

**STUDIES ON BIOSYNTHESIS OF PHOSPHATIDYLETHANOLAMINE
IN *ESCHERICHIA COLI* MEMBRANE**

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INTRODUCTION

Biological membranes play a crucial role in almost all cellular phenomena. The most striking characteristic of membrane is that the components which constitute membrane structure hold membrane functions concurrently. Bacterial membrane which has no intracellular membrane may further reveal the character, because various functions such as nutrient transport [1-2], oxidative phosphorylation [3-4] and lipopolysaccharide synthesis [5] etc. localize the cytoplasmic membrane.

Biological membranes are composed mainly of phospholipids and proteins. About 60% of *Escherichia coli* membrane is protein and 30% phospholipid [6]. The major classes of phospholipid are found to be phosphatidylethanolamine (80-90%), phosphatidylglycerol (5-15%) and small amounts of cardiolipin and phosphatidic acid [7-8]. Phosphatidylethanolamine is metabolically stable in growing cells [9]. In contrast, phosphatidylglycerol undergoes the more rapid turnover [9-11] and a precursor of a novel oligosaccharide [12]. Phosphotransferase system involved in the active transport of sugars requires phosphatidylglycerol as lipid cofactor [13]. Ohki suggests that the rapid turnover of phosphatidylglycerol coordinates with the formation of membrane components [14]. Similarly, Ballesta *et al.* show that phosphatidylethanolamine synthesis is

related to growth and cell division, whereas phosphatidylglycerol metabolism is related to other phenomena [15].

On the other hand, the physical properties of membrane are dependent on the fatty acid composition of membrane phospholipids [16-18]. It has been thought hitherto that the fatty acid compositions among individual phospholipids of *E. coli* are similar [7], but recently Kito *et al.* indicated that the fatty acid composition of individual phospholipids represented heterogeneity and varied with growth temperature and growth phase [19-21, c]. This fact shows that membrane functions are dependent on the physical properties of membrane phospholipids. Recently, the suggestion has been confirmed by many investigators [22-25].

Phospholipids biosynthesis of bacteria has been studied enzymatically with in *E. coli* [26-30], *Bacillus megaterium* [31] and *Salmonella typhimurium* [27]. The current scheme of phospholipid synthesis in *E. coli* is given in figure. Phosphatidic acid to be a common intermediate of phospholipids is formed from *sn*-glycerol 3-phosphate and acyl-CoAs by *sn*-glycerol 3-phosphate acyl transferase and monoacylglycerol 3-phosphate acyltransferase. The biosynthetic reactions (reaction 1) have been studied in detail [32-39]. Phosphatidic acid is converted to CDP-diglyceride as donor of phosphatidyl group in the presence of CTP by phosphatidate cytidyltransferase (reaction 2). The biosynthetic reaction was shown

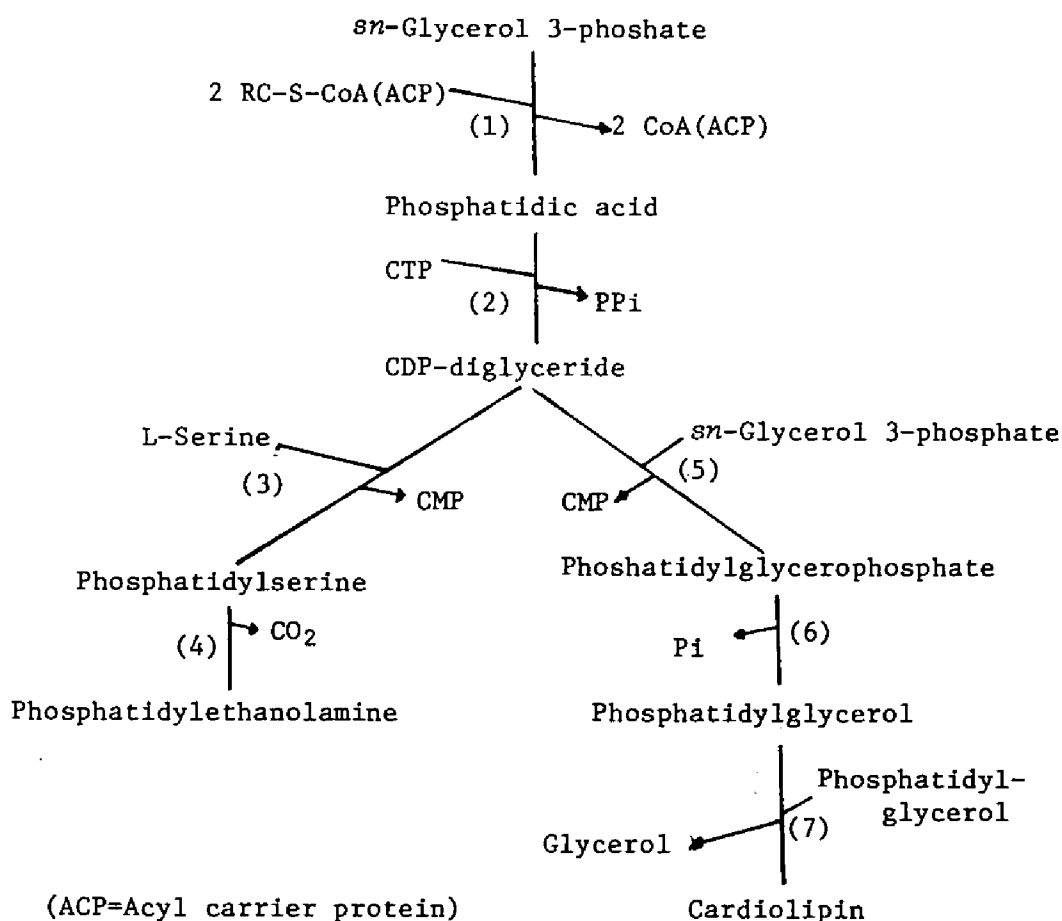


Figure. Pathways of phospholipid synthesis in *Escherichia coli*.

by Carter[40]. Kennedy *et al.* elucidated the following pathways for the synthesis of phosphatidylethanolamine(reaction 3-4) and phosphatidylglycerol(reaction 5-6)[26,41-42]. That is, CDP-diglyceride is converted to phosphatidylserine and phosphatidylglycerophosphate in the presence of L-serine and *sn*-glycerol 3-phosphate, respectively. Since phosphatidylserine decarboxylase(catalyzing

reaction 4)[43] is much more active than phosphatidylserine synthetase(catalyzing reaction 3), no accumulation of phosphatidylserine is found *in vivo*[9,26]. However, the addition of NH_2OH to be an inhibitor of many other decarboxylases results in accumulation of the lipid[58,e]. This phosphatidylethanolamine synthesizing system has been demonstrated in bacteria and yeast[44-45]. There is no evidence to indicate that the system is in animals and in plants. On the other hand, phosphatidylglycerophosphate is immediately converted to phosphatidylglycerol by the phosphatidylglycerophosphate phosphatase(catalyzing reaction 6)[42]. Therefore, no accumulation of phosphatidylglycerophosphate is also found *in vivo*[9]. The biosynthesis of cardiolipin(reaction 7) was shown by Kennedy *et al.*[47] and Rampini *et al.*[46]. These phosphatidylglycerol and cardiolipin synthesizing systems are found widely distributed in nature.

All of these enzymes responsible for the synthesis of phospholipid with the exception of phosphatidylserine synthetase are found in the particulate fraction of cell free extracts of *E.coli*[26-28, 30]. Perhaps these enzymes constitute the cytoplasmic membrane as integral protein[48]. However, it was not clear whether these enzymes are localized in the cytoplasmic membrane in a multi-enzyme complex.

The present thesis describes a membrane particulate phospho-

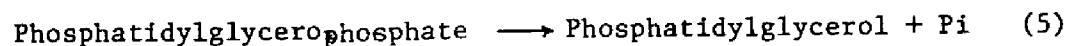
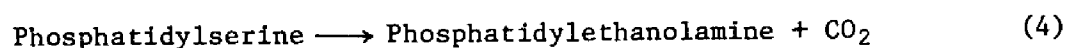
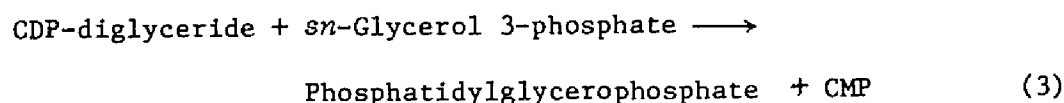
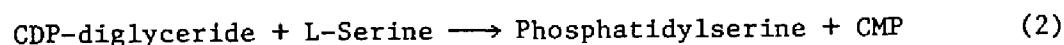
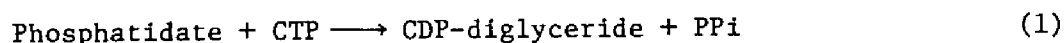
lipid synthesizing system from *E.coli*, the properties of soluble phosphatidylserine synthetase, effects of phospholipids on soluble phosphatidylserine synthetase and an exchange reaction between the radioactive phosphatidate and a corresponding moiety of endogenous phosphatidylethanolamine. Furthermore, in this thesis, occurrence of separate pools of phosphatidic acid for biosynthesis of phospholipids in *E.coli* membranes will be described.

Chapter I

A Particulate Phospholipid Synthesizing System

from *Escherichia coli* B^{b)}

The biosynthesis of phospholipids, phosphatidylethanolamine and phosphatidylglycerol, in *Escherichia coli* was shown to proceed according to Equations 1-5 by Kanfer and Kennedy[9,26], Chang and Kennedy[41-42] and Carter[40].



It was not clear whether these enzymes are localized in the cellular membrane in a multi-enzyme complex, and information on the site of phospholipid synthesis in the cells may serve as a tool in studies on the biosynthesis of the membrane.

This Chapter describes that reactions from phosphatidate to phosphatidylethanolamine and phosphatidylglycerol are catalyzed by a particulate enzyme system which functions with the phospholipid intermediates and products remaining associated with particulate material.

MATERIALS AND METHODS

Compounds and Analytical Procedures.

Palmitoyl-CoA, glycerol kinase, phosphatidate, phosphatidylethanolamine, *sn*-glycerol 3-phosphate, and preparative TLC-plates Silica Gel F254 are the products of Sigma, Pierce, Calbiochem, and E. Merck AG. DL-[U-¹⁴C]Serine and [U-¹⁴C]glycerol were purchased from New England Nuclear. *sn*-[U-¹⁴C]Glycerol 3-phosphate was enzymatically synthesized followed by purification by the procedure of Chang and Kennedy[41]. *E. coli* phosphatidylethanolamine and phosphatidylglycerol were prepared[9] from the exponentially growing cells followed by purification with preparative thin-layer chromatography with chloroform-methanol-7N ammonia (60:35:5) and chloroform-methanol-acetic acid (65:25:8) as the solvents. Protein and phosphorus were determined by the method of Lowry *et al.* [49] and Bartlett[50], respectively.

Preparation of Particulate sn-Glycerol 3-Phosphate Acyltransferase Fraction.

A particulate enzyme fraction from *E. coli* B was purified by the sucrose density gradient centrifugation as described by Kito *et al.* [32,36].

Incubation of Acyltransferase Reaction.

The incubation mixture contained 50 mM Tris-HCl buffer, pH7.2, 15 mM MgCl₂, 0.3 mM *sn*-[U-¹⁴C]glycerol 3-phosphate (1270 cpm/nmol), 50 μM palmitoyl-CoA, 3 mM dithiothreitol and 3 mg of particulate enzyme protein in a final volume of 1 ml. The incubation was run at 37°C, and 5 min after its initiation a further 50 μM palmitoyl-CoA was added. The incubation proceeded for additional 5 min and was stopped by cooling in an ice bath. The reaction mixture was then subjected to sucrose density gradient centrifugation.

Sucrose Density Gradient Centrifugation.

Samples (1 ml) were layered on 29 ml of a linear sucrose density gradient, which was formed from 10% to 40% sucrose and contained 10 mM Tris-HCl buffer, pH7.2 and 1 mM dithiothreitol. The gradients were centrifuged at 15000g for 10 min, and then 1 ml fractions were collected with an ISCO model 180 density gradient fractionator. The absorbance of each fraction was measured at 280 nm, and 0.1 ml each was used for modified paper disk assay described by van den Bosch and Vagelos[33].

Preparation of Stimulating Factor Fraction.

