel and PEI-cellulose plates and on paper chromatograms was determined by cutting strips and counting them directly in vials with 1 ml of water and 10 ml of scintillation fluid (22).

Other procedures. Enzyme preparations were concentrated by filtration through an Amicon Centrifo apparatus, using CF25 membrane cones (25,000 M_R cutoff). Digitonin micelles are retained by the membranes and concentrated with the protein.

Binding of nucleotides to charcoal (Norit A; Pfanstiehl Laboratories, Waukegan, Ill.) and subsequent elution with ammoniacal ethanol was performed according to Zimmerman (34). Protein was determined by the method of Lowry et al. (18), with bovine serum albumin as standard. When Triton X-100 or digitonin was present in samples, 0.7% (wt/vol) sodium dodecyl sulfate was included during the Lowry procedure (32) to prevent precipitation of the former two detergents.

RESULTS

Solubilization of the membrane-bound enzyme. Bile salts (cholate and deoxycholate), digitonin, and other nonionic detergents, including Triton X-45, Triton X-114, Triton X-100, Triton X-102, Brij 58, Brij 97, and Tween 80, were tested for ability to solubilize the cytidylyltransferase. Cell envelope fractions (10 mg of protein per ml in buffer A) were treated with the detergents (2%, wt/vol) for 30 to 60 min at 4°C, and the suspensions were then centrifuged for 1 h at 100,000 × *g*. Only digitonin released the activity from the membrane, with an efficiency of 70 to 90%. These results were not appreciably altered by the use of 1 M KCl, 10 mM ethylenediaminetetraacetic acid (EDTA), or a temperature of 25°C during the extraction. Treatments with Triton X-100 (2%, wt/vol) over a pH range of 5 to 9 and with urea (3 and 6 M) were ineffective in the solubilization tests.

Selective extraction of the enzyme was achieved by treatment of the cell envelope fraction with Triton X-100 (which did not extract the enzyme) followed by extraction of the residue with digitonin plus EDTA as described below for the procedure summarized in Table 2. (Treatment of the Triton X-100 pellet fraction

TABLE 2. *Purification of the cytidylyltransferase*

Fraction	Total vol (ml)	Total protein (mg)	Sp act (U/mg)[a]	Yield (%)	CTP/dCTP[b]
1. Crude extract	320	6,750	31	100	1.2
2. Envelope prepn	200	2,040	83	81	1.5
3. Triton X-100 pellet	220	1,480	160	112	1.5
4. Digitonin-EDTA extract of Triton pellet	200	260	600	75	1.2
5. DEAE-cellulose (with EDTA) pool, concentrated 8×	48	110	750	40	0.8
6. DEAE-cellulose (with Mg) pool	90	16.2	2,200	17	0.9
7. DEAE-cellulose (with Mg) pool, concentrated 12×	7.5	13.5	1,700		1.1

[a] Activity values are for CTP as nucleotide substrate. Values with dCTP were also determined (see column 6). Digitonin was included in the assays at each step to give maximal levels of activation (Fig. 2). However, some further activation (increased yield) is evident in fraction 3, which was assayed after addition of digitonin and EDTA for extraction of the enzyme.

[b] Ratio of activities with CTP and dCTP as nucleotide substrates.

with digitonin alone was not effective in solubilizing the enzyme.)

Partial purification of the cytidylyltransferase. The partial purification of the enzyme is summarized in Table 2. Cells were grown, harvested and broken and the cell envelope was isolated as described in Materials and Methods. Twenty-seven liters of culture yielded 68 g of cells (wet weight). Sonic treatment was carried out for 30 min at an amplitude setting of 7 to 8 μm with the MSE sonic oscillator. Triton X-100 was added to the envelope preparation to give a final concentration of 2% (wt/vol). The mixture was homogenized with a Potter homogenizer, stirred for 45 min, and then centrifuged at 54,000 × *g* for 5 h. The pellet was resuspended in buffer A, containing 2% (wt/vol) digitonin and 10 mM EDTA (fraction 3), homogenized, stirred for 90 min, and again centrifuged at 54,000 × *g* for 5 h. The supernatant (fraction 4) was dialyzed against buffer B (0.01 M potassium phosphate, pH 7.0, containing 4 mM EDTA and 0.5% [wt/vol] digitonin) and applied to a diethylaminoethyl (DEAE)-cellulose (Whatman DE52) column (2.1 by 38 cm) equilibrated with the same buffer. The column was washed with 70 ml of buffer B and developed with a linear KCl gradient (0 to 0.15 M KCl in 1,900 ml of buffer B). About 68% of the protein was retained on the column after the wash. The cytidylyltransferase was eluted at a salt concentration of about 0.06 M. Active fractions were pooled, and the concentration of digitonin was increased to 1% (wt/vol). The pool was then concentrated eightfold (fraction 5, Table 2), dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, containing 2 mM $MgSO_4$, 5 mM 2-mercaptoethanol, and 0.5% digitonin, and chromatographed on a DEAE-cellulose column in buffer containing Mg (Fig. 1). The pooled fraction from this column was concentrated 12-fold, and digitonin was added to increase the concentration by 0.75%. The preparation was dialyzed against buffer A and stored frozen at −70°C (fraction 7, Table 2).

