Partial Purification and Properties of Phosphatidylserine Synthetase from \textit{Escherichia coli}

\textbf{SUMMARY}

CDP-diglyceride: L-serine phosphatidyltransferase (phosphatidylserine synthetase) of \textit{Escherichia coli} is tightly associated with ribosomes in crude cell-free extracts. The synthetase has now been separated from ribosomes by extraction with solutions containing 5 M NaCl and has been purified 100-fold. The partially purified enzyme is devoid of contaminating hydrolytic activities and nearly free of RNA. The enzyme catalyzes exchange reactions of CMP with CDP-diglyceride, and of serine with phosphatidylserine. Furthermore, the enzyme catalyzes the formation of CDP-diglyceride from phosphatidylserine and CMP, although the equilibrium strongly favors synthesis of phosphatidylserine. A phosphatidyl-enzyme intermediate may thus be involved in the action of this enzyme. Under the conditions employed in the assay system, however, this intermediate seems to be unstable, since the synthetase also hydrolyzes phosphatidylserine and CDP-diglyceride at a slow rate.

Most of the phosphatidylserine synthetase activity found in extracts of \textit{Escherichia coli} is firmly bound to ribosomes (1). This enzyme therefore differs strikingly from other enzymes of phospholipid metabolism, all of which are attached to the inner, cytoplasmic membrane (1-4). Enzymes for the synthesis of phospholipids from gram-positive bacteria and from mammalian tissues are also generally associated with membranous structures (5, 6).

In the present work the interaction of the enzyme with ribosomes has been examined in greater detail. The synthetase can be dissociated from the bulk of the ribosomal RNA by cesium chloride density-gradient centrifugation (7, 8), or by extraction into aqueous polyethylene glycol in the presence of 5 M NaCl (9, 10).

We have obtained preparations of phosphatidylserine synthetase which are enriched over 100-fold relative to crude extracts and are free of contaminating hydrolytic activities. This permitted a study of certain catalytic features of the enzyme, not previously feasible. The synthetase catalyzes exchange reactions both of CMP with CDP-diglyceride and of L-serine with phosphatidylserine. The formation of phosphatidylserine from CDP-diglyceride and L-serine appears to be reversible. These observations suggest the possibility that the synthesis of phosphatidylserine proceeds through a phosphatidyl-enzyme intermediate, as shown below (Equations 1 and 2). Since the purified synthetase also appears to catalyze the cleavage of phosphatidylserine and of CDP-diglyceride, the phosphatidyl-enzyme intermediate presumably reacts with water at a slow rate in the absence of serine or CMP (Equation 3).

\begin{align*}
\text{CDP-diglyceride} + E &\rightleftharpoons \text{phosphatidyl-enzyme} + \text{CMP} \quad (1) \\
\text{Phosphatidyl-enzyme} + \text{L-serine} &\rightleftharpoons \text{phosphatidylserine} + E \quad (2) \\
\text{Phosphatidyl-enzyme} + \text{H}_2\text{O} &\rightarrow \text{phosphatidic acid} + E \quad (3)
\end{align*}

\textbf{EXPERIMENTAL PROCEDURE}

**Materials and Methods**—CDP-dipalmitin was prepared as described previously (11) from dipalmitoylphosphatidic acid. Triton X-100 (octyphenoxypolyethoxylate) was obtained from Rohm and Haas, Philadelphia. Enzymatic Synthesis of CDP-Dipalmitin, Trinitiated in Cytidine Motely—CDP-dipalmitin (0.33 mM) and [\text{G-3H}] CMP (0.33 mM and 100 cpm per amole) were incubated for 4 hours at 37°C with 3 mg of partially purified phosphatidylserine synthetase (Table I, Fraction 3) in a final volume of 6 ml. The reaction mixture also contained 0.1 mM potassium phosphate of pH 7.4 and 0.1% Triton X-100. The incubation was terminated by the addition of 4.5 ml of chloroform-methanol (2:1, v/v) and was acidified with 0.4 ml of 0.1 M HCl. The system was thoroughly mixed, and the phases were separated by a brief centrifugation at 25°C, as described previously for the chemical preparation of cytidine diphosphate diglyceride (11). The chloroform phase (3 ml) was washed twice with 30-ml portions of distilled water without disturbing the interface at which protein and some CDP-diglyceride had precipitated. The washed chloroform phase and the residual water above the interface (2 ml) were then dissolved with 4 ml of methanol, forming a single solvent phase. Precipitated protein was removed at this stage by a 10-min centrifugation at 3000 x g. About 20% of the radioactive CMP originally included in the incubation mixture was recovered in the washed chloroform phase.

