Cytidine triphosphate:phosphatidic acid cytidyltransferase in *Escherichia coli*

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ABSTRACT An enzyme has been found in particulate fractions of *Escherichia coli* that catalyzes the incorporation of cytidine triphosphate (CTP) into lipid in the presence of exogenous phosphatidic acid and Mg^{++}. The product has been identified enzymatically and by chromatography as cytidine diphosphate diglyceride. The reaction is optimal at a pH of 6.5 and Mg^{++} concentration of 5–10 mM. The apparent K_m for CTP is 7×10^{-4}M and for phosphatidic acid, 2×10^{-3}M. The reaction rate falls off rapidly with time and ceases entirely after 1 hr as the result of inactivation of the system by Mg^{++}.

SUPPLEMENTARY KEY WORDS CDP diglyceride · phospholipid biosynthesis · physical state of reaction mixture

CYTIDINE diphosphate diglycerides (CDP diglycerides) are liponucleotides that play a central role in the biogenesis of complex lipids. CDP diglycerides have been identified as the immediate precursors of phosphatidyl inositol (2, 3) and phosphatidyl glycerophosphate (4) in mammalian tissues, and of phosphatidyl serine (5) and phosphatidyl glycerophosphate (5, 6) in bacteria. In *Escherichia coli* CDP diglycerides are thought (5, 7) to be obligatory intermediates in the de novo synthesis of all complex phospholipids.

Chang and Kennedy (7) have shown by pulse-labeling experiments in intact *E. coli* that phosphatidic acid is formed rapidly and has a high rate of turnover. Presumably it serves as a precursor for other glycerophosphatides by first reacting to form CDP diglyceride according to the following reaction.

Phosphatidic aicd + CTP ⇄ CDP diglyceride + P–P_i (1)

Enzymes that catalyze this reaction in guinea pig liver (8) and in embryonic chick brain (9) have recently been described, but a similar enzyme has not been demonstrated in bacteria.

We describe here an enzyme in membrane fractions from *E. coli* that catalyzes reaction 1. Although we have been unable to demonstrate net synthesis of CDP diglyceride, it seems likely that this enzyme, CTP: phosphatidic acid cytidyltransferase (cytidyltransferase), is required for de novo phospholipid biosynthesis in this organism.

MATERIALS AND METHODS

Reagents

Cytidine nucleotides were products of Schwarz Bio-Research, Inc., Orangeburg, N.J. Phosphatidic acid was prepared from freshly purified egg lecithin (10) by a modification of the method of Kates (11), with carrot chloroplasts as the source of phospholipase D; final purification was obtained by elution of the free phosphatidic acid from silicic acid columns with 5% methanol in chloroform. It was stored as the Tris salt in chloroform at −15°C under N_2 and gave a single spot on silica gel thin-layer chromatography in chloroform–pyridine–formic acid 50:20:7, with an R_f identical with that of synthetic dipalmitoyl phosphatidic acid. The phosphatidic acid had an ester:phosphorus ratio of 2.10:1. Before use chloroform was removed under a stream of N_2 and the lipid was suspended in water at the desired concentration by brief sonication.

Synthetic dipalmitoyl phosphatidic acid and CDP dipalmitin were kindly donated by Dr. E. P. Kennedy.

A preliminary account of this work has been published (1).

Abbreviations: CMP, CDP, and CTP, cytidine monophosphate, diphosphate, and triphosphate, respectively; cytidyltransferase, cytidine triphosphate: phosphatidic acid cytidyltransferase.

Enzyme

E. coli ML 308 organisms were grown on a rotary shaker at 37°C in a minimal salts medium (12) with succinate as the carbon source. Cells were grown overnight from a small inoculum and harvested in log phase. After rapid chilling the cells were centrifuged and washed once with ice-cold 0.01 M potassium phosphate buffer at pH 6.5. The washed cells from 500 ml of culture were suspended in 10–20 ml of ice-cold buffer and disrupted with 4–5 min of sonic irradiation from a Branson model S75 sonifier. Intact cells were removed by centrifugation at 3000 *g* for 10 min. The resulting supernatant fraction was centrifuged for 30 min at 100,000 *g* in a Spinco model L ultracentrifuge. The pellet was washed once by suspension in cold buffer and recentrifugation, and finally suspended by hand homogenization in 5 ml of cold buffer. Although such preparations retained some enzyme activity upon storage at 4°C, fresh cells were routinely grown for each experiment. Typical pellet suspensions prepared in this manner contained 1–3 mg of protein per ml.