E.coli B was grown under the conditions as described by Kito *et al.*[36] and was harvested by centrifugation in the exponential growth

phase. After washing with 10 mM potassium phosphate buffer, pH7.1, the cells were resuspended in the same buffer supplemented with 10 mM dithiothreitol and disrupted by a sonifier (20 KHz) for 3 min. Large debris and particulate materials were removed by centrifugation at 30000g for 45 min and discarded. To the resulting supernatant solution, solid ammonium sulphate was added to bring the solution to 0.5 saturation. The precipitate was collected by centrifugation, dissolved in 10 mM Tris-HCl buffer, pH8.1, supplemented with 1 mM EDTA and 1 mM dithiothreitol, and then dialyzed against the same buffer overnight at 1°C. The dialyzed solution (40 mg of protein/ml) was stored at -20°C.

Heat-Treatment of Stimulating Factor Fraction.

Stimulating factor fraction was boiled for 10 min. After cooling, the supernatant was separated from the coagulated protein by centrifugation at 10000g for 30 min. By this procedure, approximately 20% of the original protein remained in the supernatant.

Analysis of Lipids.

Radioactive reaction products were extracted with chloroform and methanol. The extracts to which carrier phospholipids had been added were washed with 2M KCl and water[9], and dried under nitrogen. The radioactive lipids were separated by thin-layer chromatography

on plates of Silica Gel (Merck) with the solvents described in "Analytical Procedure". The lipids were located by iodine vapour and the appropriate areas were scraped from the plate. The radioactivity was measured by suspending the scrapings into toluene scintillation solution. Alternatively, the phospholipids were extracted with chloroform and methanol from the scrapings and deacylated by the procedure described by Ballou *et al*[51]. The water soluble compounds were co-chromatographed with authentic compounds using phenol-water-NH₄OH (48g:12ml:0.2ml) as the solvent. After a strip from the chromatogram was passed through the paper chromatogram scanner, the positions of the compounds were located with a spray for phosphate[52].

RESULTS

Particulate Fraction-Bound Radioactive Phosphatidate.

It has been observed by Pieringer *et al* [53], Ailhaud and Vagelos[54], Kito and Pizer[32] and van den Bosch and Vagelos[33] that the products of *E.coli* acyltransferase reaction are lysophosphatidate and phosphatidate. An investigation was undertaken to see whether these lipid intermediates were released free in the solution or remained bound to the particulate enzyme. The acyltransferase reaction was stopped by cooling as described under "Materials and Methods", and the incubation mixture was fractionated by sucrose density gradient centrifugation. Approximately 50% of the total

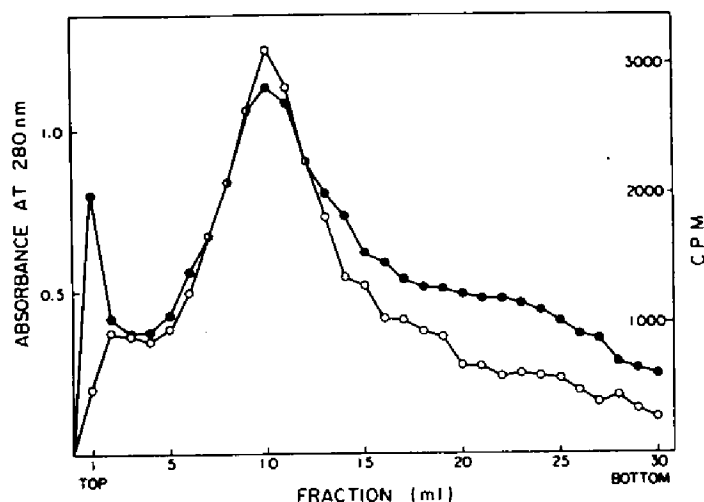


Fig. 1. *Sucrose density gradient centrifugation of the incubation mixture of acyltransferase reaction.*

Details of the experiment are given in the text. (●), absorbance ; (○), radioactivity.

radioactivity was found in the major particulate fraction (Fig. 1), and the residual radioactivity was sedimented with large particles to the bottom. Then, the fractions from 6 to 11 were collected and diluted with 10 mM Tris-HCl buffer, pH 7.2, supplemented with 15 mM $MgCl_2$ and 1 mM dithiothreitol to reduce the sucrose concentration. The particulate material in those fractions were centrifuged at 30000g for 60 min and washed twice with the same buffer. All the radioactivity was found to be sedimented with the particulate protein in the pellet (Table 1). The radioactive compounds were analyzed by thin-layer chromatography followed by deacylation as described

Table 1. *Centrifugation of particulate fraction-bound radioactive products.*

The fraction from 6 to 11(see Fig.1) was combined (2 mg protein, 42700 cpm of lipids), followed by centrifugation at 30000g for 60 min, and 10 mM Tris-HCl buffer, pH7.2, supplemented with 15 mM MgCl₂ and 1 mM dithiothreitol was used for washing. Radioactivity was determined by paper disk method.

Fraction	Radioactivity	Protein
	(cpm)	(mg)
Combined fractions	42700	2.0
Supernatant	585	Nil
1st washing	180	Nil
2nd washing	144	Nil
Pellet	41400	1.9

under "Materials and Methods". The radioactivity was found to be distributed in the phosphatidate fraction(67%), monoglyceride(25%) and phosphatidylethanolamine(8%) as shown in Table 2. Phosphatidate comprised the major portion of the phosphatidate fraction, which consisted of lysophosphatidate and phosphatidate as described previously[32]. Monoglyceride was probably the product from lysophosphatidate by the dephosphorylating enzyme reaction as described by van den Bosch and Vagelos[33]. Radioactive phosphatidylethanolamine appeared to be formed by an exchange reaction between the radioactive phosphatidate and a corresponding moiety of endogenous phosphatidylethanolamine which will be described in the subsequent section. The radioactive lipids could not be liberated from the complex by the

Table 2. *Particulate fraction-bound radioactive lipids.*

Lipids(6807 cpm) were extracted with a mixture of chloroform and methanol(2:1) containing authentic lipids from the particulate fraction-bound radioactive products(see Table 1), followed by lipid analysis as given under "Materials and Methods". Figures in parentheses show a percentage.

Lipid	cpm
Phosphatidate fraction ^{a)}	4110 (67)
Monoglyceride	1574 (25)
Phosphatidylethanolamine	475 (8)

a) Mixture of phosphatidate and lysophosphatidate: The content of lysophosphatidate which was analyzed as monoglyceride after dephosphorylation[33] was less than 15% relative to phosphatidate.

treatment of EDTA, urea and trypsin(unpublished data). Thus, all the products by acyltransferase reaction were firmly bound to the particulate fraction.

Synthesis of Phosphatidylethanolamine and Phosphatidylglycerol from Particulate Fraction-Bound Phosphatidate.

It is of interest to see that the particulate fraction-bound phosphatidate-¹⁴C can be utilized for the synthesis of phosphatidylethanolamine and phosphatidylglycerol *in situ*. If the enzymes responsible for the biosynthesis of phospholipids from phosphatidate are localized in the particulate fraction-phosphatidate-¹⁴C complex phosphatidylethanolamine or phosphatidylglycerol will be formed by the addition of CTP and either L-serine or sn-glycerol 3-phosphate

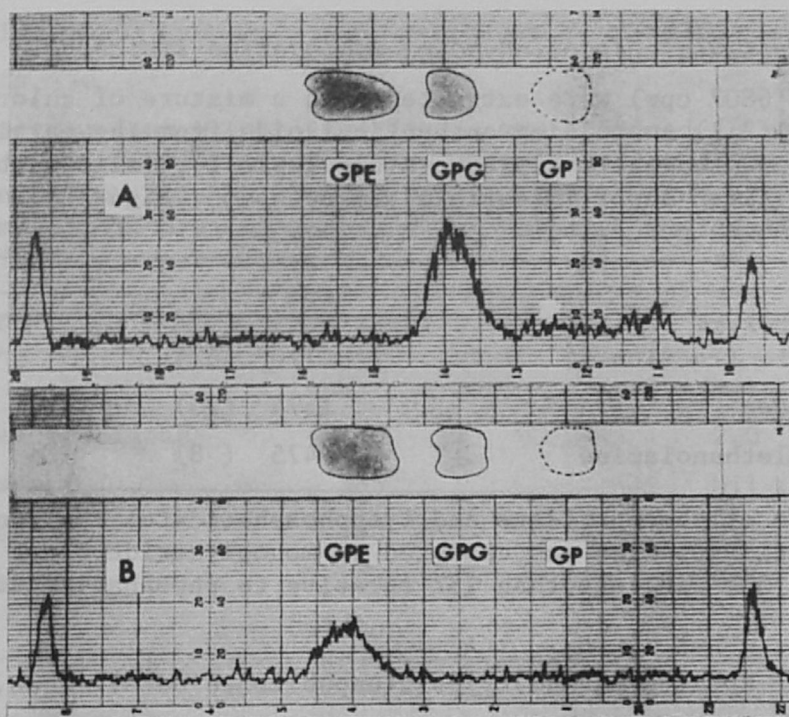


Fig.2. Paper chromatography of the deacylated products.

The deacylated products from phosphatidylglycerol- ^{14}C (A) and phosphatidylethanolamine- ^{14}C (B) formed from the particulate fraction-bound phosphatidate- ^{14}C . Key; 1, glycerophosphorylethanolamine; 2, glycerophosphorylglycerol; 3, *sn*-glycerol 3-phosphate.

to the complex. When the particulate fraction-phosphatidate- ^{14}C complex was incubated with CTP and *sn*-glycerol 3-phosphate, the formation of radioactive phosphatidylglycerol was observed, which was confirmed by thin-layer chromatography followed by deacylation method(Fig.2A). A stoichiometry was observed between the decrease in radioactive phosphatidate and the formation of radioactive phosphatidylglycerol(Table 3, Experiment 1). The stimulating factor

Table 3. Requirement for the synthesis of phosphatidylglycerol from particulate fraction-bound phosphatidate.

The particulate fraction-bound phosphatidate- ^{14}C complex or - bound unlabeled phosphatidate complex which was formed in the presence of labeled or unlabeled *sn*-glycerol 3-phosphate in the incubation mixture of acyltransferase reaction was separated by sucrose density gradient centrifugation as given under "Materials and Methods". The complex obtained as pellet by centrifugation of the fractions from 6 to 11 (see the legend of Table 1) was suspended in 10 mM Tris-HCl buffer, pH 7.2, supplemented with 15 mM MgCl_2 and 1 mM dithiothreitol at 0°C . Complete system contained 33 mM Tris-HCl buffer, pH 7.2, 15 mM MgCl_2 , 1 mM CTP and either, in Experiment 1, 0.9 mM *sn*-glycerol 3-phosphate and the particulate fraction-phosphatidate- ^{14}C complex (220 μg of protein with 910 cpm in phosphatidate fraction) or in Experiment 2, 0.1 mM *sn*-[$\text{U-}^{14}\text{C}$]glycerol 3-phosphate (166000 cpm/nmol) and the particulate fraction-unlabeled phosphatidate complex (264 μg of protein) in a total volume of 0.6 ml. Stimulating factor fraction (4 mg of protein) was added to the reaction mixture in Experiment 2. The incubation carried out in duplicate at 37°C for 20 min was terminated by the addition of chloroform and methanol (2:1) followed by analysis of lipid products as given under "Materials and Methods".

Condition	Phosphatidate fraction	Phosphatidyl-glycerol formed
Experiment 1	(cpm)	(cpm)
Complete	580	269
Minus CTP	906	29
Minus <i>sn</i> -glycerol 3-phosphate	923	39
Experiment 2		
Complete	—	2844
Complete plus stimulating factor	—	10600

fraction from cell free extracts of *E.coli* B stimulated the phosphatidylglycerol formation about 4-fold (Table 3, Experiment 2). In the case of the CTP-dependent synthesis of radioactive phosphatidyl-

Table 4. Requirement for the synthesis of phosphatidylethanolamine from particulate fraction-bound phosphatidate.

Complete system contained 33 mM Tris-HCl buffer, pH 7.2, 15 mM MgCl₂, 1 mM CTP, stimulating factor fraction (4 mg of protein) and either, in Experiment 1, 1 mM L-serine and the particulate fraction-phosphatidate-¹⁴C complex (320 µg of protein with 1450 cpm in phosphatidate fraction) or, in Experiment 2, 0.1 mM DL-[3-¹⁴C]serine (14230 cpm/n mole of L-serine) and the particulate fraction-unlabeled phosphatidate complex (340 µg of protein) in a final volume of 0.6 ml. The incubation carried out at 37°C for 20 min was terminated by the addition of chloroform and methanol (2:1) followed by analysis of lipid products as given under "Materials and Methods".