The activities with CTP and dCTP as substrates were recovered in the same fractions, and the ratio of the two activities remained quite constant throughout the purification (Table 2, Fig. 1).

Other activities in the partially purified enzyme preparation. The levels of various other enzymes related to phospholipid metabolism were measured at different stages of the transferase purification. As expected, phosphatidylserine decarboxylase (11), phosphatidylglycerophosphate synthetase (7, 13), and CDP-diglyceride hydrolase (26) activities were largely extracted from the envelope by Triton X-100 treatment. About 2% of the former two activities and about 6% of the hydrolase activity remained in fraction 4. Thus, a major advantage of the procedure of Table 2 is the step of differential extraction. The decarboxylase and hydrolase were not detectable in fraction 7 (<0.02% of initial levels).

Fraction 7 was also free of PP_i hydrolase and CTP hydrolases. These enzymes were measured by paper chromatography and thin-layer chromatography (see Materials and Methods and Table 1) of [^{32}P]PP_i (systems 3 and 4, Table 1) and [^{3}H]CTP (system 5, Table 1) after incubation at concentrations of 2 mM in the presence of enzyme. Less than 2% of each compound was degraded during a 40-min incubation with 36 μg of fraction 7 under the conditions of the cytidylyltransferase assay.

Properties of the cytidylyltransferase. The assay conditions listed in Materials and Methods for the cytidylyltransferase reaction were optimal with respect to pH and concentra-

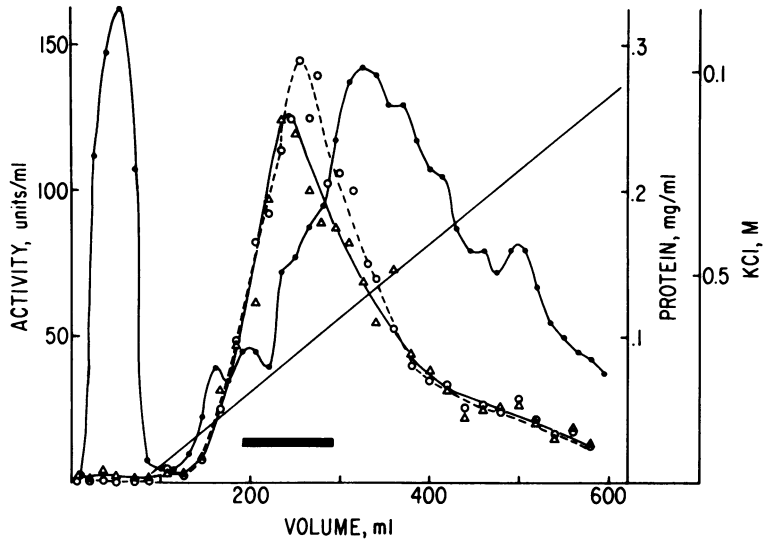

FIG. 1. *Ion-exchange chromatography of solubilized cytidylyltransferase. Fraction 5 (Table 2; 112 mg of protein in 48 ml) was dialyzed against 0.01 M potassium phosphate, pH 7, containing 2 mM $MgSO_4$, 5 mM 2-mercaptoethanol, and 0.5% digitonin and was applied to a DEAE-cellulose column (1.5 by 21 cm) equilibrated with the same buffer except that the digitonin concentration was 0.25%. After washing with 7 ml of buffer, the column was developed with a linear salt gradient (0 to 0.1 M KCl in 570 ml). Fractions (4.8 ml) were collected at a flow rate of 18 ml/h and analyzed for protein (●) and cytidylyltransferase activity with CTP (○) and dCTP (△) as nucleotide substrates. Fractions were pooled as indicated by the bar. The narrow solid line represents KCl concentration.*