In the preparation of "spheroplast" membranes, log phase cells were harvested and washed once in 0.1 M Tris buffer (pH 8.0) containing 20% sucrose, and then resuspended in the same medium at $^1/_{10}$ the original volume. Neutralized EDTA was added to a final concentration of 0.001 M, the cells were warmed to 37°C, and lysozyme (Nutritional Biochemicals Corp.) was added to a final concentration of 0.1 mg/ml. After a 15 min incubation, the "spheroplasts" were harvested by centrifugation at 10,000 *g* for 15 min, the supernate was discarded, and the tube was carefully wiped dry. The cells were lysed by vigorous suspension of the pellet in 10 ml of cold dilute buffer (potassium phosphate 0.01 M, pH 6.5) and the lysate was centrifuged for 60 min at 100,000 *g*. The supernate was decanted and sonicated briefly to fragment the viscous DNA; the pellet was washed by suspension and recentrifugation and finally suspended in 10 ml of buffer. Recovery of "pellet" protein by this method was similar to that from sonication, but recovery of "supernatant" protein was only $^1/_3$ that from sonicated cells, which indicates considerable loss during the lysozyme incubation.

Enzyme Assay

The assay was identical with that previously described (8) and measured the incorporation of CTP-^{3}H into chloroform-extractable material. Final volume of the assay was typically 0.5 ml and, except where indicated, all components (including enzyme) except the cation were premixed and warmed to 37°C; the reaction was initiated by the addition of Mg^{++}. Assays were always run in duplicate, and zero time controls repeatedly gave negligible values. Assays routinely contained 0.1–0.4 mg of pellet protein.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (13); lipid phosphorus, by Bartlett's procedure (14) as modified by Newman, Lui, and Zilversmit (15); and esters, by the method of Stern and Shapiro (16).

RESULTS

Particulate fractions obtained from sonically disrupted *E. coli* incorporated CTP-^{3}H into lipid (Table 1). Under the conditions of the assay, incorporation of radioactivity into chloroform-extractable material was entirely dependent upon added phosphatidic acid and required the addition of Mg^{++} salts (Table 1).

Specificity of the Reaction for CTP

Since net synthesis of lipid was not demonstrated (see below), it was important to rule out the possibility of a simple exchange reaction by which CMP may be incorporated into lipid (3). Under conditions in which CTP was readily converted to a lipid form, no incorporation of CMP-^{3}H could be demonstrated (Table 2).

Identification of the Product as CDP Diglyceride

An incubation was carried out for 1 hr at 37°C with twice the usual volumes of reagents. The final washed chloroform phase, containing labeled lipid product, unreacted phosphatidic acid, and bacterial pellet lipids, was dried under N_2 and redissolved in a small volume

TABLE 1 INCORPORATION OF CTP-^{3}H INTO LIPID BY PARTICULATE ENZYME IN *E. coli*

Omissions from Complete System	CTP-^{3}H Incorporated into Lipid
	mμmoles
None	2.8
$MgCl_2$	<0.1
Phosphatidic acid	<0.1

The complete system contained potassium phosphate buffer (0.16 M), phosphatidic acid (2 mM), CTP-^{3}H (2 mM) with a specific activity of 523 cpm/mμmole, 0.2 ml of *E. coli* particulate fraction and $MgCl_2$ (4 mM) in a final volume of 0.5 ml. Incubation was for 1 hr at 37°C.