Condition	Phosphatidate fraction	Phosphatidylethanolamine formed
Experiment 1	(cpm)	(cpm)
Complete	395	951
0 time control	1242	173
Minus L-serine and stimulating factor	1112	386
Minus CTP, L-serine and stimulating factor	1040	355
Minus CTP, L-serine and stimulating factor plus 0.5 mM ATP	546	860
Experiment 2		
Complete	—	2685
Minus stimulating factor	—	214
Minus CTP	—	46

ethanolamine which was identified by thin-layer chromatography followed by deacylation (Fig. 2B), the stimulating factor fraction was also required, and approximately a 16-fold stimulation was observed (Table 4). The addition of Na₂SO₄ and octanol which were reported to be a stimulator of phosphatidylserine synthetase [26] to

Table 5. Effect of the concentration of *sn*-glycerol 3-phosphate and L-serine on the synthesis of phosphatidylglycerol and phosphatidylethanolamine.

The 0.6 ml incubation mixture contained 33 mM Tris-HCl buffer, pH7.2, 15 mM MgCl₂, 1 mM CTP, stimulating factor fraction (4 mg of protein), particulate fraction-unlabeled phosphatidate complex (220 µg protein) and either *sn*-[U-¹⁴C]glycerol 3-phosphate (16600 cpm/nmol) or L-[U-¹⁴C]serine (14230 cpm/nmol) varied as shown. The incubation carried out 37°C for 20 min was terminated by the addition of chloroform-methanol (2:1) followed by analysis of lipid products as given under "Materials and Methods".

Substrate	Concentration	Phosphatidyl- glycerol formed	Phosphatidylethanol- amine formed
	(mM)	(cpm)	(cpm)
<i>sn</i> -Glycerol 3-phosphate	0.1	10600	
	0.05	8720	
	0.01	3800	
L-Serine	0.1		1635
	0.05		1130
	0.01		457

the complete system, however, inhibited the formation of phosphatidylethanolamine about 80% (data not shown). On the other hand, to explain the occurrence of radioactive phosphatidylethanolamine in the absence of CTP (Table 2), the possibility was investigated that the particulate fraction-bound phosphatidate could be used as a substrate for an exchange reaction between the radioactive moiety of the particulate fraction-bound phosphatidate-¹⁴C and endogenous phosphatidylethanolamine. The exchange reaction was observed to be

Table 6. *Heat-stability of stimulating factors.*

Stimulating factor fraction was boiled as described under "Materials and Methods". The 0.6 ml incubation mixture contained 33 mM Tris-HCl buffer, pH 7.2, 15 mM MgCl₂, 1 mM CTP, particulate fraction-unlabeled phosphatidic acid complex (240 µg protein), stimulating factor fraction (native, 4 mg ; boiled, 720 µg) and either in Experiment 1, 0.1 mM *sn*-[U-¹⁴C]glycerol 3-phosphate (16600 cpm/nmol), or in Experiment 2, 0.1 mM L-[U-¹⁴C]serine (14230 cpm/nmol). The incubation carried out at 37°C for 20 min was terminated by the addition of chloroform-methanol (2:1) followed by analysis of lipid products as given under "Materials and Methods".

Stimulating factor fraction	Phosphatidylglycerol formed	Phosphatidylethanolamine formed
	(cpm)	(cpm)
Experiment 1		
Untreated	12860	
Boiled	13170	
Experiment 2		
Untreated		1915
Boiled		762

stimulated by ATP (Table 4) as will be described in a subsequent Chapter V. The effect of the concentration of *sn*-glycerol 3-phosphate and L-serine on the synthesis of phosphatidylglycerol and phosphatidylethanolamine was determined as shown in Table 5. The *K_m*s of *sn*-glycerol 3-phosphate and L-serine were 25 and 50 µM, respectively.

As may be seen from Table 6, the stimulating factor for the formation of phosphatidylethanolamine was heat-labile, while the stimulating factor for phosphatidylglycerol formation was completely heat-stable. These results suggest that the biosynthesis of

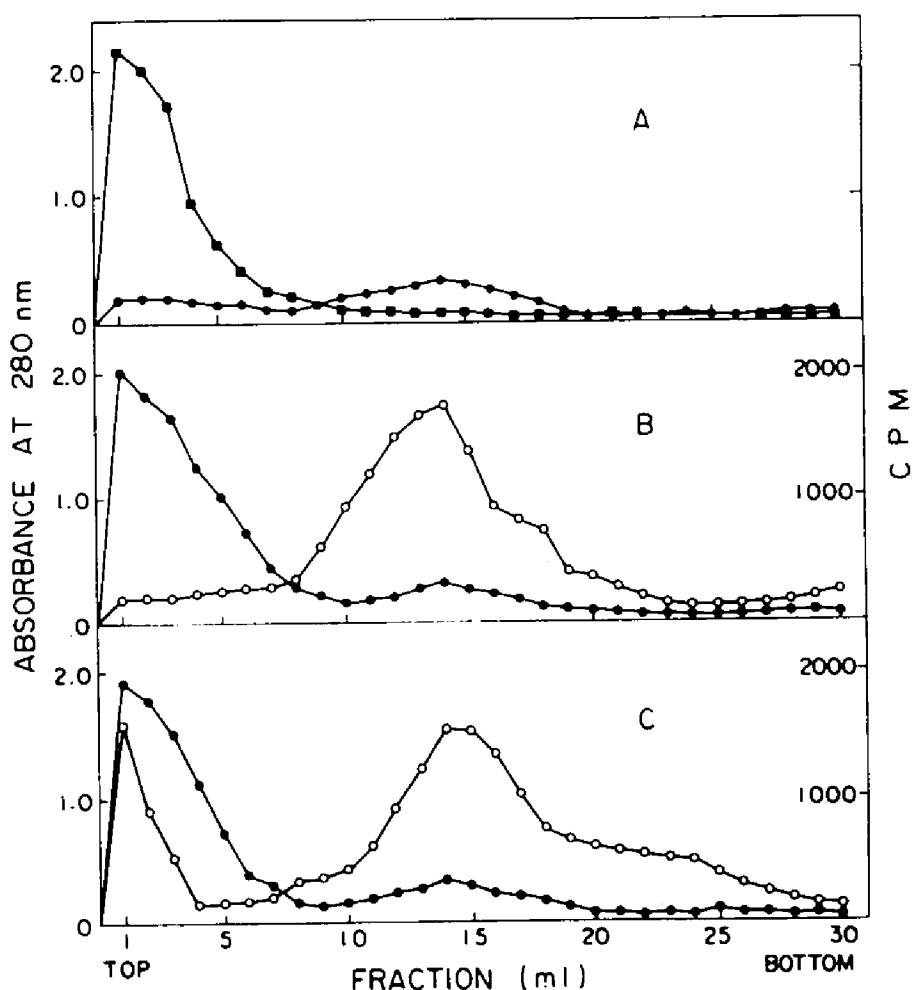


Fig.3. Sucrose density gradient centrifugation of the incubation mixture of phospholipid products.

The particulate fraction-unlabeled phosphatidate complex was prepared as described in the legend of Table 3. (A): absorbance at 280 nm of particulate fraction-phosphatidate complex, 245 μ g of protein, (●) and 1 min heated stimulating factor fraction, 920 μ g of protein, (■). (B) and (C): the 1 ml reaction mixture contained 33 mM Tris-HCl buffer, pH 7.2, 15 mM $MgCl_2$, 1 mM CTP, particulate fraction-unlabeled phosphatidate complex (245 μ g protein), 1 min heated stimulating factor fraction (920 μ g protein), and either (B), 0.1 mM *sn*-[U- ^{14}C]glycerol 3-phosphate (16600 cpm/nmol), or (C), 0.1 mM L-[U- ^{14}C]serine (14230 cpm/nmol): (●), absorbance and (○), radioactivity. The mixture was incubated at 37°C for 20 min. After cooling the radioactivity and protein was analyzed by sucrose density gradient centrifugation. Radioactivity was determined by paper disk method.

phosphatidylethanolamine and phosphatidylglycerol in a particulate enzyme system is controlled by at least two different soluble factors.

Particulate Fraction-Bound Phospholipid Products

An attempt was made to examine whether the lipid products were bound to the particulate fraction. An incubation mixture containing phosphatidylglycerol- ^{14}C synthesized from the particulate fraction-bound phosphatidate and *sn*-[U- ^{14}C]glycerol 3-phosphate was fractionated by sucrose density gradient centrifugation. As is shown in Fig. 3B, the radioactivity was coincident with the peak of the particulate material. Similarly, in the case of phosphatidylethanolamine- ^{14}C formation, the radioactive product was bound to the particulate material (Fig. 3C). Since the radioactivity found in the top fraction (Fig. 3C) which was precipitated on paper disks by trichloroacetic acid could not be extracted by chloroform and methanol, this may be a case that DL-[U- ^{14}C]serine was incorporated into compound other than lipids.

DISCUSSION

Kaback and Stadman suggested that the membrane may be the site at which phospholipid biosynthesis take place in the intact cell[1]. The enzymes responsible for the biosynthesis of phosphatidylglycerol and phosphatidylethanolamine from *sn*-glycerol 3-phosphate and fatty

acyl-CoA were found to be localized in a particulate fraction. The synthesis of phospholipids from phosphatidate may proceed in a particulate phospholipid synthesizing system through the formation of phospholipid intermediates, and the final products remained bound to the particulate material, while *sn*-glycerol 3-phosphate, the first phospholipid intermediate in lipid synthesis, is formed by a soluble enzyme[55-56].

The soluble factors which stimulate the formation of phosphatidylethanolamine and phosphatidylglycerol independently appear to contribute to the control of membrane lipid composition. Purification and characterization of the factors to elucidate their function on the particulate enzyme system will be described in the following Chapter.

Chapter II

Participation of Soluble Phosphatidylserine Synthetase in Phosphatidylethanolamine Biosynthesis in *Escherichia coli* Membrane^{d)}

Differences in the fatty acid composition among phospholipid species of *Escherichia coli* [19-21,c] suggest a complicated mechanism for the biosynthesis of phospholipid molecular species. With respect to the polar head group, however, the pathway for the synthesis of phosphatidylethanolamine and phosphatidylglycerol has been well established[26,32,40-42]. The enzymes responsible for their biosyntheses are localized in the cytoplasmic membrane[27-28,b] with the exception of phosphatidylserine synthetase which was described to occur in a soluble and a ribosome-bound forms[57]. In order to understand the mechanism by which the respective phospholipids are synthesized from the key intermediate, phosphatidic acid, it would be necessary to investigate mutual interaction of those enzymes in the membrane. This Chapter deals with the purification of soluble phosphatidylserine synthetase and its participation in the system of the phosphatidylethanolamine biosynthesis in the membrane.

MATERIALS AND METHODS

Assay of Phosphatidylserine Synthetase and Phosphatidylglycero - phosphate Synthetase.

The standard assay contained 33 mM potassium phosphate buffer, pH7.2, 15 mM MgCl₂, 0.11 mM L-[U-¹⁴C]serine (7900 cpm/nmol), 1.5 mM CDP-diolein (Serdary), 0.1% Cutscum and appropriate amounts of enzyme for the assay of phosphatidylserine synthetase. *sn*-[U-¹⁴C] Glycerol 3-phosphate (8300 cpm/nmol) was substituted for L-[U-¹⁴C] serine for the assay of phosphatidylglycerophosphate synthetase. The volume of the incubation was 0.6 ml. The reaction was allowed to proceed for 10 min at 37°C and terminated by the addition of 15 ml chloroform-methanol (2:1 v/v) and 100 µg phosphatidylserine (Cal-biochem). After being washed twice with 30 ml 2M KCl(pH2.0) and water, chloroform layer was transferred to a scintillation vial and dried under nitrogen. The radioactivity was measured in toluene scintillation on a Packard liquid-scintillation counter. A unit of activity is defined as the amount of enzyme which forms 1 nmole of product per min.

Purification of Soluble Phosphatidylserine Synthetase.