tion of each component, as tested with membrane-bound enzyme. Concentrations of Triton X-100 greater than 1% (wt/vol) inhibited the reaction. The pH and concentration dependencies were very similar with CTP or dCTP as nucleotide substrate, except for the effect of AMP. A maximum stimulation by AMP of about threefold was found with CTP as substrate, but ≤1.25-fold was found with dCTP. This difference can be explained by inhibition of the specific CDP-diglyceride hydrolase by AMP, as described by Raetz et al. (26; the enzyme does not hydrolyze dCDP-diglyceride). The solubilized enzyme (fraction 7) showed similar dependencies on concentrations of assay components, suggesting that release from the membranes had no major effect in this regard.

A number of other agents were tested for effects in the assay. Sodium dodecyl sulfate (0.1%, wt/vol) and EDTA in excess of Mg inhibited completely. Addition of Mg in excess of EDTA restored activity, with a 2.5 to 3 mM Mg excess being optimal. Thus, the *E. coli* enzyme has a requirement for divalent cations, as reported by Carter (6). *E. coli* phospholipid (0.5%, wt/vol), digitonin (0.5%, wt/vol), and Brij 97 (0.5%, wt/vol) increased activity by about 50%. Urea (3 M) had no effect.

In other experiments, potassium phosphate was replaced in the envelope preparation by 0.1 M Tris-hydrochloride (pH 8) or 0.1 M sodium phosphate (pH 7). These ions supported activity at levels about 65% that seen with the potassium salt. Depressed levels could be restored to original by addition of potassium ion.

Activity was about 32% of optimal in the absence of Triton X-100 (Fig. 2). Addition of digitonin in the absence of Triton X-100 caused slight inhibition, but stimulated the reaction in the presence of Triton (Fig. 2).

Stability of the cytidylyltransferase. At 0 to 4°C, the solubilized enzyme (fraction 4) was stable for at least several weeks. Addition of Mg in excess of EDTA provided some additional stability. The solubilized enzyme was also stable to freezing and thawing and was stable for at least several months frozen at −70°C.

Stability at elevated temperatures was also tested for the membrane-bound and solubilized activities. The membrane-bound enzyme lost no activity after 30 min at 57°C. Fraction 4 enzyme lost 70% of its activity after 30 min at 57°C, but only 19% when Mg was added in excess of EDTA.

Factors influencing stability of the enzyme at 63°C are summarized in Table 3. Phospholipid stabilized, whereas Triton X-100 destabilized. The Triton destabilization could be overcome, at least partially, by added phospholipid, but not by glycerol, sucrose, or ethylene glycol (each

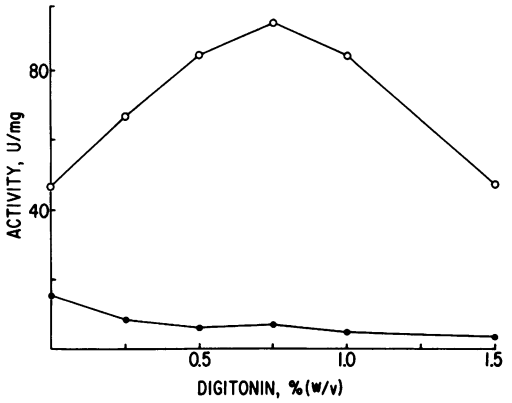

FIG. 2. *Influence of digitonin concentration on cytidylyltransferase activity. Samples (0.22 mg) of an E. coli A324 envelope preparation were assayed with digitonin at the indicated concentrations in the presence* (○) *and absence* (●) *of 0.5% (wt/vol) Triton X-100.*

20%, wt/vol). Stability was good at pH ≤7, but dropped rapidly above pH 7. Enzyme solubilized from the envelope directly with digitonin behaved like fraction 4 enzyme in the stability tests summarized in Table 3.