TABLE 2 CYTIDINE NUCLEOTIDE SPECIFICITY

Nucleotide Added	Nucleotide-^{3}H Incorporated Into Lipid
	mμmoles
CTP-^{3}H (1 μmole)	2.8
CMP-^{3}H (1 μmole)	0.1

The incubation was identical to that in Table 1, except CMP-^{3}H (specific activity 294 cpm/mμmole) was substituted for CTP-^{3}H where indicated.

of chloroform. Aliquots were applied to two silica gel thin-layer plates beside synthetic CDP dipalmitin. After development of the chromatograms the plates were divided into 1 cm strips. The silicic acid was scraped directly into counting vials and suspended in a gel counting solution consisting of 0.4% PPO (2,5-diphenyloxazole), 0.005% POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene), and 3% thixotropic gel powder (Cab-O-Sil, Packard Instrument Co., Inc.) in toluene; radioactivity was determined in a Packard liquid scintillation counter. By this method the cytidine-containing lipid was shown to cochromatograph with synthetic CDP dipalmitin in chloroform–methanol–acetic acid–water 50:28:4:8 and in isobutyl ketone–acetic acid–water 40:30:7 with an R_f of 0.61 and 0.27, respectively.

The labeled lipid material was also identified enzymatically. Kanfer and Kennedy (5) described a soluble enzyme in *E. coli* that catalyzes the following reaction.

$$\text{CDP diglyceride} + \text{L-serine} \rightarrow \text{phosphatidyl serine} + \text{CMP} \quad (2)$$

Since the lipid product to be identified was labeled in the cytidine moiety, if it is CDP diglyceride there should be a serine-dependent loss of ^3H from the lipid phase in the presence of the enzyme that catalyzes reaction 2.

An experiment to test this is described in Table 3. With a crude ammonium sulfate fraction of soluble protein from *E. coli* as enzyme source (5), there was almost complete loss of radioactivity from lipid in the presence of L-serine. Unlabeled CDP dipalmitin was required as "carrier" to prevent serine-independent breakdown of the labeled lipid, presumably by nonspecific phosphodiesterase. This experiment confirms the chromatographic identification of the cytidine lipid as CDP diglyceride.

pH Optimum

The enzyme had optimal activity at a pH of 6.5 (Fig. 1). Above 7.0 and below 5.8 the rate of reaction dropped rapidly.

TABLE 3 ENZYMATIC IDENTIFICATION OF LABELED LIPID PRODUCT

Tube	Lost from Lipid Phase
	cpm
1. L-Serine omitted	0
2. Complete	691

An aliquot of chloroform phase containing 826 cpm of labeled product from an experiment similar to that described in Table 1 was added to each of two tubes and the chloroform removed under N_2. To each tube we then added 1 μmole of CDP dipalmitin, 10 μmoles of Na_2SO_4, 0.02 ml of octanol, and approximately 1 mg of enzyme in a total volume of 1 ml. Tube 2 also contained 1 μmole of L-serine. After incubation for 1 hr at 37°C the lipid fraction was reisolated and radioactivity was determined.

Cation Requirement

As demonstrated above (Table 1), the synthesis of CDP diglyceride showed an absolute requirement for added divalent cation. Mg^{++} gave the highest activity but was somewhat inhibitory at high levels (Fig. 2). Mn^{++} could substitute for Mg^{++} but gave only $^1/_4$–$^1/_3$ the rate observed with Mg^{++}; Ca^{++} and the monovalent cations Na^+, K^+, and NH_4^+ were virtually inactive. In preliminary experiments carried out with Tris buffer at a pH of 7.4 (data now shown), some incorporation of CTP-^3H into lipid was observed when high concentrations (0.1–0.2 M) of K^+ or NH_4^+ (but not of Na^+) were

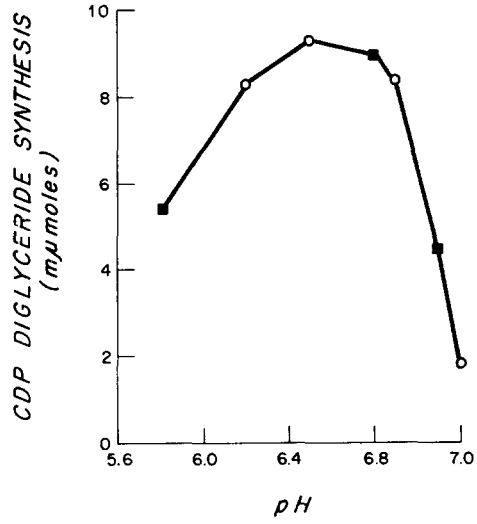

FIG. 1. Effect of pH upon CDP diglyceride synthesis. Each tube contained potassium phosphate buffer (0.2 M), phosphatidic acid (1 mM), CTP-^3H (1 mM), 0.2 ml of enzyme, and $MgCl_2$ (4 mM). The pH was determined directly on the incubation mixture. Tubes were incubated for 30 min at 37°C. Results of two separate experiments are shown; the absolute activities were corrected to agree at a common pH.