Over 97% of the tritiated lipid obtained in this manner chromatographed with chemically synthesized CDP-dipalmitin on Silica Gel F thin layer plates (E. Merck, Darmstadt) in the solvent system chloroform-methanol-water-acetic acid (25:15:4:2, v/v). Final purification of the tritiated CDP-diglyceride was achieved by chromatography on a column of DEAE-cellulose under the conditions described previously (11). An additional 2 mg of CDP-dipalmitin were added as carrier just prior to the chroma-
Purification of phosphatidylserine synthetase of Escherichia coli

<table>
<thead>
<tr>
<th>Fraction</th>
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<th>Total protein</th>
<th>Specific activity</th>
<th>Yield %</th>
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<td>4. Polymer partitioning</td>
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<td>5. (NH4)2SO4</td>
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<td>6. Phosphocellulose</td>
<td>1700</td>
<td>110</td>
<td>1310</td>
<td>22</td>
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</tbody>
</table>

*a Done in portions as described in the text.*

Enzymatic Synthesis of (Dipalmitoyl)phosphatidyl[1-14C]Serine—CDP-dipalmitin (0.23 mM) and DL-[1-14C]serine (1 mM and 600 cpm per nmole) were incubated for 6.5 hours at 37° with 0.2 mg of partially purified phosphatidylserine synthetase (Fraction 6, Table I) in a final volume of 60 ml. The incubation mixture also contained 0.1 M potassium phosphate of pH 7.4 and 0.1% Triton X-100. The reaction was terminated by the addition of 48 ml of chloroform-methanol (2:1, v/v) and 8 ml of 1 N HCl. The phases were mixed and the chloroform layer was extracted with distilled water as described above for the preparation of tritiated CMP. Seventy per cent of the CDP-dipalmitin present initially was converted to (dipalmitoyl)phosphatidyl-[1-14C]serine.

The radioactive phosphatidylserine was further purified by chromatography on DEAE-cellulose at 4° under conditions identical with those previously described for CDP-dipalmitin (11). The product obtained in this manner had a ratio of ester to serine of 1.5:1. The product was over 95% pure on a radiochemical basis when chromatographed on Silica Gel F thin layer plates (E. Merck, Darmstadt) in the solvent chloroform-methanol-water-acetic acid (25:15:4:2, v/v). Ninhydrin and phosphate spray reagents (12) revealed a single component coincident with the major peak of radioactivity. When the product was treated with partially purified phosphatidylserine deacylase from E. coli (13) more than 90% of its radioactivity was released as CO2. The final preparation of phosphatidylserine was stored as the Tri salt in aqueous suspension. It was stable for several months at 0° or when frozen at -20°.

Enzyme Assays—Phosphatidylserine Synthetase—This activity was measured as described previously (1), except that all incubations were performed at 37°, and bovine serum albumin (0.1 mg per ml) was included in the final incubation mixture when purified preparations were assayed. This level of albumin has no effect on the activity of crude fractions of the synthetase.

CDP-Diglyceride Exchange—Three general methods were used to assay this reaction. In Method I the incorporation of tritiated CDP into lipid was measured directly, whereas in Method II the loss of radioactivity from water-soluble CDP was determined. Method III, which was only feasible with the purified enzyme, depended on the release of water-soluble, tritiated CDP from labeled CDP-diglyceride, stimulated by nonradioactive CDP-diglyceride (15). In all cases a unit of activity was defined as the amount of enzyme which catalyzed 1 nmole of exchange per min.

CDP-Diglyceride Hydrolase—CDP-dipalmitin, labeled with tritium in the cytidine moiety, was incubated with hydrolase and the release of water-soluble CDP was measured (15). The assay mixture contained 0.25 ml of CDP-dipalmitin (0.33 mM per nmole), 0.1 M potassium phosphate of pH 7.4, 0.1% Triton X-100, and enzyme in a final volume of 0.06 ml. Incubations were performed at 37° for 20 min in plastic tubes, and the reaction was terminated by the addition of 3 ml of chloroform-methanol (2:1, v/v), containing 0.01 M HCl. After the further addition of 2 ml of water, the phases were thoroughly mixed. The water-soluble CDP formed was determined from the radioactivity of the aqueous layer, units of activity being expressed as nmole per min. With enzyme preparations purified more than 100-fold, bovine serum albumin was included in the incubation mixture at a level of 0.7 mg per ml, an amount of protein which had no effect on the activity of crude hydrolase fractions.

Serine-Phosphatidylserine Exchange—A procedure analogous to Method I for CDP-exchange was employed in measuring this activity. (Dipalmitoyl)phosphatidyl-[1-14C]serine (600 cpm per nmole) was used as the substrate. Each incubation contained 0.33 mM (dipalmitoyl)phosphatidyl[1-14C]serine, 0.6 M NaCl, 0.1 mg per ml of bovine serum albumin, 0.1% Triton X-100, 30 mM potassium phosphate, and 10 mM Tris HCl of pH 7.4, in a final volume of 0.06 ml. The release of water-soluble [14C]from phosphatidylserine after a 60-min incubation at 37° was measured in the absence and in the presence of 0.33 mM L-serine. The difference was taken as a measure of serine-phosphatidylserine exchange. The rate of exchange was 4 to 5 times greater than the rate of hydrolysis observed in the absence of added L-serine. The amount of water-soluble radioactivity formed during the incubation was determined as described for CDP-diglyceride hydrolase.