The time course of treatment at 63°C is shown in Fig. 3. The membrane-bound activity showed an apparent increase at first, and then decayed slowly. No such activation occurred with the solubilized enzyme. The activities with CTP and dCTP as substrate exhibited similar stabilities.

Identification of product. To identify CDP-diglyceride, the reaction was carried out with CTP (20-fold-increased specific radioactivity relative to usual assay) and phosphatidic acid substrates, and with 540 μg of fraction 7 enzyme, in a volume of 2 ml for 40 min. Unlabeled CDP-dipalmitin (3 mg) was then added as carrier. Phospholipid was extracted into chloroform (27) and applied to a column (1.3 by 7.5 cm) of DEAE-cellulose in chloroform-methanol-water (2:3:1, vol/vol). The column was eluted in an ammonium acetate (pH 7.4) gradient, as described by Raetz and Kennedy (27). All of the chloroform-soluble radioactivity was eluted with 271-nm-absorbing material at the position expected for CDP-dipalmitin, with 92% recovery. Radioactivity from the peak fractions chromatographed with authentic CDP-dipalmitin on silica gel plates in systems 1 and 2 (Table 1). The chromatographically purified, enzymatically synthesized CDP-diglyceride was also treated with the specific CDP-diglyceride hydrolase (26); 88% of the radioactivity was rendered water soluble by the hydrolase, except in the presence of AMP (5 mM), a known inhibitor of the hydrolase.

TABLE 3. *Heat treatment of cytidylyltransferase*[a]

Experimental conditions	Percent activity remaining with given substrate	
	CTP	dCTP
Additions during heat treatment		
None	62	56
Brij 97	14	17
Triton X-100	5	10
E. coli phospholipid	91	100
Triton X-100 + *E. coli* phospholipid	48	47
pH during heat treatment		
3	118	
4	108	
5	144	
6	117	
7	65	
8	4	9
9	2	6

[a] Fraction 4 enzyme was incubated at 63°C for 10 min after the indicated additions and pH adjustments. Portions were then assayed for cytidylyltransferase with both CTP and dCTP as nucleotide substrate. Activity is reported as the percentage of that observed for enzyme subjected to the indicated conditions but assayed before the 63°C incubation. The digitonin, Triton X-100, and Brij 97 concentrations were 2% (wt/vol); phospholipid was 0.5% (wt/vol). pH adjustments were made by addition of potassium hydroxide or phosphoric acid.

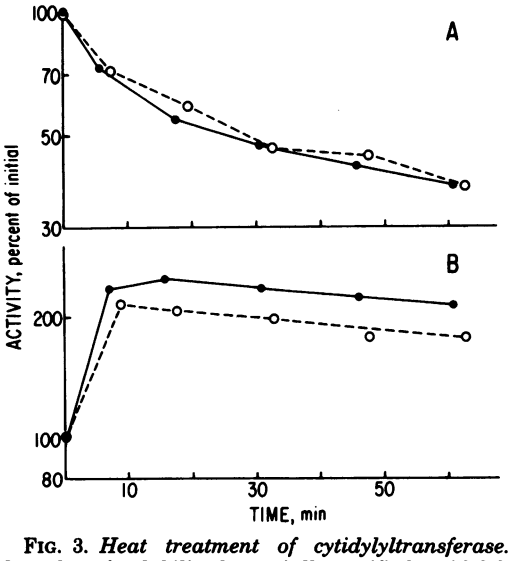

FIG. 3. *Heat treatment of cytidylyltransferase. Samples of solubilized, partially purified cytidylyltransferase (fraction 7, Table 2) (A) and E. coli cell envelope (fraction 2, Table 2) (B) were heated at 63°C. At the indicated times, portions were withdrawn and assayed for cytidylyltransferase activity with CTP* (●) *and dCTP* (○) *as nucleotide substrates.*

The radioactive product from reaction with dCTP and phosphatidic acid as substrates, after extraction into chloroform, chromatographed with authentic dCDP-dipalmitin on silica gel systems 1 and 2 (Table 1) and, as expected, was resistant to degradation by the CDP-diglyceride hydrolase.