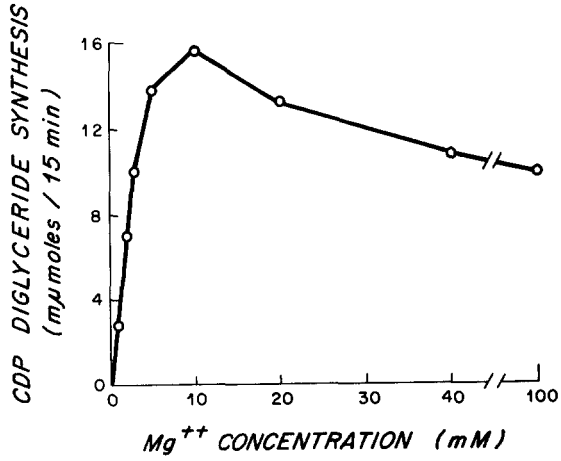

FIG. 2. Effect of Mg^{++} concentration. Incubations as in Table 1 except that imidazole buffer of pH 6.5 was used. The assay was carried out for 15 min at 37°C.

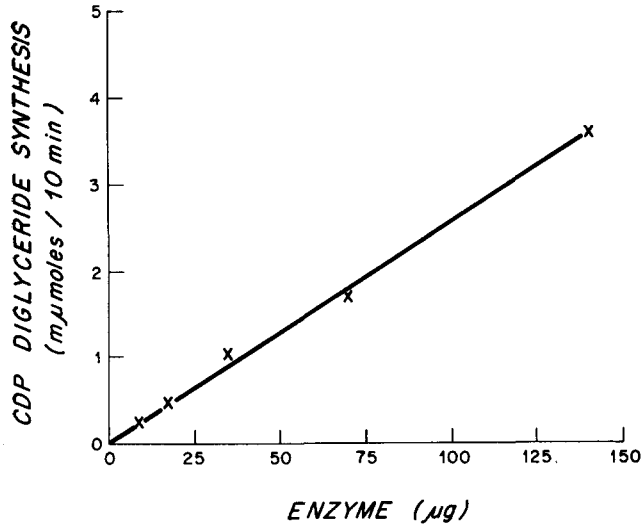

FIG. 3. Effect of enzyme concentration. The assay mixture was identical with that in Table 1 except that the concentration of $MgCl_2$ was 20 mM. Incubation was for 10 min at 37°C.

used in place of Mg^{++}; this effect of monovalent cations was markedly reduced at the optimal pH of the reaction.

Effect of Enzyme and Substrate Concentrations

The initial rate of synthesis of CDP diglyceride was a linear function of enzyme concentration over a wide range (Fig. 3).

The rate of reaction increased with increasing substrate concentration at low levels, but at sufficiently high concentrations both substrates were slightly inhibitory (Fig. 4). Approximate K_m values for each of the substrates were determined from double reciprocal plots of the data. With CTP constant at 10^{-3}M, the apparent K_m for phosphatidic acid was 2×10^{-3}M. With phosphatidic acid constant at 2×10^{-3}M, the K_m for CTP was 7×10^{-4}M.

Location of Enzyme

Initial experiments to locate the enzyme were done with high-speed supernatant and pellet fractions prepared from sonicates of *E. coli*. Under these conditions activity was repeatedly observed in both fractions (Table 4) although the absolute and relative amounts varied greatly from preparation to preparation. In an attempt to obtain membrane fractions under gentler conditions, spheroplast lysates were prepared. With these preparations activity was observed only in the "pellet" fraction (Table 4); however, it should be noted that there was much lower recovery of "soluble" protein by this method (see Methods), and selective loss of a soluble enzyme that is separate from the particle-bound enzyme cannot be ruled out. Because of uncertainty as to the significance of the "soluble" enzyme activity, all studies described here were done on pellet fractions prepared after sonication of the cells.