A buffer system different from that employed in all other assays was used for serine-phosphatidylserine exchange, because of the solubility properties of (dipalmitoyl)phosphatidylserine. Small amounts of glycerol or 2-mercaptoethanol, which were sometimes added with the enzyme sample, did not interfere with the assay. There was no hydrolysis of phosphatidylserine in the absence of added enzyme, even after prolonged incubation at 37°.

A unit of activity was defined as described above.

Other Procedures Protein concentration was determined by the method of Lowry et al. (16) and, in some cases, by a modification applicable to solutions containing polyethylene glycol and 2-mercaptoethanol (10).

RESULTS

Association of Phosphatidylserine Synthetase with Purified Ribosomes—The method of Trnub et al. (8) was used to purify ribosomes from frozen cells of E. coli B. Phosphatidylserine synthetase was enriched about 2.5-fold in washed ribosomal preparations relative to extracts of broken cells. When the
purified ribosomes were sedimented in sucrose gradients containing 10 mM MgCl₂, synthetase sedimented with the ribosomal material. Thus the association of the enzyme with ribosomes, noted previously (1), is not disrupted when ribosomes are isolated and washed for use in cell-free protein synthesis. When purified ribosomes were dissociated into 50 S and 30 S subunits in buffers containing 0.1 mM MgCl₂, enzymatic activity was present on both subunits (data not shown), as previously observed in crude preparations (1).

**Dissociation of Phosphatidylserine Synthetase from Ribosomes in Presence of CsCl**—About 40% of the “intrinsic” proteins of ribosomes (the “split protein” fraction) can be removed by isopycnic density-gradient centrifugation in CsCl (7, 8). These are tightly bound proteins which are not washed off by salt solutions of intermediate ionic strength (7, 8). Treatment of ribosomes purified as described above with CsCl gives rise to nonfunctional “core” particles consisting of RNA and residual ribosomal proteins (7, 8). Under the conditions of the experiment of Fig. 1, the core particles, which have a low buoyant density because of their RNA content, banded near the bottom of the gradient, as indicated by the peak of absorbance at 260 nm. The split proteins, which have a lower buoyant density, float in a thin disc at the top of the gradient. As shown in Fig. 1, all of the phosphatidylserine synthetase activity was recovered in the split protein fraction. No activity was detected in the region of the gradient containing the ribosomal cores. These results demonstrate that the enzyme does not depend on the bulk of the ribosomal RNA or core protein for activity.

It has not yet been possible to determine whether the enzyme is one of the major ribosomal proteins. The analytical techniques used to separate these proteins irreversibly inactivate the synthetase. A major ribosomal protein common to both subunits, however, has not been reported (17).

Since the synthetase has an affinity for cytosine-containing liponucleotides, it might simply bind to the cytosine residues of ribosomal RNA through its active site. If this were the case, extraction from ribosomes might actually result in a substantial increase in the total activity. This phenomenon has been observed in the case of the “latent” ribonuclease associated with the 30 S subunit (18, 19). Since no activation was observed in the results of Fig. 1, however, the binding of phosphatidylserine synthetase to ribosomes appears to involve a region of the enzyme other than the active site.

**Preparation of Phosphatidylserine Synthetase from Cell-free Extracts—CsCl treatment is not feasible on a preparative scale. An alternative method of purification summarized in Table I was therefore employed for further studies. Frozen cells of E. coli B were obtained from the Grain Processing Co., Muscatine, Iowa. Fifty grams of cells were suspended in a Waring Blendor in 400 ml of 10 mM potassium phosphate buffer of pH 7.4, containing 10 mM 2-mercaptoethanol and 2 mM disodium EDTA. All procedures were carried out between 0 and 4°.

After four passages through a Martin Gaulin press at 9000 p.s.i., the sucrose 20% solution of broken cells was adjusted to a volume of 500 ml with the same buffer (Table I, Fraction 1) and was centrifuged for 1 hour at 20,000 × g. The supernatant (Fraction 2) was rapidly mixed with 150 ml of an aqueous solution of 5% streptomycin sulfate (w/v) in 25% Triton X-100 (v/v). This served to dissolve membrane fragments while simultaneously precipitating nucleic acids and ribosomes. Exposure of the enzyme to Triton X-100 at this stage also resulted in higher recoveries in the polymer partitioning step described below. The mixture was held on ice for 1 hour, after which the heavy precipitate was harvested by a 20-min centrifugation at 10,000 × g. The clear supernatant was discarded. The pellet was dissolved with the aid of a Potter homogenizer in 20 mM potassium phosphate of pH 7.4, containing 10 mM 2-mercaptoethanol, 2 mM disodium EDTA, and 5 mM NaCl in a final volume of 200 ml (Fraction 3). The pH was adjusted after the addition of the NaCl.