Reaction mechanism. Kinetic analysis of the forward reaction is shown in Fig. 4. Converging lines in this type of plot are consistent with a sequential mechanism, i.e., both nucleotide and phosphatidic acid binding before release of either product. K_m^{CTP} and V_{max} values, obtained from secondary plots of slope and intercept, were 0.28 ± 0.02 mM and 1,800 U/mg, respectively. K_m values obtained for phosphatidic acid were erratic, but in the range of 1 mM. Similar kinetic analysis with dCTP as nucleotide substrate also yielded converging lines in double-reciprocal plots and K_m^{dCTP} and V_{max} values of 0.90 ± 0.09 mM and 2,400 U/mg, respectively.

The enzyme catalyzed a pyrophosphate-dependent release of radioactivity from [^{3}H]CDP-dipalmitin (Fig. 5). The dependence upon added PP_i distinguishes this activity from hydrolysis. To confirm that this activity represents true pyrophosphoryolysis of CDP-dipalmitin, i.e., reversal of the cytidylyltransferase reaction with formation of labeled CTP, a reaction was carried out with [^{3}H]CDP-dipalmitin (2 mM; 100,000 cpm/μmol) and [^{32}P]PP_i (8 mM; 25,000 cpm/μmol) in a volume of 0.1 ml containing 36 μg of fraction 7 enzyme. After 40 min, water-soluble radioactivity was adsorbed onto charcoal; the charcoal was washed, and bound radioactivity was eluted with ammoniacal ethanol as in Materials and Methods. The product was found to contain ^{3}H and ^{32}P in a ratio of 3.85:1. The theoretical ratio for labeled CTP, based on the specific radioactivities of CDP-diglyceride and PP_i, is 4.0:1. The eluted material migrated with CTP on PEI-cellulose plates in system 5 (Table 1), with an unaltered ratio of ^{3}H to ^{32}P.

The observed initial rate for the reverse reaction, expressed in terms of specific activity of fraction 7 enzyme, was 2,600 U/mg. This compared to 1,700 U/mg for the forward reaction (Table 2).

For further information on the mechanism of the reaction, the rate of isotope equilibration (exchange) between various components of the system was studied. A plausible sequence for the readily reversible synthesis of CDP-diglyceride

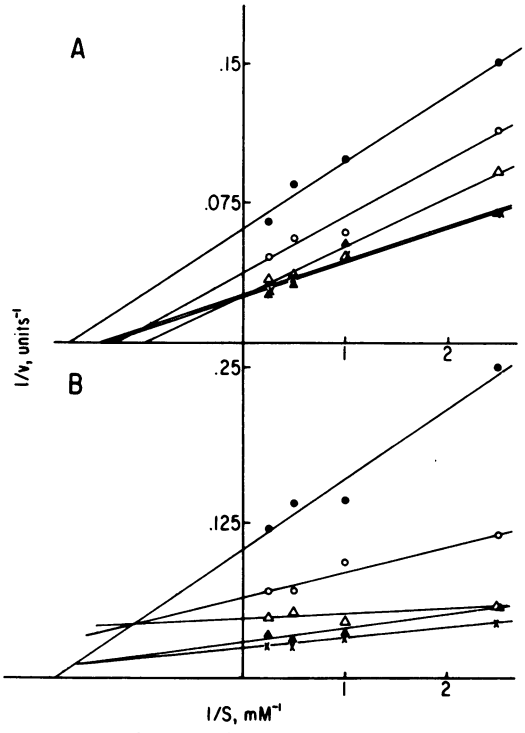

FIG. 4. *Influence of CTP and phosphatidic acid concentrations on cytidylyltransferase activity. (A) Double-reciprocal plots of reaction velocity versus phosphatidic acid concentration. Fixed CTP concentrations were 0.2 (●), 0.4 (○), 1 (△), 2 (▲), and 4 (×) mM. (B) Double-reciprocal plots of reaction velocity versus CTP concentration. Fixed phosphatidic acid concentrations were 0.2 (●), 0.4 (○), 1 (△), 2 (▲), and 4 (×) mM. For both (A) and (B), 18 μg of fraction 7 (Table 2) enzyme was present in each tube. Lines were fitted to the data points by the method of least squares.*