Time Course of the Reaction

The incorporation of CTP-^{3}H into lipid was repeatedly observed to be linear with time over only 10–20 min and to stop entirely after 1 hr (Fig. 5). Chloroform extraction of the reaction mixture after a 1 hr incubation and chromatography of the lipids showed no significant loss of phosphatidic acid (data not shown). However, the addition of fresh phosphatidic acid at this point led to a further burst of synthesis (Table 5) at almost the initial rate; additional CTP had a slight stimulatory effect, and

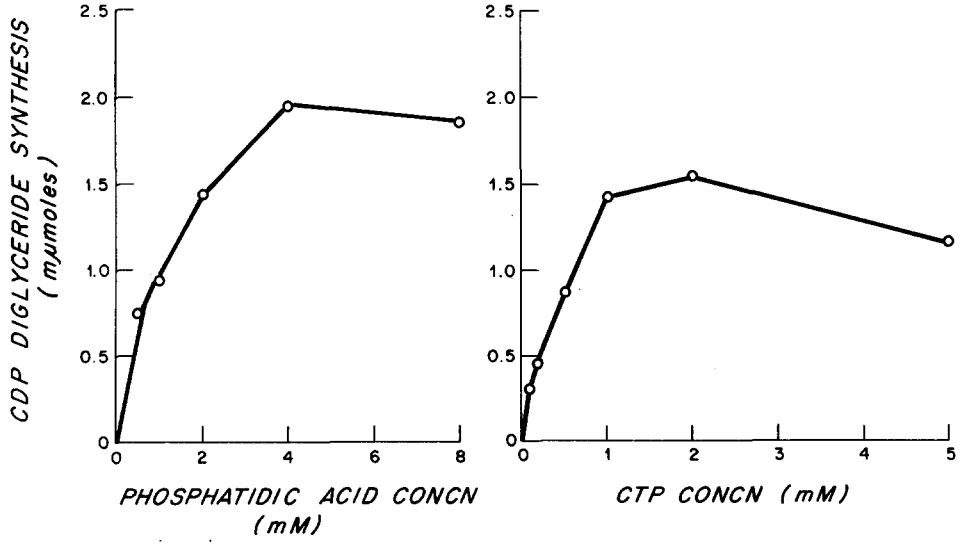

FIG. 4. Effect of substrate concentration. The concentration of phosphatidic acid was varied with CTP constant at 1 mM (left) and that of CTP was varied with phosphatidic acid constant at 2 mM (right). Other conditions as in Table 1. Incubation was for 20 min at 37°C.

TABLE 4 LOCATION OF CYTIDYLTRANSFERASE ACTIVITY IN *E. coli*

Method of Preparing Enzyme	Fraction Tested	CTP-^{3}H Incorporated into Lipid
		mμmoles
Sonication	Supernate	0.7
	Pellet	1.5
Spheroplast lysis	Supernate	0
	Pellet	1.3

The fractions were prepared (see Methods) so that equal volumes represented the yield from equal numbers of cells. 0.1 ml of each fraction was tested in a mixture identical to that in Table 1. Incubation was for 30 min at 37°C.

TABLE 5 LIMITING REAGENT IN THE TIME COURSE OF CDP DIGLYCERIDE SYNTHESIS

Tube	Additions at 60 min	CTP-^{3}H Incorporated into Lipid	
		0–60 min	60–120 min
		mμmoles	
1	None	2.7	—
2	None	—	<0.1
3	Phosphatidic acid, 0.5 μmole	—	2.2
4	CTP, 0.5 μmole	—	0.5
4	Enzyme, 0.2 ml	—	<0.1

Five identical pairs of tubes were incubated, each tube containing potassium phosphate of pH 6.5 (0.2 M), phosphatidic acid (1 mM), CTP-^{3}H (1 mM) with a specific activity of 140 cpm/mμmole, 0.2 ml of enzyme preparation, and $MgCl_2$ (4 mM). At 1 hr the reaction was stopped in one set; additions were made to the others, as indicated, and the reaction was permitted to proceed for another hour. Synthesis during the 2nd hr was determined as the difference between the total cpm and that observed at 1 hr.

fresh enzyme alone led to no increase in synthesis. This anomalous behavior is apparently the result of the gradual formation of insoluble Mg phosphatidate (see below). To explain the effects noted above, we performed an experiment in which either enzyme or phosphatidic acid was preincubated with Mg^{++} and CTP for 1 hr before addition of the remaining component (Table 6). In each case there was complete loss of activity. That this was not due simply to heat inactivation of the enzyme was demonstrated in the control tube in which phosphatidic acid and enzyme were preincubated for 1 hr, and Mg^{++} was then added. Under these conditions no loss of activity was observed.