**Extraction with Polyethylene Glycol—Partition between aqueous phases of polyethylene glycol and dextran in the presence of 5 mM NaCl has been used to separate enzymes from their nucleic acid substrates (10). In a similar procedure, to separate the synthetase from the bulk of the nucleic acids, 34 ml of 20% (w/w) Dextran T-500 (Pharmacia) and 68 ml of 30% (w/w) polyethylene glycol (Carbowax, PEG 6000) were rapidly mixed with Fraction 3. The sample was stirred for 30 min at 4°, and the phases were separated by a 10-min centrifugation at 10,000 × g. The upper polyethylene glycol phase, which contained the enzyme was aspirated without disturbing the interface and was set aside in an ice bath. The lower dextran-rich phase, which contained most of the nucleic acid, was washed once more with 100 ml of a fresh, polyethylene glycol phase, previously equilibrated with dextran in the same buffer system used above. After centrifugation the second polyethylene glycol extract was mixed with the first (Fraction 4) and the washed dextran phase was discarded. At this stage of purification the enzyme was stable for several weeks when stored in an ice bath; a precipitate which formed on storage could be removed by low speed centrifugation.

**Precipitation with (NH₄)₂SO₄—Solid (NH₄)₂SO₄ (70 g) was slowly added to 300 ml of Fraction 4. The mixture was stirred for 20 min at 4° and was then centrifuged for 20 min at 10,000 × g.** (This amount of (NH₄)₂SO₄ was saturating in the presence of NaCl, which remained from the previous polymer partition step.) A small, concentrated polyethylene glycol phase floated on top of a larger aqueous-salt phase after centrifugation. The synthetase was precipitated by the (NH₄)₂SO₄ and was concentrated at the interface after centrifugation along with a substantial amount of protein. The enzyme was recovered from the

![Fig. 1. Dissociation of phosphatidylserine synthetase from ribosomal cores by isopycnic density gradient centrifugation in CsCl. All operations were performed at 4°. A sample of washed ribosomes (0.5 ml), which contained 10 mM MgCl₂, 10 mM Tris-HCl of pH 7.5, 20°, 30 mM (NH₄)Cl, and 6 mM 2-mercaptoethanol, with an absorbance of 240 at 260 nm, was mixed with 4.3 ml of 5 mM CsCl containing 20 mM Tris-HCl of pH 7.6 (4°). To this solution were added 0.2 ml of 1 M MgCl₂ and 0.02 ml of 1 M 2-mercaptoethanol. The final mixture was centrifuged for 36 hours at 150,000 × g in a 5.5-ml Beckman nitrocellulose tube. A Beckman model L3-50 centrifuge and an SW 50.1 rotor were employed. Fractions were collected as described previously (1) and were analyzed for phosphatidylserine synthetase (synthetase) and optical density at 260 nm. Although samples diluted for enzyme assay contained as much as 0.3 mM CsCl, these levels did not interfere with the measurement of activity.**
interface by carefully removing both the upper, polyethylene glycol phase and the lower, aqueous phase. The protein precipitate, which adhered to the walls of the centrifuge tube, was then redissolved in a final volume of 77 ml of 0.5 M potassium phosphate of pH 7.4, which contained 10% glycerol (v/v) and 10 mM 2-mercaptoethanol. A small amount of material which did not redissolve was removed by a 10-min centrifugation at 10,000 × g. The clear supernatant (Fraction 5) was stored in an ice bath. The enzymatic activity of Fraction 5 was stable for several weeks. Based on the optical density at 260 nm, over 95% of the nucleic acid present in Fraction 3 was removed.

Chromatography on Phosphocellulose—The phosphatidylserine synthetase was further purified by ion exchange chromatography on phosphocellulose. In a typical preparation, Fraction 5 (10 ml) was diluted 4-fold with distilled water and was applied to a column (1.2 × 10 cm) of phosphocellulose (Whatman P-11), equilibrated at 4°C with buffer containing 0.1 M potassium phosphate of pH 7.4 and 10% glycerol. Washing of the phosphocellulose with acid and base prior to use, according to the method of Kurland et al. (20), was essential. The enzyme, which was retained by the phosphocellulose at the high concentration of NaCl (0 to 1.8 M), was eluted with a linear gradient of NaCl (0 to 1.8 M in 140 ml) prepared in the same buffer (Fig. 2). Fractions containing the enzyme, which was eluted at a concentration of 1 M NaCl, were pooled and stored in an ice bath (Fraction 6).

It is noteworthy that the enzyme did not adsorb to phosphocellulose prior to extraction from the ribosomal RNA. Apparently the same sites which bind the enzyme to nucleic acid also bind it to phosphocellulose. The enzyme was clearly not homogeneous after the phosphocellulose step (Fig. 2), but numerous hydrolytic activities present in crude extracts were removed.

After extraction from ribosomes, enzymatic activity was rapidly lost upon dialysis of the sample against phosphate buffers of low ionic strength (less than 0.1 M). Fractions 4 and 5 precipitated at low salt concentrations, and the enzymatic activity could not be recovered.

Exchange of CMP Moiety of CDP-diglyceride with Free CMP—When tritiated CMP was incubated with unlabeled CDP-dipalmitin in the presence of enzyme fractions containing phosphatidylserine synthetase but in the absence of L-serine, radioactive CMP was incorporated into CDP-diglyceride (Table I). The rate of appearance of radioactivity into the lipid (CDP-diglyceride) fraction equaled the rate at which it was lost from the water-soluble CMP pool (data not shown). When unlabeled CDP-diglyceride was omitted from the incubation mixture, no significant incorporation of CMP into lipid could be detected. When unlabeled L-serine was added (Experiment 2) labeling of CDP-diglyceride from CMP was also almost completely abolished, as predicted by Scheme 1.