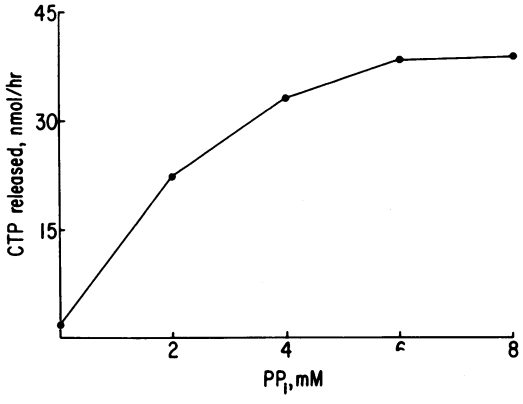

FIG. 5. *Enzyme-catalyzed pyrophosphoryolysis of CDP-diglyceride. The enzyme preparation (fraction 7, Table 2; 36 μg) was incubated as described in Materials and Methods for the reverse of the cytidylyltransferase reaction, with the indicated PP_i concentrations. [^{3}H]CTP present in the supernatant was determined after precipitation of lipid and added albumin by trichloroacetic acid (see Materials and Methods).*

might be a ping-pong mechanism (8) involving the following reactions:

$$\text{CTP} + \text{enzyme} \rightleftharpoons \text{CMP} - \text{enzyme} + \text{PP}_i \quad (1)$$

$$\text{CMP} - \text{enzyme} + \text{phosphatidic acid} \rightleftharpoons \text{CDP-diglyceride} + \text{enzyme} \quad (2)$$

The ability of the enzyme to catalyze reaction (1) was tested by incubation with [^{3}H]CTP and [^{32}P]PP$_i$. No significant ^{32}P labeling of CTP was observed (test 1, Table 4). However, when unlabeled phosphatidic acid was also added to the system, extensive exchange of the terminal pyrophosphate moiety of CTP with [^{32}P]PP$_i$ occurred. Results with [^{3}H]dCTP as substrate were similar (test 2, Table 4). The finding that exchange of PP$_i$ with CTP (or dCTP) occurred only in the presence of phosphatidic acid argues against a ping-pong mechanism such as that shown in reactions (1) and (2) and favors a sequential mechanism, in which both substrates must bind to the enzyme before the release of either product (8).

Since PP$_i$ can exchange with the pyrophosphate moiety of CTP, but no exchange occurs between the cytosine moieties of CTP and CDP-diglyceride (in the presence or absence of phosphatidic acid; test 3, Table 4), it is likely that PP$_i$ is released before CDP-diglyceride in the forward reaction sequence (cf. reference 8; whichever product shows exchange into substrate in the absence of the other is the first one released).

Test 4 of Table 4 was designed to measure isotope exchange from the perspective of the reverse reaction. Little or no exchange was observed between phosphatidic acid and CDP-dipalmitin in the presence or absence of PP$_i$.

Competition between CTP and dCTP. Figure 6 shows double-reciprocal plots for inhibition of the CTP substrate reaction with dCTP and vice versa. The apparent K_i values for CTP and dCTP from these plots were 0.48 ± 0.37 and 0.75 ± 0.23 mM, respectively, and the inhibition in each case was competitive. This supports the idea that the same enzyme binds both CTP and dCTP.

In addition, during chromatography on DEAE-cellulose, in a system essentially similar

TABLE 4. *Enzyme-catalyzed exchange reactions*[a]

Exchange tested[b]	Incubation time (min)	Concn of reaction components (mM)					Apparent exchange (%)[c]
		CTP	dCTP	PP$_i$	Phosphatidic acid	CDP-dipalmitin	
1. CTP-PP$_i$ (±phosphatidic acid) [^{3}H]C-R-P-P-P ↔ ^{32}P-^{32}P	36	2		2			0
		2		2	2		9.4[d]
		2		5			0.6
		2		5	2		12.6
2. dCTP-PP$_i$ (±phosphatidic acid) [^{3}H]C-dR-P-P-P ↔ ^{32}P-^{32}P	36		2	2			0
			2	2	2		10.0[d]
			2	5			0
			2	5	2		8.5
3. CTP-CDP-dipalmitin (±phosphatidic acid) C-R-P-P-P ↔ [^{3}H]C-R-P-P-digl	40	2				1	0.1
		2			2	1	1.0
		2				2	0
		2			2	2	0.2[e]
4. CDP-dipalmitin-phosphatidic acid (±PP$_i$) C-R-P-P-digl ↔ P-[^{14}C]digl	30				2	1	0.2
				5	2	1	0

[a] All incubations were carried out with 36 μg of fraction 7 enzyme under the conditions described in Materials and Methods.