E.coli B was grown under the conditions as described by Kito *et al.* [32] and harvested during the middle exponential growth phase. 60 g wet weight cells was suspended in 360 ml of 10 mM potassium phosphate buffer, pH7.2, containing 5 mM dithiothreitol and cells were disrupted at 0°C with a sonicator, Kaijo Denki T-A-4201, (20 KHz) for 25 min. Cell debris was removed by centrifugation for 45 min at 13000g

(all procedures were performed at 0-5°C). The supernatant fluid was stirred for 30 min after the addition of 10mM Tris-HCl buffer, pH8.1, and 5 mM EDTA. Solid ammonium sulfate was added to the solution to give a final concentration of 50%. The precipitated material was removed from the ammonium sulfate solution by centrifugation for 20 min at 13000g. The precipitate was dissolved in 130 ml of 10 mM Tris-HCl buffer, pH8.1, containing 1 mM EDTA and 0.5 mM dithiothreitol, and dialyzed against the same buffer. To the dialyzed solution (175 ml) 50 mM MgCl₂ was added. After incubation for 20 min at 25°C, the suspension was centrifuged for 35 min at 13000g. Approx. 60% of the total phosphatidylserine synthetase activity and most of the phosphatidylglycerophosphate synthetase activity were found in the precipitate with MgCl₂. The supernatant fluid containing the rest of the enzyme activity was dialyzed against 50 mM potassium phosphate buffer, pH7.2, containing 0.5 mM dithiothreitol (buffer A), and then applied to a column of DEAE-Sephadex A-50, 2.6 x 100 cm, containing 17g ion-exchange Sephadex previously equilibrated with buffer A. After washing with a column volume of buffer A containing 0.05M NaCl(500ml), the chromatogram was developed with a 2000 ml linear gradient of NaCl ranging from 0.05 to 0.3M. The fraction (95-115) containing the enzyme with high specific activity were collected (Fig.4A) and dialyzed against buffer A. The protein solution was placed on a column of DEAE-Sephadex, 1.5 x 30 cm. The column was developed with a 1200 ml linear gradient of NaCl from 0.05 to 0.18 M. The elution profile was

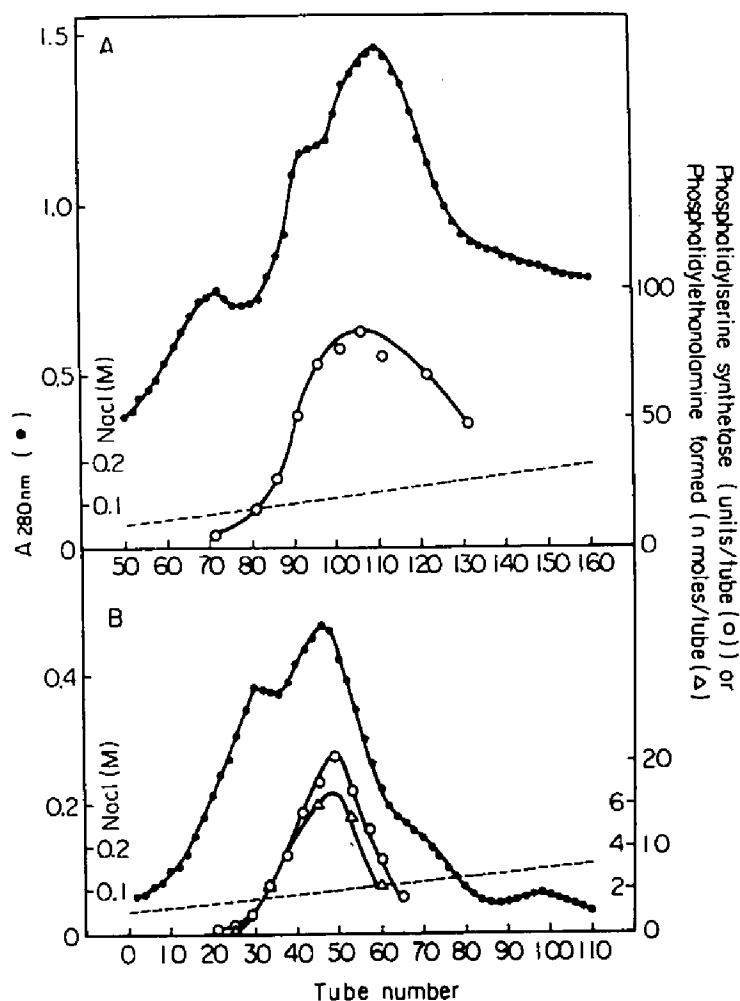


Fig. 4. DEAE-Sephadex chromatography of soluble phosphatidylserine synthetase.

(A) First, (B) second DEAE-Sephadex chromatography. Each tube contained 9 ml. Standard assay conditions were used. Phosphatidylserine synthetase (○); phosphatidylethanolamine formation in the membrane-phosphatidic acid complex (Δ); absorbance at 280 nm (●); NaCl concentration (—)

shown in Fig.4B. The enzyme activity became unstable in a dilute salt solution after DEAE-Sephadex treatments. Attempts to stabilize the enzyme perfectly were unsuccessful. The specific activities obtained by first and second DEAE-Sephadex chromatograms were 8.4 and 5.1 U/mg protein. The recovery of the total activity was 9.7%.

Ribosomal Phosphatidylserine Synthetase.

E.coli B cells (3g wet wt) harvested during the middle exponential growth phase were sonically disrupted with 25 ml of 10 mM Tris-HCl buffer, pH8.1, containing 10 mM MgCl₂ and 60 mM KCl. The extracts were centrifuged for 30 min at 30000g 15 min after being incubated with beef pancreas DNAase (4 µg protein/ml) for 15 min at 1°C. Then, supernatant was centrifuged for 120 min at 158000g. The ribosomal pellet (ratio of absorbance 235 nm/260 nm = 0.57) was suspended in the same buffer as above. The phosphatidylserine synthetase activity of this preparation was 13.5 U/mg protein.

Membrane-Phosphatidic Acid Complex.

The membrane-phosphatidic acid complex whose phosphatidic acid had been enzymatically synthesized *in situ* from *sn*-glycerol 3-phosphate and palmitoyl-CoA was prepared as described in the preceding Chapter. The incubation mixture contained 50 mM Tris-HCl buffer, pH 7.2, 15 mM MgCl₂, 1 mM *sn*-glycerol 3-phosphate, 0.14 mM palmitoyl-CoA

6mM dithiothreitol and *E.coli* B membrane particulate fraction[32,36] in a final volume of 1 ml. The incubation was run at 37°C, and 5 min after its incubation a further 0.12 mM palmitoyl-CoA was added. The incubation proceeded for additional 5 min and terminated by cooling in an ice bath. The reaction mixture was layered on 29 ml of a 10 to 40% sucrose density gradient made up in 10 mM Tris-HCl buffer, pH8.1, 10 mM MgCl₂ and 1 mM dithiothreitol at 1°C. The gradients were centrifuged for 10 min at 15000g and then 1 ml fractions were collected with an ISCO model 180 density-gradient fractionator. The fractions of protein peak of which profile was the same as the preceding Chapter were collected, diluted with the same buffer as above to reduce sucrose concentration and centrifuged for 60 min at 15000g. The membrane-phosphatidic acid complex obtained as pellet was suspended in the same buffer.

Assay of Phosphatidylethanolamine Synthesis from Membrane-Bound Phosphatidic Acid.

The standard assay contained 33 mM potassium phosphate buffer, pH 7.2, 15 mM MgCl₂, 1 mM CTP, 0.11 mM L-[U-¹⁴C]serine (7900 cpm/nmol), soluble phosphatidylserine synthetase and membrane-phosphatidic acid complex (228 µg protein) in a final volume of 0.6 ml. The incubation carried out for 10 min at 37°C was terminated by the addition of 15 ml chloroform and methanol(2:1,v/v) and 200 µg phosphatidylethanol-

amine (Sigma) and 100 μ g phosphatidylserine. After being washed twice with 30 ml of 2M KCl and water, chloroform layer was dried under nitrogen. The radioactive lipids were separated by thin-layer chromatography on plates of silica gel (Merck, Art 5721) with the solvent of chloroform-methanol-acetic acid (65:25:8) or chloroform-methanol-7N ammonia (60:35:5). The lipids were located by iodine vapour and the appropriate areas were scraped from the plates. The radioactivity was measured by suspending the scrapings into toluene scintillation solution.

Sucrose-Density-Gradient Centrifugation.

Samples (1 ml) to examine association of soluble phosphatidylserine synthetase to the membrane-phosphatidic acid complex layered on 29 ml of a linear sucrose density gradient, which was formed from 10-40% sucrose and contained 10 mM Tris-HCl buffer, pH8.1, and 1 mM dithiothreitol. The gradients were centrifuged for 20min at 15000g, and then 1.4 ml fractions were collected with an ISCO model 180 density gradient fractionator.

Determination of Protein.

Protein was determined by the method of Lowry *et al.* [49]. Bovine serum albumin was used as standard.

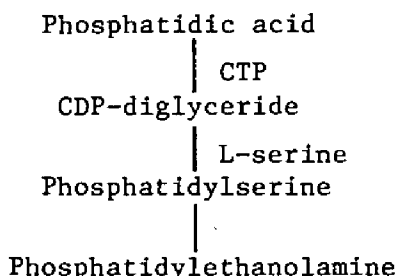
RESULTS

Soluble Phosphatidylserine Synthetase Reaction.

Soluble phosphatidylserine synthetase was found to catalyze the reaction to produce phosphatidylserine from CDP-diolein and L-serine (data not shown). K_m for L-serine was 150 μ M. The nonionic detergent Cutscum activated the enzyme greatly as described by Kanfer and Kennedy[26] and was added to the standard assay mixture. No phosphatidylglycerophosphate was formed by substitution of L-[U- 14 C]serine for *sn*-[U- 14 C]glycerol 3-phosphate.

Effect of Soluble Phosphatidylserine Synthetase on the Phosphatidylethanolamine Synthesis in the Membrane-Phosphatidic Acid Complex.

As described in the preceding Chapter, phosphatidic acid formed enzymatically from palmitoyl-CoA and *sn*-glycerol 3-phosphate remained associated with the membrane (membrane-phosphatidic acid complex). The formation of membrane phosphatidylethanolamine with a corresponding decrease in the amount of phosphatidic acid of the membrane-phosphatidic acid complex was proved to proceed in the presence of CTP, L-serine and soluble factor[Chapter I] similarly to the evidence by Thomas *et al.*[29](scheme 1). It was apparent in the elution profile shown in Fig.4B that the peak of this soluble factor coincided with that of soluble phosphatidylserine synthetase. As is shown in Fig.5,



Scheme 1. *Pathway of biosynthesis of phosphatidylethanolamine.*

soluble phosphatidylserine synthetase increased the phosphatidylethanolamine synthesis from the membrane-bound phosphatidic acid. Such a requirement of the soluble enzyme suggests the removal of this enzyme from the membrane-phosphatidic acid complex during preparation of this complex. The formation of small amounts of phosphatidylethanolamine without the addition of the soluble enzyme may be due to endogenous phosphatidylserine synthetase activity which will be described in the subsequent section. As described in "Materials and Methods", approx. 60% of total phosphatidylserine synthetase activity was precipitated with MgCl_2 during purification. However, this fraction was less effective to the phosphatidylethanolamine formation than the soluble enzyme, since only 0.14 nmole phosphatidylethanolamine per ml was found to be formed by the addition of 1.7 units of this enzyme activity compared with that by the soluble enzyme (Fig.5)

Effect of Ribosomal Phosphatidylserine Synthetase on the Phosphatidyl-

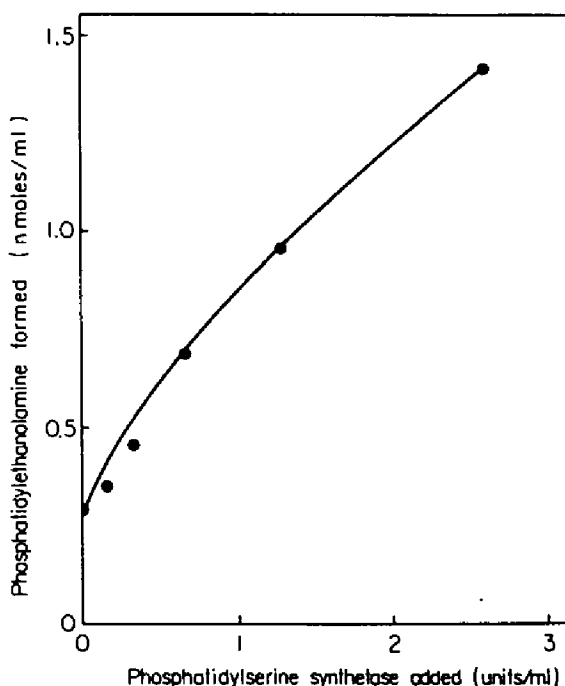


Fig. 5. Effect of soluble phosphatidylserine synthetase on phosphatidylethanolamine synthesis in the membrane-phosphatidic acid complex.

Standard assay conditions were used except that the amounts of the enzyme were varied as shown.

ethanolamine Synthesis in the Membrane-Phosphatidic Acid Complex.

The ribosomal phosphatidylserine synthetase was found to have a K_m of 150 μM for L-serine which is the same as that of the soluble enzyme. A requirement of ribosomal enzyme for the phosphatidylethanolamine synthesis in the membrane-phosphatidic acid complex was examined. As is shown in Table 7, the amounts of phosphatidylethanolamine formed in the presence of the ribosomal enzyme was 8 to 15% of those formed in the presence of the soluble enzyme. The

Table 7. *Requirements of soluble and ribosomal phosphatidylserine synthetase for phosphatidylethanolamine synthesis.*

The phosphatidylethanolamine synthesis in the membrane-phosphatidic acid complex was determined under the standard conditions except that various enzyme preparations were used. Figures in parentheses are shown by subtracting the values obtained with no addition of the enzymes.