The radioactive lipid formed from [3H]CMP was identified as CDP-diglyceride by the following criteria: (a) chromatography on DEAE-cellulose (Fig. 3) and (b) on thin layer plates of silicic acid (11); (c) incubation of the isolated radioactive product with fresh phosphatidylserine synthetase in the presence of unlabeled L-serine. This resulted in a rapid release of tritiated, water-soluble CMP.

**Nucleotide Specificity of CMP-CDP-Diglyceride Exchange Reaction**—If the CMP moiety of CDP-diglyceride exchanges with free CMP, it is expected that CDP-diglyceride, tritiated in the CMP moiety, should release water-soluble radioactivity when incubated with excess nonradioactive CMP. In the experiment of Table III, a 7.5-fold stimulation of tritium release was observed when CMP, present in a 10-fold excess, was incubated with tritiated CDP-diglyceride and partially purified enzyme. The radioactive, water-soluble product obtained in this manner migrated with an authentic standard of CMP under the conditions of paper chromatography described in an earlier communication (15). The background level of water-soluble tritium formed in the absence of added CMP resulted both from residual, contaminating membrane-bound hydrolase (15) present in the enzyme sample, and from the intrinsic hydrolase activity of the phosphatidylserine synthetase itself.

Only CMP and dCMP greatly stimulated the release of radio-

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**Table II**

<table>
<thead>
<tr>
<th>Incorporation of [3H]CMP into CDP-diglyceride</th>
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<tr>
<td><strong>Experiment conditions</strong></td>
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<tr>
<td><strong>Radioactive lipid</strong></td>
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<tr>
<td>Complete system</td>
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<tr>
<td>Omit enzyme</td>
</tr>
<tr>
<td>Omit CDP-dipalmitin</td>
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<td>Zero time control</td>
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**Fig. 2. Chromatography of phosphatidylserine synthetase extracted from ribosomes on a column of phosphocellulose. Elution with a linear gradient of NaCl at a flow rate of about 47 ml per hour was performed as described in the text. Protein concentration was determined by the method of Lowry et al. (16). Fractions of about 2.5 ml were collected.**
activity from CDP-diglyceride tritiated in the cytidine moiety. All other nucleotide monophosphates shown in Table III had negligible effects. This degree of nucleotide specificity is comparable to that observed previously (11) for the formation of phosphatidylserine from L-serine and various nucleoside diphosphatidylglycerides.

Serine-Phosphatidylserine Exchange—Enzyme preparations purified 130-fold (Table I, Fraction 6) were found to catalyze the exchange of free L-serine with the serine moiety of phosphatidylserine. The results of Table IV show that unlabeled L-serine, in the presence of enzyme, greatly stimulated the release of radioactive serine from phosphatidyl[1-14C]serine. Conversely, radioactive L-serine was incorporated into nonradioactive phosphatidylserine by the purified enzyme. The serine-phosphatidylserine exchange reaction took place in the absence of added CMP.

The specificity of the serine exchange reaction for L-serine closely corresponded to that reported by Kanfer and Kennedy (21) for the formation of phosphatidylserine from L-serine and CDP-diglyceride. For instance no detectable exchange could be demonstrated when D-serine or ethanolamine were substituted for L-serine.

Direct evidence for such a reversal is presented in Table V. These results demonstrate that fractions of purified phosphatidylserine synthetase (Table I, Fraction 6) catalyze the incorporation of tritiated CMP into a chloroform-soluble substance when phosphatidylserine is present. The identity of this radioactive product was established as CDP-diglyceride by thin layer chromatography (11) in the solvent chloroform-methanol-water-acetic acid (25:15:4:2, v/v).

Hydrolysis of CDP-diglyceride by Phosphatidylserine Synthetase—Membranes of E. coli contain an active, highly specific enzyme that hydrolyzes CDP-diglyceride (15). This hydrolase is very different in its intracellular distribution, chromatography, and other properties from phosphatidylserine synthetase. However, phosphatidylserine synthetase also appears to have a distinct and significant activity in the hydrolysis of CDP-diglyceride that cannot be separated from the synthetase during chromatography.

Reversal of Phosphatidylserine Synthesis—Table IV shows that excess CMP stimulates the release of serine (identified by paper chromatography as described in Table IV) from phosphatidylserine in the presence of the purified enzyme. This suggests that the formation of phosphatidylserine from CDP-diglyceride and L-serine is reversible.