[b] Abbreviations in the schematic representations of the exchange tests are: C, cytosine moiety; R, ribose moiety; dR, deoxyribose moiety; P, phosphate moiety in ester linkage; digl, diglyceride moiety. Radioactive labels in the molecules are indicated by ^{3}H and ^{14}C notations preceding the moieties containing the labels and by the ^{32}P notation.

[c] Methods for measuring apparent exchange are described in Materials and Methods. Values for controls with no enzyme were subtracted in every case.

[d] The ^{32}P- and ^{3}H-labeled material isolated by adsorption to charcoal (see Materials and Methods) migrated quantitatively with authentic CTP (test 1) or dCTP (test 2) during PEI-cellulose thin-layer chromatography (system 5, Table 1).

[e] Water-soluble radioactivity was identified in this case as CMP by PEI-cellulose thin-layer chromatography (system 4, Table 1). Presumably this was produced by hydrolysis of the [^{3}H]CDP-dipalmitin.

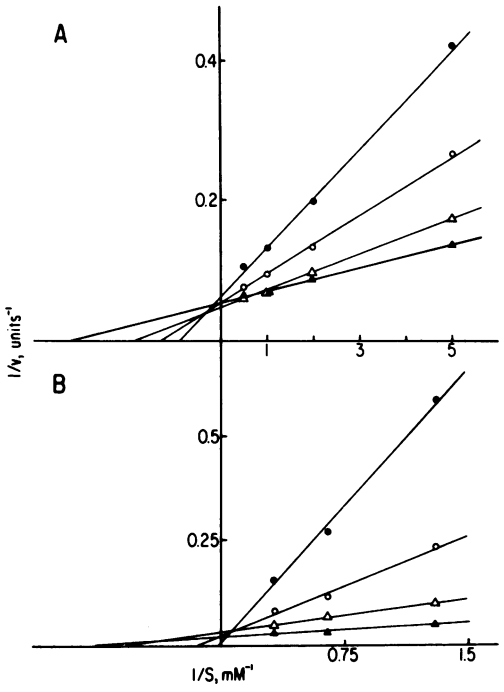

FIG. 6. *Effects of CTP and dCTP on cytidylyltransferase activity measured with dCTP and CTP (respectively) as radioactively labeled nucleotide substrate. (A) Double-reciprocal plots of reaction velocity with varied concentrations of CTP and fixed concentrations of unlabeled dCTP. The fixed dCTP concentrations were 3 (●), 1.5 (○), 0.5 (△), and 0 (▲) mM. (B) Double-reciprocal plots of reaction velocity with varied concentrations of dCTP and fixed concentrations of unlabeled CTP. The fixed CTP concentrations were 3 (●), 1.5 (○), 0.5 (△), and 0 (▲) mM. For both (A) and (B), 9 μg of fraction 7 (Table 2) enzyme was added to each tube. Lines were fitted to the data points by the method of least squares.*

to that of Fig. 1 except on a smaller scale (0.56-by 5-cm column size), it was found that introducing CTP (2 mM) into the buffer delayed elution of the enzyme (to about 0.048 M KCl in the salt gradient) without affecting the elution of bulk protein. Significantly, the activities toward both CTP and dCTP as substrates were delayed in identical manner.

Other properties. Several groups (21, 30) have reported that phospholipid synthesis in *E. coli* is subject to stringent control. In this regard, we found that guanosine 5′-diphosphate 3′-diphosphate (1.0 and 2.5 mM) did not inhibit the cytidylyltransferase.

The reverse reaction of CDP-dipalmitin pyrophosphorolysis was progressively inhibited as the CDP-dipalmitin concentration was increased. At 4.35 mM CDP-dipalmitin, no activity was measurable.

N-ethylmaleimide (6 mM) did not inhibit the cytidylyltransferase.

DISCUSSION

The CTP:phosphatidic acid cytidylyltransferase has been purified about 70-fold from cell-free extracts and freed of activities, such as CDP-diglyceride hydrolase, CTP hydrolases, and pyrophosphate hydrolase, which might interfere with characterization and kinetic analysis. If the cytidylyltransferase is present in the cell in levels comparable to those of other enzymes of phospholipid biosynthesis (17), further purification of several hundred-fold would be required for homogeneity. Attempts at further purification have been hampered by the apparent tendency of the enzyme to aggregate with itself and with other envelope proteins.