	Addition of phosphatidylserine synthetase	Phosphatidylethanolamine formed
	U	nmol
None		0.190(0)
Soluble enzyme	0.92	0.598(0.408)
	1.84	0.707(0.517)
Ribosomal enzyme	0.92	0.222(0.032)
	1.85	0.266(0.076)
Soluble enzyme plus ribosomal enzyme	0.92 + 0.92	0.407(0.217)

phosphatidylethanolamine synthesis was approx. 50% inhibited by the addition of the ribosomal enzyme to the soluble enzyme. The inhibition can be accounted for a decrease in the amounts of the soluble enzyme by its binding to ribosomes rather than by the presence of inhibitors in the ribosome preparation (Table 8). Hence, the soluble enzyme appears to participate in the biosynthesis of phosphatidylethanolamine in the membrane.

Association of Soluble Phosphatidylserine Synthetase to the Membrane-Phosphatidic Acid Complex.

Table 8. *Binding of soluble phosphatidylserine synthetase to ribosomes.*

After mixing 23.5 U soluble phosphatidylserine synthetase and ribosomes, 2.2 mg protein, with 29.7 U enzyme activity in 6 ml of 10 mM potassium phosphate buffer, pH7.2, the mixture was centrifuged for 120 min at 158000g to separate the soluble and ribosomal enzymes after incubation for 30 min at 5°C. The enzyme activities were determined under the standard assay conditions.

Phosphatidylserine synthetase	Enzyme activity		
	before mixing	mixture	after centrifugation
	U/ml		
Soluble enzyme	3.92	8.87	1.47
Ribosomal enzyme	4.94		6.70

Association of soluble phosphatidylserine synthetase to the membrane-phosphatidic acid complex was examined by sucrose density-gradient centrifugation. The sedimentation profile of the membrane-phosphatidic acid complex indicates the presence of small amounts of endogenous phosphatidylserine synthetase activity which was firmly bound to the membrane and gradually being released(Fig.6A). It is uncertain whether this activity is inherent or an artifact arising during preparation of the membrane-phosphatidic acid complex. The soluble enzyme appears to be loosely associated with the membrane, since trailing peak of the enzyme activity was observed during sedimentation(Fig.6B).

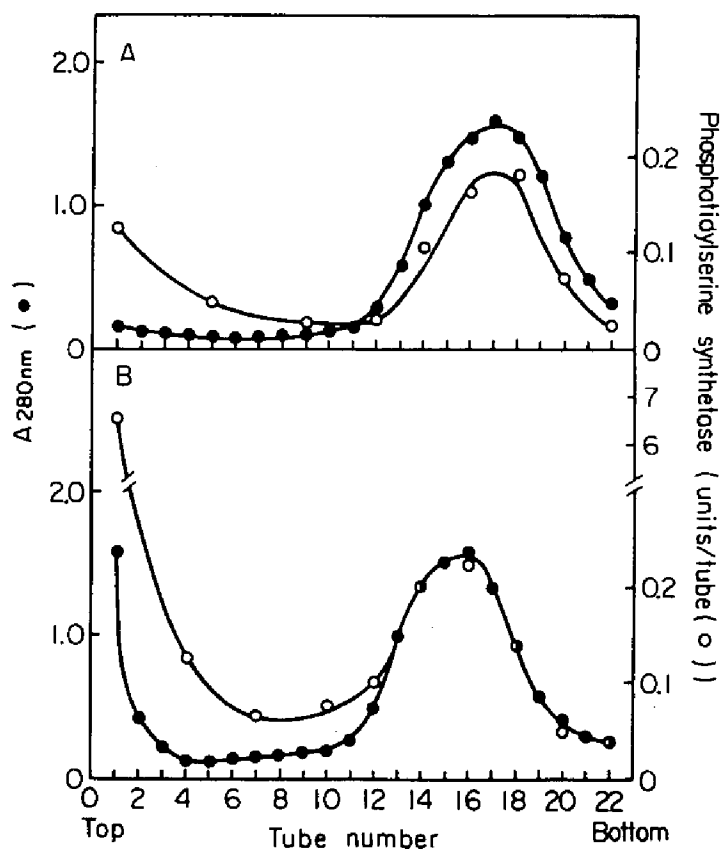
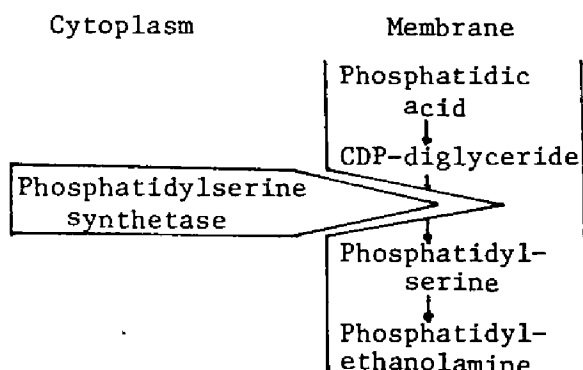


Fig. 6. Association of soluble phosphatidylserine synthetase with the membrane-phosphatidic acid complex.

(A) The membrane-phosphatidic acid complex, 2.9 mg protein.
 (B) The membrane-phosphatidic acid complex, 2.9 mg protein, 33 mM potassium phosphate buffer, pH 7.2, 5 mM $MgCl_2$ and 12 U soluble phosphatidylserine synthetase in a final volume of 1.5 ml were incubated for 1 min at 37°C, then cooled in ice bath and applied to a sucrose density gradient as under "Materials and Methods". After centrifugation, 1.4 ml fractions were collected. Standard assay conditions were used. Phosphatidylserine synthetase activity (○) and absorbance at 280 nm. (●)



Scheme 2. *Participation of soluble phosphatidylserine synthetase in the phosphatidylethanolamine synthesis in the membrane.*

DISCUSSION

Kanfer and Kennedy have found phosphatidylserine synthetase in the extracts of *E.coli* B[26], and very low activities of this enzyme have been observed in the membrane[27-28]. Raetz and Kennedy have described a large portion of the enzyme activity is firmly associated with ribosomes[57]. In our experiments, approx.60% of the total phosphatidylserine synthetase activity was precipitated with $MgCl_2$, and the rest was obtained in a soluble form. However, the soluble enzyme was found to act more effectively with regard to the phosphatidylethanolamine synthesis from the membrane-bound phosphatidic acid *via* CDP-diglyceride than the ribosomal enzyme. Phosphatidylserine synthetase hypersensitive to temperature has been found in a mutant thermosensitive for the synthesis of phosphatidylethanolamine[e]. Hence, this enzyme may be a peripheral

protein[48] and participate in the phosphatidylethanolamine synthesis in *E.coli*(Scheme 2).

It has become apparent that the soluble enzyme tends to be bound to ribosomes. Nevertheless, the role of the ribosomal enzyme remains obscure.

Chapter III

Regulatory Effects of Phospholipids on Soluble Phosphatidylserine Synthetase of *Escherichia coli*.^{g,h)}

Phosphatidylserine synthetase occurs in a soluble form and participates in the biosynthesis of phosphatidylethanolamine by its association with the membrane of *Escherichia coli* as described in Chapter II. However, the other enzymes responsible for the biosynthesis of phospholipids are firmly bound to the membrane[27-28,b]. It was thought of interest to see how the soluble enzyme functions in the membrane. From the viewpoint of lipid metabolism in the membrane, the soluble enzyme was assumed to regulate the phosphatidylethanolamine synthesis in unique fashion. The effects of major phospholipid classes of *Escherichia coli* membrane on the kinetic parameters of the soluble enzyme are described in this Chapter.

MATERIALS AND METHODS

Reagents.

CDP-diolein, CDP-dipalmitin, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine and cardiolipin were the products of Serdary Research Lab. Palmitic and *cis*-vaccenic acids were purchased from Applied Science Lab. Linoleic acid was the product of

Nutritional Biochemical Corp. L-[U-¹⁴C]Serine and L-[3-¹⁴C]serine were purchased from the Radiochemical Center. Silica gel TLC plates (Art 5721) were the products of Merck.

Soluble Phosphatidylserine Synthetase.

Soluble phosphatidylserine synthetase was purified from *E.coli* B according to the method described in Chapter II. Enzyme unit was defined as Chapter II.

Membrane-Phosphatidic Acid Complex.

The membrane-bound phosphatidic acid which had been synthesized from *sn*-glycerol 3-phosphate and palmitoyl-CoA with the isolate membrane of *E.coli* B was prepared as Chapter II.

Assay of Phosphatidylserine Synthetase.

The reaction mixture contained 33 mM potassium phosphate buffer, pH 7.2, 15 mM MgCl₂, 0.11 mM L-[U-¹⁴C]serine (11455 cpm/nmol), 1.5 mM CDP-diolein and an appropriate amount of soluble phosphatidylserine synthetase in a volume of 0.6 ml. Prior to initiation of the reaction by the addition of CDP-diolein, the mixture was incubated for 1 min at 37°C. The reaction was allowed to proceed for 10 min at 37°C and terminated by the addition of chloroform-methanol(2:1) and 200 µg phosphatidylserine. After being washed twice with 30 ml 2M KCl, pH 2.0,

and distilled water, chloroform layer was dried under nitrogen and separated by thin-layer chromatography with the solvent of chloroform-methanol-acetic acid(65:25:8). The area of phosphatidylserine was scraped and transferred to a scintillation vial.

Preparation of Lipid Dispersion.

Phospholipids suspended in 83 mM potassium phosphate buffer, pH 7.2, and fatty acid in distilled water at concentrations of 0.25% were sonicated for 3 min with a sonicator, Kaijo Denki TA-4201(20 KHz) by cooling in an ice bath.

Analytical Methods.

Protein was determined by the method of Lowry *et al*[49]. Bovine serum albumin was used as standard. Radioactivity was measured in the toluene scintillation solution on a Packard liquid-scintillation counter.

RESULTS

Formation of Membrane-Bound CDP-diglyceride from Membrane-Bound Phosphatidic Acid.

All of the reaction steps for the biosynthesis of phosphatidylethanolamine from phosphatidic acid were assumed to proceed in the membrane[27-28,b]. Hence, soluble phosphatidylserine synthetase may act on membrane-bound CDP-diglyceride and free L-serine to form

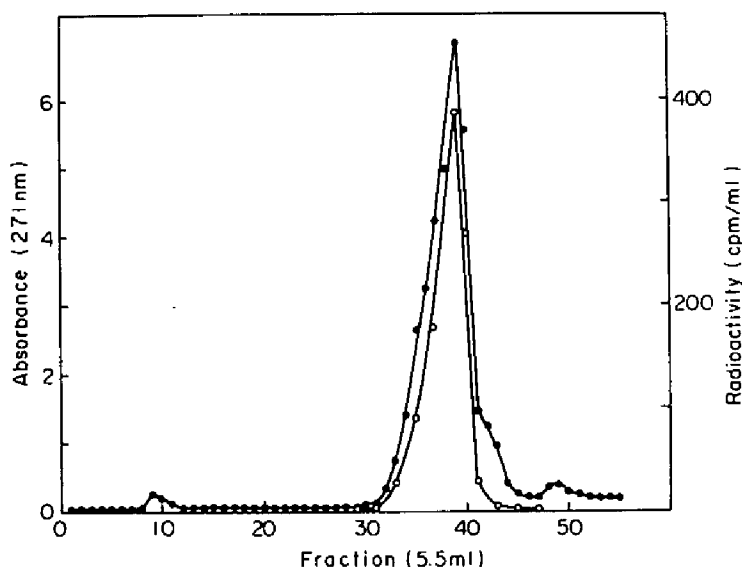


Fig. 7. *Formation of CDP-diglyceride from membrane-bound phosphatidic acid.*

The reaction mixture contained 33 mM potassium phosphate buffer, pH7.2, 15 mM MgCl_2 , 13 nmoles $[\text{U-}^{14}\text{C}]\text{CTP}$ (370 $\mu\text{Ci}/\mu\text{mol}$) and membrane-phosphatidic acid complex (1.35 mg protein) in a volume of 1 ml. The reaction was carried out for 2 min at 37°C and terminated by the addition of 15 ml chloroform-methanol (2:1, v/v) and 30 mg CDP-di-palmitin. After being washed with 30 ml water, pH1.5, and twice with distilled water, chloroform layer to which 15 ml methanol and 4.5 ml water had been added was applied to a column of DEAE-cellulose (acetate form), 1.5 x 30 cm, containing 7g ion-exchange cellulose in chloroform-methanol-water (2:3:1). The chromatogram was developed with a 500 ml linear gradient of ammonium acetate, pH7.4, ranging from 0 to 0.2 mM. Absorbance at 271 nm (●), radioactivity (○).

phosphatidylserine in the membrane. It was examined whether membrane-bound CDP-diglyceride was formed from membrane-bound phosphatidic acid and CTP by membrane-bound phosphatidate cytidyltransferase [27-28].