The rate at which the inhibitory Mg effect became manifest was dependent on the cation concentration. At the usual concentration (4 mM) used in these studies, no loss of activity was observed when Mg^{++} was added shortly before the enzyme (Table 7), although preincubation for 1 hr before enzyme addition led to complete loss of activity (Table 6). However, at much higher cation concentrations (40 mM), over 90% of the control

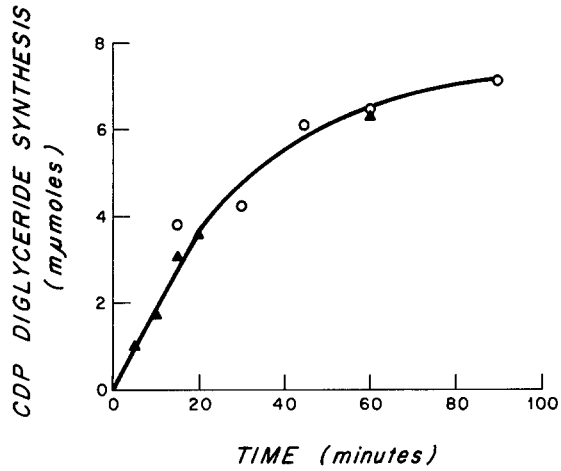

FIG. 5. Time course of CDP diglyceride synthesis. Incubation conditions as in Table 4. Results of two separate experiments are shown.

TABLE 6 EFFECT OF Mg^{++} ON CYTIDYLTRANSFERASE ACTIVITY

Preincubation	Additions at Zero Time	CDP Diglyceride Synthesis
		mμmoles
None	Other reagents, then enzyme, then Mg^{++} (4 mM)	6.2
Complete system minus Mg^{++}	Mg^{++} (4 mM)	6.6
Complete system minus enzyme	Enzyme	0.1
Complete system minus phosphatidic acid	Phosphatidic acid	0.1

The complete system contained potassium phosphate buffer of pH 6.5 (0.2 M), phosphatidic acid (1 mM), CTP-^{3}H (1 mM), $MgCl_2$ (4 mM), and 0.2 ml of enzyme in a final volume of 0.5 ml. Tubes with the indicated omissions were preincubated at 37°C for 1 hr, the missing component was then added, and the reaction was allowed to proceed for 30 min. Results are compared with those for a control tube (no preincubation). Since the reaction shows a complete dependence on added phosphatidic acid, enzyme, and cation, no synthesis of product could occur during the preincubation in any of the tubes.

activity was lost when Mg^{++} was added immediately before rather than after the enzyme (Table 7).

DISCUSSION

As described by Kennedy and coworkers (5, 6), the biosynthesis of phospholipids in *E. coli* follows two pathways, both dependent on CDP diglyceride.

CDP diglyceride + L-serine → phosphatidyl serine + CMP (2)

CDP diglyceride + α-glycerophosphate → phosphatidyl glycerophosphate + CMP (3)

Phosphatidyl serine formed in reaction 2 is decarboxylated to yield phosphatidyl ethanolamine (5), the princi-

TABLE 7 EFFECT OF Mg^{++} CONCENTRATION ON INACTIVATION OF CYTIDYLTRANSFERASE

Order of Addition of Reagents	CDP Diglyceride Synthesis
	mμmoles
A	
(1) Other reagents	
(2) Enzyme	6.2
(3) Mg^{++} (4 mM)	
B	
(1) Other reagents	
(2) Mg^{++} (4 mM)	5.9
(3) Enzyme	
C	
(1) Other reagents	
(2) Enzyme	
(3) Mg^{++} (40 mM)	11.4
D	
(1) Other reagents	
(2) Mg^{++} (40 mM)	1.0
(3) Enzyme	