<table>
<thead>
<tr>
<th>Table IV</th>
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<tr>
<td><strong>L-Serine-phosphatidylserine exchange catalyzed by phosphatidylserine synthetase of Escherichia coli</strong></td>
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<tr>
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<td>0.033 mM L-serine</td>
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<td>0.33 mM L-serine</td>
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<tr>
<td>3.3 mM L-serine</td>
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<td>3.3 mM CMP</td>
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<table>
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<th>Table V</th>
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<tr>
<td><strong>Formation of CDP-diglyceride from phosphatidylserine and CMP by phosphatidylserine synthetase</strong></td>
</tr>
<tr>
<td>Incubation conditions</td>
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<tr>
<td>Omit phosphatidylserine synthetase</td>
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<td>Omit phosphatidylserine</td>
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</tbody>
</table>
matography on phosphocellulose (Figs. 2 and 4). The rate of hydrolysis, however, was only one-third that of CMP exchange under standard assay conditions, or 0.6% of the rate of phosphatidylserine synthesis. All assays were performed under identical conditions of pH, ionic strength, etc. Phosphatidylserine synthetase thus can donate phosphatidyl residues to water with a low efficiency in the absence of L-serine. This hydrolytic activity, however, accounts for less than 1% of the total CDP-diglyceride hydrolase activity present in crude extracts, most of which is due to the membrane-bound hydrolase (Fig. 5). The hydrolytic activity associated with the phosphatidylserine synthetase also differs from the membrane-bound CDP-diglyceride hydrolase in that it is not inhibited by AMP (15).

Association of CMP Exchange, Serine Exchange, and Hydrolase Activity with Phosphatidylserine Synthetase—The finding that L-serine prevents the incorporation of tritiated CMP into CDP-diglyceride strongly indicates that the CMP exchange reaction is catalyzed by phosphatidylserine synthetase, since L-serine is a substrate for that enzyme, and would be expected to reduce or prevent CMP exchange in the reactions of Scheme 1. When fractions containing the phosphatidylserine synthetase at various stages of purification were also assayed for CMP exchange activity, the ratio of the two activities was found to be similar (Table VI) except that the synthetase was slightly higher in the crude cell-free extract and the 20,000 × g supernatant fraction. These fractions, however, also contain substantial amounts of the membrane-bound CDP-diglyceride hydrolase, previously described (15), which might be expected to interfere with the CMP exchange assay more than with the synthetase assay. Indeed, unless care was taken to measure the initial rate of CMP exchange by means of short incubation times and high specific activities of CMP, the CMP exchange reaction was obscured entirely in crude extracts. This effect of the hydrolase in crude extracts (i.e. Fraction 1) is shown in Fig. 6, in which all of the CDP-diglyceride in the system appeared to be degraded after 100 min. When partially purified phosphatidylserine synthetase (Fraction 3) was used for the incubation, the incorporation of tritiated CMP into CDP-diglyceride by the CMP exchange reaction occurred for a much longer time and went to a much higher level (Fig. 6), presumably because Fraction 3 is largely devoid of the hydrolase (Table VI).

Several additional lines of evidence suggest that phosphatidylserine synthesis and CMP exchange are catalyzed by the same enzyme. In particular, the sedimentation of the CMP exchange enzyme with ribosomes in crude extracts (Fig. 5) is virtually identical with that observed previously with the phosphatidylserine synthetase.

**Table VI**

Purification of CMP exchange activity with phosphatidylserine synthetase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>PS-synthetase</th>
<th>CMP exchange</th>
<th>CDP-diglyceride hydrolase</th>
<th>Ratio of PS-synthetase to CMP exchange</th>
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</thead>
<tbody>
<tr>
<td>1. Broken cells</td>
<td>10.3</td>
<td>0.17</td>
<td>8.13</td>
<td>60.6</td>
</tr>
<tr>
<td>2. 20,000 × g supernatant</td>
<td>13.0</td>
<td>0.23</td>
<td>5.70</td>
<td>56.5</td>
</tr>
<tr>
<td>3. Streptomycin precipitate</td>
<td>24.2</td>
<td>0.71</td>
<td>0.94</td>
<td>34.1</td>
</tr>
<tr>
<td>4. Polymer partitioning</td>
<td>31.6</td>
<td>0.72</td>
<td>1.89</td>
<td>43.9</td>
</tr>
<tr>
<td>5. (NH₄)₂SO₄</td>
<td>80.5</td>
<td>1.74</td>
<td>7.17</td>
<td>46.3</td>
</tr>
<tr>
<td>6. Phosphocellulose</td>
<td>1310</td>
<td>30.8</td>
<td>8.78</td>
<td>42.5</td>
</tr>
</tbody>
</table>

**Fig. 4.** Co-chromatography of phosphatidylserine synthetase, CMP exchange, and hydrolase on a column of phosphocellulose. Conditions were identical with those described in Fig. 2. CMP exchange was measured by Method I and phosphatidylserine synthetase (synthetase) and hydrolase activity were measured by the standard assays (1, 15).