Table 9. *Formation of membrane-bound CDP-diglyceride from membrane-bound phosphatidic acid.*

The reaction was performed under the conditions similar to those described in Fig.7. The reaction was terminated in an ice bath and centrifuged for 4 min at 0°C at 150000g. Fifteen ml chloroform-methanol(2:1) and 20 mg CDP-diglyceride were added to supernatant and pellet fractions. After being washed with 30 ml water,pH1.5, and distilled water, chloroform layer was dried up under nitrogen and separated by thin-layer chromatography with solvent of diisobutylketone-acetic acid-water(40:30:7). The area of CDP-diglyceride was scraped and transferred to a scintillation vial.

Fractions	CDP-diglyceride
	(cpm)
Supernatant	96
Pellet	2768

Membrane-phosphatidic acid complex and [U-¹⁴C]CTP were incubated for 2 min at 37°C and terminated with chloroform-methanol(2:1,v/v), and the chloroform layer was washed with water,pH1.5, and distilled water. Radioactive products were co-chromatographed with cold CDP-dipalmitin on DEAE-cellulose at pH7.4 with a linear salt gradient according to Raetz and Kennedy[60]. As shown in Fig.7, the radioactive lipid was eluted in the identical fractions to the peak (absorbance at 271 nm) of the carrier CDP-dipalmitin. Then, the peak fractions(31-41) were collected, dried up and separated by thin-layer chromatography with the solvent of diisobutylketone-acetic acid-water(40:30:7,by vol). The radioactivity was found to coincide in position with CDP-dipalmitin. On the other hand, the reaction mixture containing membrane-phosphatidic acid complex and [U-¹⁴C]CTP

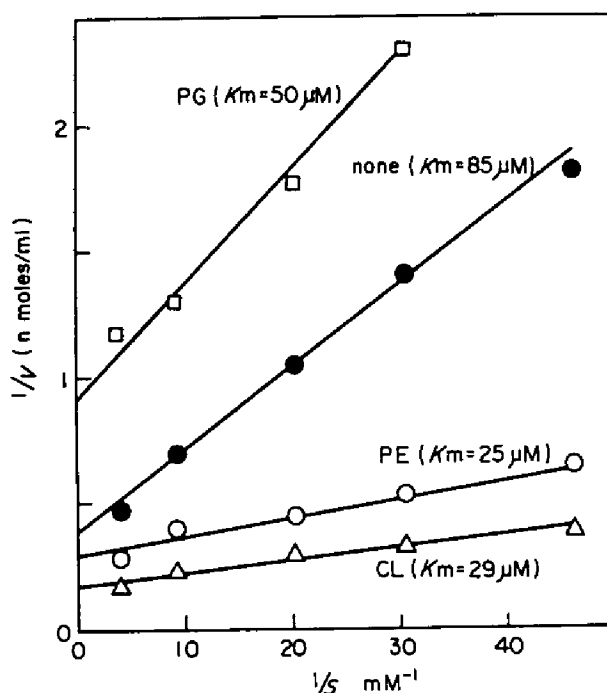


Fig. 8. Determination of K_m for L-serine in the presence of free CDP-diglyceride with or without phospholipids.

The reaction was performed under the assay conditions except that phospholipids were added at a concentration of 0.1%, and that the concentration of L-[U- ^{14}C]serine was varied as shown. None, (●); phosphatidylethanolamine, (○); phosphatidylglycerol, (□); cardiolipin, (△)

was centrifuged for 4 min at 0°C at 150000g after incubation for 2 min at 37°C , and separated to supernatant and pellet. The radioactive CDP-diglyceride was found to be localized in the pellet fraction (Table 9). Hence, CDP-diglyceride which had been formed from membrane-bound phosphatidic acid and CTP were proved to be firmly bound to the membrane. Membrane-bound CDP-diglyceride was used as the substrate of soluble phosphatidylserine synthetase, which will be described in the subsequent section.

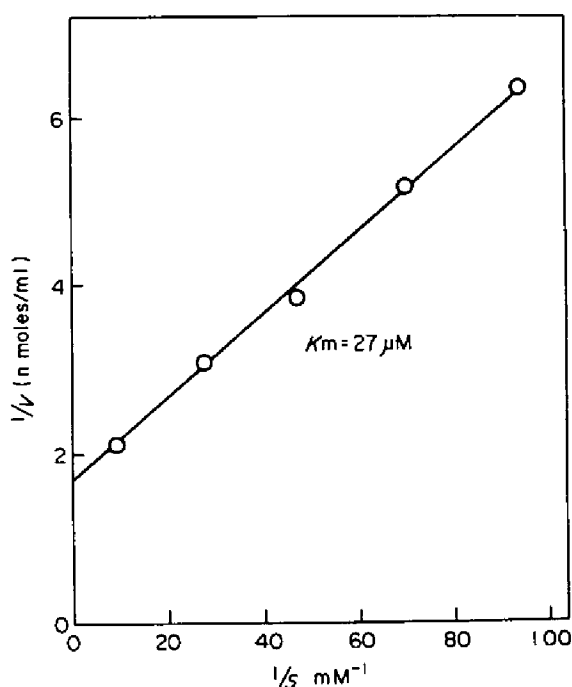


Fig. 9. Determination of K_m for L-serine in the presence of membrane-bound CDP-diglyceride.

The reaction mixture contained 33mM potassium phosphate buffer, pH7.2, 15mM $MgCl_2$, 1mM CTP, various concentrations of L-[3- ^{14}C] serine(12300 cpm/nmol), membrane-phosphatidic acid complex(96 μg protein) and 2.4 units soluble phosphatidylserine synthetase in a volume of 0.6 ml. The reaction was allowed to proceed for 10 min at 37°C and terminated by the addition of 15 ml chloroform-methanol(2:1,v/v) and 200 μg each of phosphatidylserine and phosphatidylethanolamine. After being washed twice with 30 ml 2M KCl, pH2.0, and distilled water, chloroform layer was transferred to a scintillation vial and dried.

K_m for L-Serine.

When free CDP-diglyceride was used as an acceptor of L-serine to produce phosphatidylserine, K_m of soluble phosphatidylserine synthetase for L-serine was described to be 150 μM in the presence

of detergent Cutscum [Chapter II], which is 5-fold lower than that obtained with crude enzyme[26]. In the absence of Cutscum, K_m for L-serine was found to be 85 μ M(Fig. 8). However, when membrane-bound CDP-diglyceride was substituted for free CDP-diglyceride, K_m for L-serine was 27 μ M(Fig. 9). From the evidence that K_m for L-serine in the presence of membrane-bound CDP-diglyceride was lowered 3-fold than that in the presence of free CDP-diglyceride, it is suggested that changes in the conformation of soluble phosphatidylserine synthetase may be caused by its association with the membrane to utilize membrane-bound CDP-diglyceride.

Effects of Phospholipids on Soluble Phosphatidylserine Synthetase.

Association of soluble phosphatidylserine synthetase with membrane as described in Chapter II may raise a possibility that membrane phospholipids interact with the soluble enzyme. Effects of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, the major classes of the phospholipids of *E.coli*, on the soluble enzyme were examined in the presence of free CDP-diglyceride.

In the presence of Cutscum, phospholipids behaved more or less inhibitory to the enzyme(Table 10). The enzyme may be affected by a mixed micelle of Cutscum and individual phospholipids, since different modes of the effects were observed in the absence of Cutscum. As shown in Fig. 8, cardiolipin activated the enzyme and lowered K_m

Table 10. *Effects of phospholipids on soluble phosphatidylserine synthetase with or without Cutscum.*

The reaction was performed under the assay conditions except that phospholipids and Cutscum were added at concentration of 0.1%.

Addition	Phosphatidylserine formed	
	with Cutscum	without Cutscum
	nmoles/ml	
None	18.04	1.67
Phosphatidylethanolamine	12.66	2.47
Phosphatidylglycerol	5.14	0.55
Cardiolipin	5.29	4.32

for L-serine 85 μ M to 29 μ M. Phosphatidylethanolamine slightly activated the enzyme and decreased K_m from 85 μ M to 25 μ M. However, in the presence of phosphatidylglycerol, the enzyme was strongly inhibited, and K_m for L-serine was 50 μ M. Phosphatidylserine exhibited no effect. Though the assays were performed with preincubation of the enzyme and individual phospholipids for 1 min, similar results were obtained without preincubation.

As shown in Table 11, the compounds which constitute hydrophilic moieties of phospholipids such as glycerol, *sn*-glycerol 3-phosphate and ethanolamine showed essentially no effect at the concentrations similar to those of phospholipids used. The enzyme was slightly activated by unsaturated fatty acids.

Table 11. *Effect of various compounds on soluble phosphatidylserine synthetase.*

The reaction was performed under the assay conditions except that various compounds were added at concentrations of 1.3 μ moles/ml.

Additions	Phosphatidylserine formed (nmoles/ml)
None	2.15
Ethanolamine	1.98
Glycerol	2.19
<i>sn</i> -Glycerol 3-phosphate	1.63
Palmitic acid	1.98
<i>cis</i> -Vaccenic acid	3.08
Linoleic acid	3.65

Desensitization of Phospholipids Effects by Heat Treatment.

It was examined whether the effects of phospholipids were caused by their binding to the soluble enzyme.

The enzyme was completely desensitized by treatment for 5 min at 40°C against the effects of cardiolipin and phosphatidylethanolamine without loss of the activity (Table 12). Phosphatidylglycerol may bind to the different site on the enzyme from those for cardiolipin and phosphatidylethanolamine, since heat treatment failed to change the phosphatidylglycerol effect. The enzyme was completely inactivated by treatment for 5 min at 50°C, while the crude enzyme was rather stable at least for 30 min at 37°C[e].

Table 12. *Desensitization of phospholipids effects by heat treatment.*

The mixture containing 50 mM potassium phosphate buffer, pH7.2, 91 mM $MgCl_2$, 0.5 mM dithiothreitol and soluble phosphatidylserine synthetase(9.1 units) in a volume of 1 ml was incubated for 5 min at 40°C. The reaction was carried out under the assay conditions except that the enzyme treated as above was used, and that phospholipids were added at a concentration of 0.1%. The activity obtained in the absence of phospholipids without heat treatment was regarded as 100%.

Heat treatment	Addition	Phosphatidylserine formed (nmoles/ml)	Activity %
0 min	None	2.08	100
	Cardiolipin	5.67	273
	Phosphatidyl- ethanolamine	3.08	148
	Phosphatidyl- glycerol	0.59	28
5 min	None	1.62	78
	Cardiolipin	2.04	98
	Phosphatidyl- ethanolamine	1.98	95
	Phosphatidyl- glycerol	0.37	18

DISCUSSION

Detergent Cutscum which has been used as an activator for the assay of phosphatidylserine synthetase[26] was found to increased K_m for L-serine. Hence, the results obtained in the absence of Cutscum appear to be worthwhile to describe natural properties of the enzyme.

Soluble phosphatidylserine synthetase does not require phospholipids for its activity, but appears to require them for its functioning on the phosphatidylethanolamine biosynthesis. From the evidence that K_m for L-serine in the presence of free CDP-diglyceride with the coexistence of either phosphatidylethanolamine or cardiolipin was compatible with that in the presence of membrane-bound CDP-diglyceride, it is strongly suggested that those phospholipid species may alter the conformation of the soluble enzyme after its association with the membrane.

An intracellular L-serine concentration has been described to be 80-90 μM in *E.coli*[61], which may saturate the soluble enzyme associated with the membrane(K_m , 27 μM). On the other hand, K_m of the enzyme in the free state was 85 μM and decreased to 25-29 μM in the presence of phosphatidylethanolamine and cardiolipin. Such changes in K_m values are assumed to bear a close relationship to the pool size L-serine.

Effects of phospholipid species on the soluble enzyme may depend on their polar head groups, since no relationship was observed between fatty acid compositions of phospholipid species and either activation or inhibition of the enzyme(Table 13). The individual compounds corresponding to the polar head groups or hydrocarbon chains of phospholipids showed essentially no effect on the soluble

Table 13. *Fatty acid composition of phospholipid species used.*

Fatty acid composition was determined by the method of Kito *et al.* [20]. Values are expressed as weight percentage of total fatty acids.