The cytidylyltransferase differs from a number of other enzymes (11, 13, 26) in that, despite being located in the inner membrane of the *E. coli* cell envelope (4, 33), it is not solubilized by most nonionic detergents (notably Triton X-100).

The specific effect of digitonin in solubilizing the enzyme is also puzzling. Digitonin has an aggregation number of 60 and micelle weight of 70,000 (12). There are some reports of its use with other particulate enzymes (16, 20), but it has been used relatively little for enzyme solubilization, and little is known about its mode of action. Advantages to the use of digitonin are that it is nonionic and that (in this case) the enzyme is very stable in its presence.

McCaman and Finnerty have reported that the cytidylyltransferase activity in another organism, *Micrococcus cerificans*, is resistant to solubilization by a variety of treatments, including detergents, phospholipases, urea, organic solvents, and salts (19). The activity from embryonic chicken brain is also resistant to solubilization (25). It is possible that digitonin would be effective in these cases.

The cytidylyltransferase in our hands shows an absolute requirement for Mg (other divalent cations were not tested), in agreement with Carter (6). The enzyme from other sources (5, 15, 19, 23, 25) also requires Mg or some other divalent cation. The preferences for K^+ and nonionic detergent reported here have been previously observed (15, 19, 23, 25).

Several lines of evidence suggest that the same enzyme utilizes both CTP and dCTP as alternative nucleotide substrates, and that it utilizes them with similar efficiency in vitro. The dependencies on assay conditions (for both the membrane-bound and partially purified activities) were similar for both substrates, and var-

ious nonspecific agents exerted similar effects in both cases. The activities toward the two substrates were purified together (Table 2). In both the crude envelope and partially purified preparations, heat treatment caused apparent alterations in activity which were parallel for the two substrates (Fig. 3). The presence of CTP during DEAE-cellulose chromatography retarded the elution of the enzyme, presumably because CTP binds to the enzyme and increases the negative-charge density of the eluting species; significantly, the activities toward both CTP and dCTP were retarded in identical fashion. There was no distinction between CTP and dCTP utilization in terms of reaction mechanism. Double-reciprocal plots of initial velocity kinetic data yielded intersecting lines indicative of a sequential mechanism, with both CTP and dCTP as nucleotide substrate, and the parallel exchange reactions

$$\text{CTP} \leftrightarrow \text{PP}_i \text{ (+ phosphatidic acid)}$$

$$\text{dCTP} \leftrightarrow \text{PP}_i \text{ (+ phosphatidic acid)}$$

could be demonstrated. K_m and V_{max} values with CTP and dCTP were virtually identical. Additional evidence that the same enzyme binds CTP and dCTP was obtained from kinetic studies which revealed that CTP and dCTP are competitive substrates.

The kinetic data for the cytidylyltransferase suggest a sequential, rather than a ping-pong, reaction mechanism (8). The exchange reaction studies support this, since exchange between dCTP/CTP and PP_i does not occur unless phosphatidic acid is present. PP_i is probably released before CDP-diglyceride in the forward reaction sequence (8; whichever product shows exchange into substrate in the absence of the other is the first one released). The lack of any exchange for the reverse reaction test involving phosphatidic acid and CDP-dipalmitin ($\pm PP_i$) is surprising; in view of the fact that high concentrations of CDP-dipalmitin inhibit the reverse reaction, it may be that CDP-diglyceride competes for the phosphatidic acid binding site (8), thus interfering with exchange. In addition to the kinetic and exchange studies, another argument against a ping-pong mechanism for the enzyme is the absence of CDP-dipalmitin hydrolysis.

Some qualifications should be noted concerning these analyses of mechanism: first, such analyses are ideally done with homogeneous enzyme preparations; however, our partially purified preparation appears suitable because it is free of interfering activities such as CDP-diglyceride hydrolase, CTP hydrolases, and PP_i hydrolase; second, the application of classical kinetic analysis can provide useful insights, but may be an oversimplification for enzymes utilizing lipid substrates incorporated in detergent micelles; third, we have not rigorously proved that the same enzyme catalyzes the forward, reverse, and exchange reactions described; and finally, we cannot rule out the possibility that the need for phosphatidic acid in the CTP-PP_i exchange is an allosteric effect rather than a true mechanistic requirement.

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