**Fig. 5.** Association of CMP exchange enzyme with ribosomes in extracts of broken cells. A sample of sonically disrupted *Escherichia coli* B (0.5 ml), which contained 10 mg of protein per ml, 10 mM Tris-HCl of pH 7.4, and 2 mM MgCl₂, was layered on top of a 10-m1 sucrose gradient, as described previously (1). A shelf of concentrated sucrose at the bottom of the gradient trapped rapidly sedimenting membrane fragments and prevented their packing on the bottom of the tube. Fractions were assayed for the CMP exchange activity by Method I and for the CDP-diglyceride hydrolase. The absorbance of the fractions at 260 nm (l-cm path length) was also determined. The peak of ribosomal material (mainly consisting of 70 S particles) sedimented near Fraction 22.

**Fig. 6.** Incorporation of tritiated CMP into CDP-dipalmitin, catalyzed by crude, cell-free extracts or partially purified ribosomes. Method I was used to measure CMP exchange. Aliquots of Fraction 1 and Fraction 3 (Table I) were employed, which contained identical amounts (1.8 units) of phosphatidylserine synthetase.
serine synthetase (1). Furthermore, very little CMP exchange activity was found in the membrane fraction, concentrated at the bottom of the sucrose gradient on the 60% sucrose shelf in the experiment of Fig. 5. Failure to find the CMP exchange activity associated with membranes may, in part, be due to the presence of the CDP-diglyceride hydrolase (15) in the membrane fraction (Fig. 5). However, even the initial rate of CMP incorporation catalyzed by membranes was consistently low, under conditions which only a small portion of the CDP-diglyceride present during the incubation was hydrolyzed. The relatively slow rate of CMP exchange catalyzed by the membranes is noteworthy, since it suggests the other phosphatidyltransferase of E. coli (phosphatidylglycerophosphate synthetase), which is present in the membranes (1–8), does not catalyze a CMP-CDP-diglyceride exchange, and thus may have a different mode of action.

The strongest evidence that phosphatidylserine synthesis and CMP exchange occur on the same enzyme is shown in Fig. 4. From this it can be seen that the two activities chromatograph identically on a column of phosphocellulose. It is very unlikely that different proteins would have precisely the same mobility under these highly unusual conditions.

The serine phosphatidylserine exchange also chromatographed with the phosphatidylserine synthetase on the phosphocellulose column of Fig. 4 (data not shown). Thus the phosphatidylserine synthetase seems to catalyze not only the formation of phosphatidylserine, but also the exchange of CMP into CDP-diglyceride, and the exchange of serine with phosphatidylserine. At very slow rates it hydrolyzes CDP-diglyceride and phosphatidylserine as well. Because a highly reactive phosphatidylserine decarboxylase is present in the membrane of E. coli (21), the exchange reactions involving phosphatidylserine are difficult to demonstrate prior to purification on phosphocellulose.

The presence of these exchange activities in what appears to be a single enzyme leads us to postulate the sequence of reactions for the synthesis of phosphatidylserine shown in Scheme 1.

**DISCUSSION**

The present study confirms previous findings (1) that the phosphatidylserine synthetase of E. coli is tightly bound to ribosomes in cell extracts. Although the synthetase is not removed by the extensive washing procedures to which ribosomes are subjected prior to use in systems for cell-free synthesis of protein (8), the enzyme can be dissociated from the ribosomal core under certain conditions of high ionic strength. These extreme procedures also extract about 40% of the intrinsic ribosomal proteins (7, 8). The solubility properties of the extracted enzyme resemble those of ribosomal proteins, since a high concentration of salt is required to keep the extracted enzyme in solution (8).

The activity of the synthetase, measured under standard assay conditions, is neither enhanced nor significantly inhibited upon removal of the nucleic acid. Furthermore, the $K_m$ of the purified synthetase is not significantly different from that of the crude enzyme. In both cases the $K_m$ for L-serine is surprisingly high (0.8 mm), since intracellular serine concentrations in gram-negative bacteria are thought to be at least 1 order of magnitude lower (22). Serine-activating enzymes and serine-transport proteins tend to have $K_m$ values for serine which are on the order of 0.01 mm (23, 24). Thus, the relatively low affinity of the phosphatidylserine synthetase for L-serine is striking. Perhaps the assay conditions employed in vivo do not adequately reflect the state of the enzyme in vivo. Alternatively, L-serine may not be the optimal substrate for the synthetase. Other serine derivatives, such as serine-containing peptides or seryl-tRNAs should be tested as possible phosphatidyl acceptors. It has previously been shown by Lennarz and his co-workers (25) that lysyl-tRNA is involved in the biosynthesis of lysyl phosphatidylglycerol, while glycytRNA is the precursor of the glycine bridges in the cell wall of Staphylococcus aureus (26). There is some precedent for considering a role of aminomethyl-tRNA species other than in protein synthesis.

The extracted enzyme primarily differs from the ribosomally bound form in that it is very tightly adsorbed to phosphocellulose. About 1 M NaCl is required for elution of the synthetase from phosphocellulose columns. The synthetase does not bind to carboxymethylcellulose and Sephase under comparable conditions, suggesting that it is not simply a basic protein but that it has a site which recognizes phosphate moieties. It seems likely that the same site which binds the enzyme to ribosomes is responsible for its adsorption to phosphocellulose. Because extraction from ribosomes does not alter the activity of the synthetase, the binding of the synthetase to ribosomes and phosphocellulose probably involves a region of the enzyme molecule other than the active site. The interaction of the purified enzyme with polynucleotides deserves further study although a homogenous enzyme fraction would be desirable for this purpose.