Fatty acids	Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Cardio-lipin	Phosphatidyl-serine
Myristic acid	5	trace	trace	trace
Palmitic acid	52	34	1	2
Palmitoleic acid	14	trace	2	
<i>cis</i> -9,10-Methylenehexadecanoic acid	8			
Stearic acid	trace	14	1	62
Oleic acid	13*	29	8	33
Linoleic acid		20	85	
Unknown	8	3	3	3

**cis*-Vaccenic acid

enzyme. Thus, the whole molecular structure of phospholipids appears to be necessary for their functions.

At least two different sites on the enzyme for phospholipids were demonstrated by the desensitization experiments. Effects of cardiolipin and phosphatidylglycerol may raise the possibility that phosphatidylserine synthetase, finally the phosphatidylethanolamine biosynthesis, is controlled by phospholipid species of the alternate biosynthetic pathway in the membrane.

Chapter IV

Occurrence of Separate Pools of Phosphatidic Acid for Biosynthesis of Phospholipids in *Escherichia coli* Membrane ^{f)}

Differences in fatty acid compositions of different phospholipid species in *Escherichia coli* have been reported by Kito *et al.* [19-21,c]. The phospholipids in this organism are formed by acylation of *sn*-glycerol 3-phosphate to form phosphatidic acid, which is precursor of phospholipids. The present Chapter describes that separate pools of phosphatidic acid are available for the syntheses of individual phospholipids. This may explain the differences observed in the compositions of fatty acids in different phospholipid species.

MATERIALS AND METHODS

Growth Condition.

E. coli B was grown at 17 and 37°C in modified M-9 medium supplemented with 0.1% casamino acids and 0.2% glucose[32]. Growth was followed by measuring the absorbance at 660 nm.

Pulse Labeling with [1-¹⁴C]Acetate.

Cells were labeled with sodium [1-¹⁴C]acetate (2.0 µCi/µmol, 1 µCi/ml of culture ; New England Nuclear) in the middle

exponential growth phase (100 ml of A₆₆₀ 1.0). After 3 min, labeling was stopped by addition of 5% cold trichloroacetic acid.

Extraction and Separation of Lipids.

Trichloroacetic acid precipitates were suspended in 1 ml of water, and 5 ml of methanol was added, followed by 10 ml of chloroform. After standing for an hour at 25°C, the lipid extracts were washed twice with 2M KCl and once with water. The lipids were separated by thin-layer chromatography on plates of Silica Gel (Merck Art 5721) with chloroform-methanol-acetic acid (65:25:8) as solvent.

Analysis of Fatty Acids of Phospholipids.

Phosphatidylethanolamine and phosphatidylglycerol obtained were esterified in 3% solution of methanolic HCl in the water bath of 93°C for 2 hr. Fatty acid methylesters were extracted from the reaction mixture with ether three times and washed out the remaining HCl with the same volume of distilled water. The extracted labeled fatty acid methylesters were collected using a 0.6 x 300 cm glass column packed with 15% DEGS on Neopack 1A at 187°C. The fatty acid methylesters were analysed by gas-liquid chromatography.

Preparation of Phosphatidylserine Synthetase and Phosphatidyl-

glycerophosphate Synthetase.

Phosphatidylserine synthetase and phosphatidylglycerophosphate synthetase were prepared as described in Chapter II.

Phosphatidylethanolamine and Phosphatidylglycerol Synthesis from Membrane-Bound Phosphatidic Acid Complex.

Standard assay of phosphatidylethanolamine synthesis from membrane-phosphatidic acid complex, prepared as described in Chapter I, contained 33 mM potassium phosphate buffer, pH7.2, 15 mM MgCl_2 , 1 mM CTP, membrane-phosphatidic acid complex (123 μg protein), 0.11 mM L-[U- ^{14}C]serine (7900 cpm/nmol) and 14.6 units of soluble phosphatidylserine synthetase in 0.6 ml incubation mixture. In the case of phosphatidylglycerol synthesis, 0.1 mM *sn*-[U- ^{14}C]glycerol 3-phosphate (8300 cpm/nmol) and 0.4 unit of phosphatidylglycerophosphate synthetase instead of L-serine and soluble phosphatidylserine synthetase, respectively, were contained. Reactions were terminated by adding 15 ml of chloroform and methanol (2:1), and then 200 μg of phosphatidylethanolamine (Sigma), 200 μg of phosphatidylglycerol (Serdary), and 100 μg of phosphatidylserine (Calbiochem) were added. Analysis of the radioactive reaction products was described in Chapter I.

Determination of Protein.

Protein was determined by the method of Lowry *et al.* [49].

RESULTS

Fatty Acid Composition of Bulk, and Newly Synthesized Phospholipids of E.coli B.

E.coli B was grown at 17 and 37°C. Both cultures were pulse labeled with sodium[1-¹⁴C]acetate for 3 min in the middle of the exponential growth phase (A_{660} 1.0), and then the fatty acid composition of newly synthesized phospholipids was analyzed as under "Materials and Methods". Differences were found in the fatty acid compositions, especially in the palmitoleic and *cis*-vaccenic acids contents of phosphatidylethanolamine and phosphatidylglycerol (Table 14). The results are in agreement with those by Merlie and Pizer (personal communication). *cis*-9,10-Methylenehexadecanoic acid is synthesized from palmitoleic acid esterified in the phospholipid molecules[58]. Counting it as palmitoleic acid the fatty acid compositions of newly synthesized phospholipids were found to be similar to those of bulk phospholipids. Thus, the basic fatty acid composition of phospholipid species appears to remain unchanged under similar growth conditions. If there is a common phosphatidic acid pool for syntheses of individual phospholipids, phosphatidylethanolamine and phosphatidylglycerol should have the same fatty acid composition. The similarity in the fatty acid compositions of phosphatidylethanolamine and phosphatidylglycerol in cells in the stationary phase of growth[19] seems to exclude the possibility

Table 14. *Fatty acid composition of bulk, and newly synthesized phospholipids of E.coli B.*

Cells were labeled with sodium [1-¹⁴C]acetate as described under "Materials and Methods". The isolation and analysis of lipids are described in that section. Values are expressed as percentages.

Fatty acid	Fatty acid composition			
	Phosphatidyl- ethanolamine		Phosphatidyl- glycerol	
	New	Bulk	New	Bulk
17°C				
Myristic acid	0.7	2.0	0.8	1.0
Palmitic acid	29.1	30.7	26.4	25.1
Palmitoleic acid	28.1	25.5	13.7	13.5
<i>cis</i> -9,10-Methylenehexa- decanoic acid	0.3	2.0	0.6	1.1
<i>cis</i> -Vaccenic acid	41.8	39.8	58.5	59.3
37°C				
Myristic acid	3.8	5.0	1.0	1.7
Palmitic acid	40.1	41.8	40.1	39.3
Palmitoleic acid	30.2	21.3	21.9	17.4
<i>cis</i> -9,10-Methylenehexa- decanoic acid	0.7	5.2	0.3	2.2
<i>cis</i> -Vaccenic acid	25.2	26.7	36.7	39.4

that the enzyme systems for syntheses of the different phospholipids show different substrate specificities with CDP-diglyceride molecular species. However, the differences cannot be adequately explained as due simply to subsequent rearrangement of fatty acids, since the reacylation activity is not operative[59] or low during the lag

period after shift-down of the growth temperature in *E.coli*[20].

Separate Pools of Phosphatidic Acid in the Membrane-Phosphatidic Acid Complex.

Therefore, studies were made on the possible existence of separate pools of phosphatidic acid, which primarily determine the fatty acid compositions of different phospholipid species. The membrane-phosphatidic acid complex was prepared as described in the preceding Chapter I and II. The phosphatidic acid of this complex is synthesized *in situ* from *sn*-glycerol 3-phosphate and palmitoyl-CoA by membrane-bound *sn*-glycerol 3-phosphate acyltransferase and mono-acylglycerol 3-phosphate acyltransferase[32,34]. The pathways for the synthesis of phosphatidylethanolamine and phosphatidylglycerol from phosphatidic acid in *E.coli* have been well established[26,32,40-42]. The enzymes responsible for the biosynthesis of phospholipids are localized in *E.coli* membrane[27-28,b]. Hence, membrane-bound phosphatidic acid was converted by the membrane-bound enzymes to phosphatidylethanolamine in the presence of CTP and L-serine, and to phosphatidylglycerol in the presence of CTP and *sn*-glycerol 3-phosphate, respectively[Chapter I,II]. The maximum activities for syntheses of phosphatidylethanolamine and phosphatidylglycerol from phosphatidic acid *via* CDP-diglyceride in the complex were obtained on addition of soluble phosphatidylserine synthetase and phosphati-

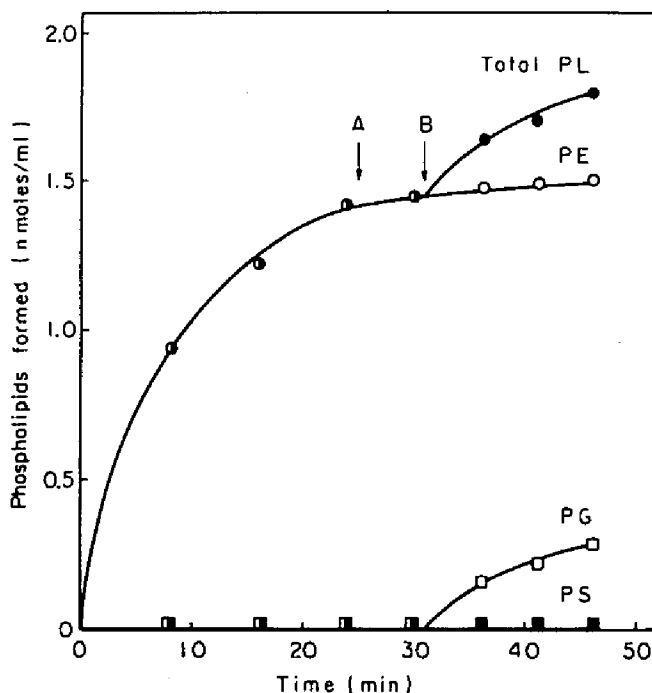


Fig. 10. *Separate pools of phosphatidic acid in the membrane-phosphatidic acid complex.*

Standard assay condition were used except that the volume of the incubation mixture was increased. Phospholipid formation was followed by extracting 0.6 ml samples from 4.5 ml of assay mixture with 15 ml of chloroform-methanol(2:1) at intervals during the reaction at 37°C. At the point indicated by the arrow (A), 119 μ moles of L-[U- 14 C]serine(7900 cpm/nmol) and 14.7 units of soluble phosphatidylserine synthetase were added. At the point (B), 120 μ moles *sn*-[U- 14 C]glycerol 3-phosphate(8300 cpm/nmol) and 0.22 unit of phosphatidylglycerophosphate synthetase were added. Analysis of radioactive phospholipids were described in "Materials and Methods". (o), phosphatidylethanolamine; ($\square$), phosphatidylglycerol; ($\blacksquare$), phosphatidylserine; ($\bullet$), total phospholipids.

phosphatidylglycerophosphate synthetase, respectively. Thus, if phosphatidylethanolamine and phosphatidylglycerol are synthesized *via* CDP-diglyceride from a common pool of phosphatidic acid, phosphatidyl-

glycerol should not be formed when the pool has been consumed for synthesis of phosphatidylethanolamine. However, as shown in Fig.10, when phosphatidylethanolamine synthesis in the membrane-phosphatidic acid complex had reached a plateau, addition of *sn*-[U-¹⁴C]glycerol 3-phosphate and phosphatidylglycerophosphate synthetase resulted in synthesis of phosphatidylglycerol. This result might be explained by supposing that phosphatidylethanolamine synthesis is inhibited by the product. However, this possibility is excluded by the high content of phosphatidylethanolamine in the membrane-phosphatidic acid complex. The small increase in the formation of phosphatidylglycerol, compared with that of phosphatidylethanolamine may be due to breakdown of phosphatidic acid pool during incubation. The synthesis of phosphatidylethanolamine(0.25 nmole) was also observed after phosphatidylglycerol synthesis had been completed(1.8 nmoles).

DISCUSSION

These results appear to differ from those obtained with *B. megaterium* by Patterson and Lennarz[31]. This difference may be because these workers used Cutscum, since detergents probably disturb the localization of CDP-diglyceride formed *in situ* from exogenous phosphatidic acid and CTP. The separate pools phosphatidic acid probably formed by the separately localized acyltransferase, under metabolic control coupled to the syntheses of individual phospholipids in the membrane of *E.coli*.