Buffer, CTP, and phosphatidic acid were mixed as in Table 6, then 0.2 ml of enzyme and sufficient $MgCl_2$ to give the indicated final concentration were added at 1 min intervals. Incubation was for 30 min at 37°C.

pal phosphatide of *E. coli* (17). This lipid does not show a rapid turnover (18) and presumably functions as a stable component of bacterial membrane systems. Phosphatidyl glycerophosphate from reaction 3 is dephosphorylated to phosphatidyl glycerol (19). The phosphatidyl glycerol formed in growing *E. coli* has been shown by pulse-labeling experiments to turn over rapidly (18), although its ultimate fate remains obscure. Stanacev, Chang, and Kennedy (20) have described an enzyme in *E. coli* capable of converting this lipid to cardiolipin, while in gram-positive organisms Lennarz, Nesbitt, and Reiss (21) have demonstrated the synthesis of *O*-aminoacyl phosphatidyl glycerols from phosphatidyl glycerol plus aminoacyl–sRNA.

In mammalian liver, phosphatidyl ethanolamine is formed by the reaction of 1,2-diglycerides with CDP ethanolamine (22). This pathway appears to be inoperative in *E. coli*; experiments (7) specifically designed to demonstrate the pool of metabolically active precursors suggested that in *E. coli* phosphatidic acid and not diglyceride is the obligatory intermediate in glycerophosphatide synthesis. The physiological role of the diglyceride kinase described by Pieringer and Kunnes (23) remains unclear. The synthesis of CDP diglyceride from phosphatidic acid therefore appears to be an obligatory step in phospholipid biosynthesis in *E. coli*.

Although net formation of CDP diglyceride has not been demonstrated in these experiments, it seems likely from the foregoing considerations that the enzyme described takes part in the de novo biosynthesis of phospholipids in *E. coli*. Failure to observe incorporation of CMP-^{3}H (Table 2) and the absolute dependence of the reaction upon exogenous phosphatidic acid (Table 1) rule out the possibility of a simple exchange reaction such as that described by Paulus and Kennedy (3) in mammalian tissues.

An unresolved problem is the finding of significant amounts of enzyme activity in high speed supernatant fractions prepared from bacterial sonicates but not in similar fractions prepared from "spheroplast" lysates. It is possible that the vigorous sonication used to disrupt the cells releases some of the particulate enzyme. Conversely, a truly soluble enzyme may be lost during the process of spheroplast formation in a manner analogous to the release of certain enzymes by "cold shock" (24).

Of particular interest to us is the striking importance of the physical state of the in vitro assay system, a finding similar to that noted with the guinea pig cytidyltransferase (8). Mg^{++} apparently plays a crucial role in both systems. The experiment described in Table 6 suggests that the cation can have two separate effects that inhibit the reaction. Preincubation of the system without enzyme presumably leads to the formation of insoluble Mg salts of phosphatidic acid (a gross precipitate is observed) which can no longer react with the enzyme. Preincubation of enzyme and Mg^{++} (accompanied again by a visible increase in opacity) also inactivates the system; this suggests that Mg^{++}-induced aggregation of the particles, well known in other membrane-containing systems (25), prevents penetration of the lipid substrate to the active site. Phosphatidic acid apparently protects the membrane-bound enzyme against this effect of Mg^{++}. While particulate enzyme alone exposed to 4 mM Mg^{++} is inactivated (Table 6), similar preparations retain almost full activity when the incubation with Mg^{++} is carried out in the presence of phosphatidic acid (Table 5).

The nature of the interaction between particulate enzyme and phosphatidic acid is obscure but the interaction is apparently essential to the reaction in vitro. That this interaction is very rapid can be demonstrated at high Mg^{++} concentrations (Table 7). When additions are made at intervals as short as 1 min, full activity is observed if the enzyme and phosphatidic acid are premixed before cation addition, while >90% loss of activity occurs when the enzyme is added last. More prolonged preincubation of enzyme and lipid does not enhance this reaction (Table 6) when tested at lower (4 mM) Mg^{++} levels.

The unusual features of the cytidyltransferase reaction may, of course, represent in vitro artifacts. However, it is hoped that further experiments may help elucidate the mechanisms by which membrane-bound enzymes function in vivo.

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