Phosphatidylserine synthetase appears to function by a mechanism in which an intermediate phosphatidyl-enzyme is generated, as shown in Scheme 1. Each of the partial reactions indicated in the scheme has now been demonstrated to be catalyzed by partially purified fractions of the synthetase. Regrettfully, the synthetase has not been purified to homogeneity, so that the possibility that unrelated enzymes accompany it cannot be excluded. It is considered highly unlikely, however, that unrelated enzymes catalyze the exchange and hydrolysis reactions, since these latter reactions are almost completely abolished by added L-serine, even under conditions when only a fraction of the total CDP-diglyceride has been used up in the synthetase reaction. To explain this finding, it would be necessary to assume that hypothetical, extraneous enzymes responsible for exchange and hydrolysis have a specificity for L-serine identical with that of the synthetase, and are inhibited by this amino acid. Similarly, only CMP and dCMP stimulate the release of radioactivity from CDP-diglyceride labeled in the CMP moiety, corresponding to the known specificity of the phosphatidylserine synthetase (11). These facts, taken together with the association of all three catalytic activities during chromatography on phosphocellulose under highly unusual conditions, support the view that the reactions of Scheme 1 are catalyzed by phosphatidylserine synthetase.

The serine-phosphatidylserine exchange reaction reported here involves both the cleavage and the resynthesis of a phospholipid-phosphodiester bond. In the process a phosphatidylo moiety is donated from 1 serine molecule to another. This mechanism of phosphatidyl transfer does not involve cytidine nucleotides and does not result in de novo synthesis of phosphodiester bonds. However, the same enzyme molecule appears to catalyze both de novo phosphatidylserine synthesis and serine exchange.

The serine-phosphatidylserine exchange reaction is formally similar to the interconversion of phosphatidylethanolamine and phosphatidylethanolamine which has been described in animal tissues (27). In rat liver microsomes phosphatidylserine is made by an enzyme which transfers the phosphatidyl moiety of phosphatidylethanolamine to the hydroxyl group of serine. The E. coli
This is consistent with the data of Kanfer and Kennedy (21), who reported that the phosphatidylserine synthetase of E. coli does not donate phosphatidyl moieties of CDP-diglyceride to ethanolamine at a detectable rate.

Other reactions in which 1 phospholipid molecule serves as a source of phosphatidyl residues for the synthesis of another have been reported in the literature (28-30). For instance, cardiolipin is formed from 2 molecules of phosphatidylglycerol in bacteria, without the involvement of a cytosine-containing pyrophosphate (29, 30). Recently a number of laboratories have described "base exchange" reactions in mammalian systems which also occur in the absence of cytosine-containing coenzymes and are probably similar to the serine-phosphatidylserine exchange reaction reported here (31). The enzymatic basis and biological significance of these interesting exchange reactions has not yet been clarified.

Chang and Kennedy (32) studied glycerophosphate-phosphatidylglycerophosphate exchange with a purified phosphatidylglycerophosphate synthetase preparation from E. coli. Unlike serine-phosphatidylserine exchange, the process observed by Chang and Kennedy required the addition of CMP as well. Whether this implies an intrinsically different mechanism for phosphatidylglycerophosphate synthetase or whether it is an allosteric effect of CMP is not certain. Chang and Kennedy did not describe CMP-CDP-diglyceride exchange in their study of this enzyme.

The results of Table V conclusively demonstrate that CDP-diglyceride can be formed from CDP and phosphatidylserine by the purified phosphatidylserine synthetase. Thus phosphatidylserine synthetase is reversible, although the equilibrium favors the phosphatidylserine deacetylase. Nonetheless the intrinsic reversibility of this reaction might allow a cell to convert excess phosphatidylserine to CDP-diglyceride and indirectly to phosphatidylglycerophosphate under some circumstances. Other examples of the reversal of phospholipid synthesis have been cited in the literature (33-35). For instance, the formation of lecithin from CDP-choline and s-diglyceride appears to be reversible (33). Studies with whole membrane vesicles of E. coli have also suggested reversal of phospholipid synthesis under certain conditions, although the complexity of this system has prohibited definitive interpretations (34, 35).

Since CDP-diglyceride and phosphatidylserine are slowly hydrolyzed by the synthetase with the release of CMP and serine, respectively, the phosphatidyl-enzyme intermediate postulated in Scheme I must slowly transfer phosphatidyl moieties to water (Equation 3) under the conditions of the assay system. The finding that the phosphatidylserine synthetase can act as a hydrolyase suggests that the highly specific CDP diglyceride hydrolase, recently described in our laboratory (42) and largely associated with the membrane (Fig. 5), in theory might be a phosphatidyltransferase as well. As yet, however, no acceptor for phosphatidyl units other than water has been identified for this enzyme.

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Partial Purification and Properties of Phosphatidylserine Synthetase from *Escherichia coli*

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