Chapter V

The Formation of Phosphatidylethanolamine in the Presence of ATP by a Particulate Enzyme System from *Escherichia coli* B.^{a)}

Phosphatidylethanolamine and phosphatidylglycerol as major phospholipid classes of *E. coli* are well known to be synthesized from phosphatidic acid *via* CDP-diglyceride [26,40-42]. Accordingly, among the four nucleoside triphosphate, roles of CTP on the biosyntheses of the phospholipids have been widely studied.

This Chapter describes the formation of phosphatidylethanolamine from *sn*-glycerol 3-phosphate and palmitoyl-CoA in the presence of ATP by a particulate enzyme system from *E. coli* B.

MATERIALS AND METHODS

Compounds and Analytical Procedures.

Various compounds used this experiment, synthesis of *sn*-glycerol 3-phosphate and purification of phospholipids from *E. coli* were described in Chapter I. Protein and phosphorous were determined by the method of Lowry *et al.* [49] and Bartlett [50], respectively.

Preparation of Particulate Acyltransferases Fraction.

Preparation of particulate *sn*-glycerol 3-phosphate acyltransferase and monoacylglycerol 3-phosphate acyltransferase fraction

from *E.coli* was described in Chapter I.

Analysis of Lipids.

Analysis of the radioactive reaction products and identification of the products were described in Chapter I.

Incubation of Acyltransferase Reaction.

The 0.3 ml of incubation mixture containing 33 mM Tris-HCl buffer, pH 7.2, 13.3 mM $MgCl_2$, 333 μM *sn*-[U- ^{14}C]glycerol 3-phosphate (920 cpm/nmol), 43 μM palmitoyl-CoA and particulate enzyme (0.1 mg protein) was incubated for 20 min at 37°C. The reaction was stopped by the addition of 15 ml chloroform and methanol (2:1, v/v) containing 0.4 mg of phosphatidylethanolamine, 0.2 mg of phosphatidylglycerol and 0.1 mg of phosphatidic acid, followed by analysis of lipid products as given under "Materials and Methods" in Chapter I.

RESULTS

Formation of Phosphatidylethanolamine in the Presence of ATP by a Particulate Enzyme System.

It was shown that the particulate enzyme fraction catalyzed a palmitoyl-CoA dependent conversion of *sn*-[U- ^{14}C]glycerol 3-phosphate in phosphatidic acid and lysophosphatidic acid as described Kito and Pizer [32] (Fig. 11A).

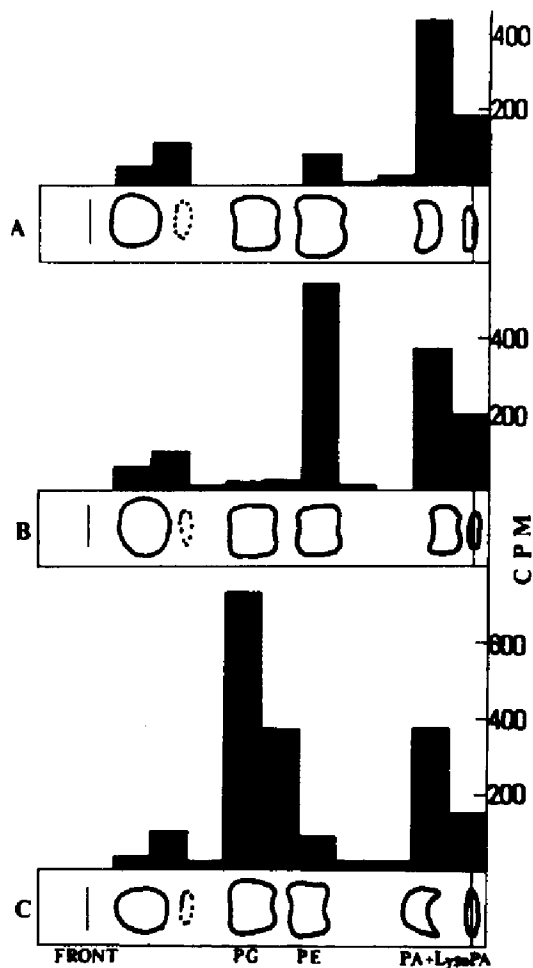


Fig. 11. *Thin-layer chromatography of the reaction products.*

The radioactive products obtained from the reaction in the absence of nucleoside triphosphate (A), in the presence of ATP (B), and of CTP (C) were chromatographed on silica gel plates with chloroform-methanol-7N ammonia (60:35:5). Lipids analysis were described in "Materials and Methods". The histograms show the distribution of radioactivity. PA, LysoPA, PE and PG indicate phosphatidic acid, lysophosphatidic acid, phosphatidylethanolamine and phosphatidylglycerol, respectively.

When 1 mM CTP was added to the incubation mixture, radioactive

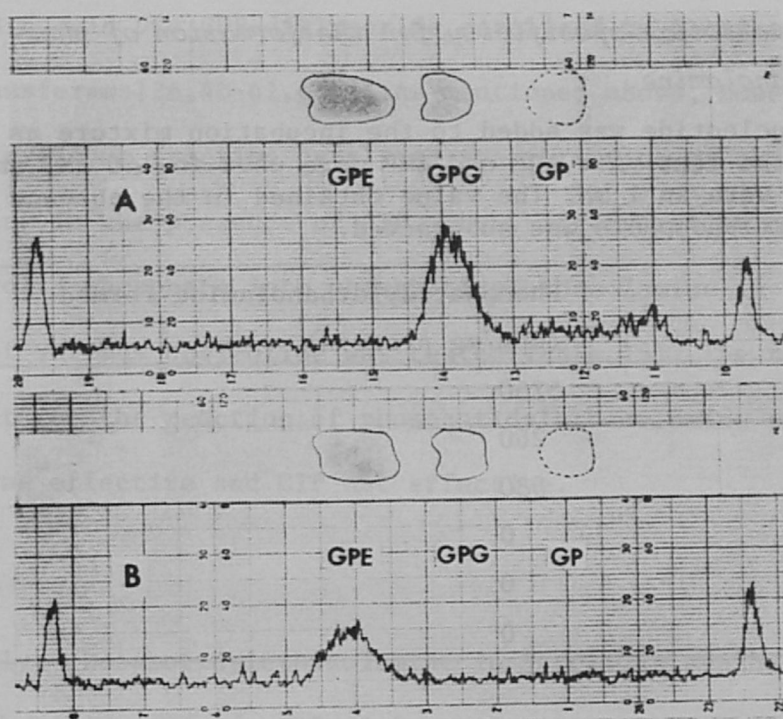


Fig. 12. Paper chromatography of the deacylated products.

The phospholipids were determined as in "Materials and Methods". The deacylated products from the radioactive phospholipids formed in the presence of ATP and of CTP correspond to (A) and (B), respectively. GP, GPG and GPE indicate *sn*-glycerol 3-phosphate, glycerolphosphorylglycerol and glycerolphosphorylethanolamine, respectively.

phosphatidylglycerol was formed (Fig. 11C), which was further confirmed by the observation that only radioactive glycerolphosphorylglycerol was obtained by the deacylation [51] of this compound (Fig. 12B). The results suggest the localization of the enzymes of the pathway for the biosynthesis of phosphatidylglycerol in the particulate enzyme used [26]. However, this particulate enzyme may lack phosphatidylserine synthetase since phosphatidylethanolamine could not be formed by

Table 15. *Nucleotide specificity for the formation of phosphatidylethanolamine.*

Each nucleotide was added to the incubation mixture as described in the text. ATP, ADP and AMP were used in 140 μ M, and CTP, GTP and UTP were in 1 mM. The value obtained in the absence of nucleoside triphosphate was subtracted.

Addition	Phosphatidylethanolamine formed (cpm/mg protein)
ATP	5250
ADP	260
AMP	530
CTP	0
GTP	0
UTP	0

the addition of L-serine with CTP to the incubation mixture. When 140 μ M ATP was substituted for CTP, radioactivity was found in the phosphatidylethanolamine area (Fig. 11B), which was then scraped and extracted with chloroform-methanol (2:1). The extract was acylated [51], and water-soluble derivatives were subjected to paper chromatography. The only radioactive compound produced by deacylation was glycerolphosphorylethanolamine (Fig. 12A). On this basis, the product formed in the presence of ATP was found to be phosphatidylethanolamine.

Nucleotide Specificity for the Formation of Phosphatidylethanolamine.

Phospholipids of *E. coli* have been known to be synthesized from

phosphatidic acid *via* CDP-diglyceride catalyzed by phosphatidate-cytidyltransferase[26,40-41,g,h]. As mentioned above, however, the particulate enzyme fraction catalyzed the formation of phosphatidylethanolamine in the presence of ATP. Then, incorporation of *sn*-[U-¹⁴C]glycerol 3-phosphate into phosphatidylethanolamine in the presence of various nucleotide was tested (Table 15). The results showed that for the reaction of phosphatidylethanolamine formation only ATP was effective and CTP not effective.

DISCUSSION

Usually, phosphatidylethanolamine in *E.coli* is synthesized from phosphatidic acid *via* CDP-diglyceride in the presence of L-serine. However, the results described above show that phosphatidylethanolamine is formed from *sn*-glycerol 3-phosphate and palmitoyl-CoA in the presence of ATP by the particulate enzyme. This phosphatidylethanolamine formation could not be observed by the removal of palmitoyl-CoA from the reaction mixture, and was not increased by the addition of L-serine or ethanolamine to the reaction mixture. These results suggest an exchange reaction between radioactive phosphatidic acid formed and phosphatidic acid moiety of phosphatidylethanolamine in the particulate enzyme in the presence of ATP.

SUMMARY

Chapter I ;

The phosphatidate formed by *sn*-glycerol 3-phosphate acyltransferase present in a particulate fraction from *Escherichia coli* remained associated with the particulate material and was converted to phosphatidylethanolamine and phosphatidylglycerol by other enzymes contained in the particulate fraction. Those enzymes required a heat-labile factor for the synthesis of phosphatidylethanolamine and a heat-stable factor for the phosphatidylglycerol. Both of these phospholipids like their precursor remained bound to the particulate fraction.

Chapter II ;

Phosphatidylserine synthetase occurs in a soluble and a ribosome-bound form in *Escherichia coli*. The soluble enzyme of *E.coli* B was partially purified by DEAE-Sephadex chromatography. The ribosomal enzyme was obtained by differential centrifugation. Two types of the enzyme had the same K_m (150 μM) for L-serine. The soluble enzyme was required for the biosynthesis of phosphatidylethanolamine from phosphatidic acid *via* CDP-diglyceride in the membrane. The ribosomal enzyme was less effective to the phosphatidylethanolamine formation than the soluble enzyme. By the sucrose

density gradient centrifugation method the soluble enzyme was proved to be loosely associated with the membrane.

Chapter III ;

Soluble phosphatidylserine synthetase participates in the biosynthesis of phosphatidylethanolamine in the membrane of *Escherichia coli*. When membrane-bound CDP-diglyceride, which had been enzymatically synthesized from membrane-bound phosphatidic acid and CTP, was used as an acceptor of L-serine to form phosphatidylserine, K_m of soluble phosphatidylserine synthetase for L-serine was 27 μM . On the other hand, when free CDP-diglyceride was substituted for membrane-bound CDP-diglyceride, K_m for L-serine was 85 μM . However, in the presence of cardiolipin, the enzyme was activated, and K_m was lowered from 85 μM to 29 μM . Phosphatidylethanolamine slightly activated the enzyme and decreased K_m to 25 μM . Phosphatidylglycerol inhibited the enzyme (K_m , 50 μM). Phosphatidylserine showed no effect. Treatment of the enzyme for 5 min at 40°C resulted in loss of sensitivity to the effects of cardiolipin and phosphatidylethanolamine except phosphatidylglycerol without changing the activity.

Chapter IV ;

Cultures of *Escherichia coli* B were pulse labeled with sodium [1- ^{14}C]acetate for 3 min. The newly synthesized individual

phospholipids were found to be different in the fatty acid composition as well as bulk phospholipids, especially in the palmitoleic and *cis*-vaccenic acids contents of phosphatidylethanolamine and phosphatidylglycerol. On the other hand, phosphatidic acid for the phosphatidylethanolamine synthesis and phosphatidic acid for the phosphatidylglycerol synthesis were present in the membrane-bound phosphatidic acid. These results suggest the possibility of occurrence of separate pools of phosphatidic acid for biosynthesis of phospholipids in *E.coli* membrane.

Chapter V ;

Phosphatidylethanolamine was formed *sn*-glycerol 3-phosphate and palmitoyl-CoA in the presence of ATP by a particulate enzyme from *Escherichia coli*. This result suggests that radioactive phosphatidylethanolamine was formed by a exchange reaction between the radioactive phosphatidate and a corresponding moiety of endogenous phosphatidylethanolamine in the particulate enzyme from *E.coli*.

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