The enzymes of phospholipid synthesis in *Clostridium butyricum*[1]

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Abstract We have examined extracts of *Clostridium butyricum* for several enzymes of phospholipid synthesis. Membrane particles were shown to catalyze the formation of CDP-diglyceride from [^{3}H]CTP and phosphatidic acid. The reaction was dependent on Mg^{2+} and stimulated by monovalent cations. CDP-diglyceride formed in vitro was found to be a substrate for both phosphatidylglycerophosphate synthetase and phosphatidylserine synthetase. The formation of phosphatidylglycerophosphate from added CDP-diglyceride and [U-^{14}C]*sn*-glycerol-3-phosphate was dependent on Mg^{2+} and Triton X-100. The dephosphorylation of endogenously-generated phosphatidylglycerophosphate to yield phosphatidylglycerol was observed to be pH-dependent. The formation of phosphatidylserine from CDP-diglyceride and L-[3-^{14}C]serine was stimulated by Mg^{2+} and Triton X-100. dCDP-diglyceride was a suitable substrate for both phosphatidylglycerophosphate synthetase and phosphatidylserine synthetase. Phosphatidylserine decarboxylase activity was barely detectable in membrane particles from *C. butyricum*. The addition of *E. coli* membrane particles provided efficient phosphatidylserine decarboxylase activity in this system. Although plasmalogens are the principal lipids of *C. butyricum*, none of the products of phospholipid synthesis formed in vitro contained measurable amounts of plasmalogens. The subcellular distribution of both phosphatidylglycerophosphate synthetase and phosphatidylserine synthetase in *C. butyricum* was also studied. Both were found to be membrane-associated—**Silber, P., R. P. Borie, and H. Goldfine.** The enzymes of phospholipid biosynthesis in *Clostridium butyricum*. *J. Lipid Res.* 1980. **21:** 1022–1031.

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Considerable progress has been made toward understanding the pathways and mechanisms of phospholipid biosynthesis in bacteria. Many of the enzymes have been characterized and several have been isolated. Most of this work has been done in *Escherichia coli* (for reviews see 1, 2). After the acylation of *sn*-glycerol-3-phosphate by transfer of an acyl group from either the acyl carrier protein or CoA, the phosphatidic acid formed has been shown to undergo the following series of reactions, which lead to phosphatidylglycerol or phosphatidylethanolamine (1, 2).

$$\text{Phosphatidic acid} + \text{CTP} \xrightarrow{Mg^{2+}} \text{CDP-diglyceride} + PP_i \quad 1)$$

$$\text{CDP-diglyceride} + \textit{sn}\text{-glycerol-3-phosphate} \xrightarrow{Mg^{2+}} \text{Phosphatidylglycerophosphate} + \text{CMP} \quad 2)$$

$$\text{Phosphatidylglycerophosphate} \rightarrow \text{Phosphatidylglycerol} + P_i \quad 3)$$

$$\text{CDP-diglyceride} + \text{L-serine} \rightarrow \text{Phosphatidylserine} + \text{CMP} \quad 4)$$

$$\text{Phosphatidylserine} \rightarrow \text{Phosphatidylethanolamine} + CO_2 \quad 5)$$

Work on other bacterial systems has shown that these pathways are generally applicable to aerobic and facultative bacteria (3–6); however, no comparable studies have been reported in anaerobic bacteria. Unlike aerobic and facultative bacteria, many strict anaerobes have been found to have plasmalogens as major components of their phospholipids. These lipids contain an alk-1-enyl ether bond at the C-1 position of the *sn*-3-glycerol-phospholipids (7, 8).

Plasmalogens are also present in mammalian tissues, where their formation is known to involve the acylation of dihydroxyacetone phosphate, substitution of a long chain fatty alcohol for the acyl chain at C-1,

Abbreviations: TLC, thin-layer chromatography.

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and after several additional steps, the removal of two hydrogens by an oxygen-requiring microsomal cytochrome b_5-linked enzyme, which desaturates a saturated glycerol ether intermediate (7). Several lines of evidence argue that this pathway is not present in anaerobic bacteria. First an oxygen requirement must necessarily be ruled out; and previous work has shown that dihydroxyacetone phosphate (9) and probably long-chain fatty alcohols are not involved in the bacterial pathway (8, 10).

Much of the previous work in this laboratory has been directed towards understanding the formation and function of bacterial plasmalogens. This study describes some of the enzymes involved in phospholipid biosynthesis in *Clostridium butyricum* with the view that such information may ultimately lead to a further understanding of an anaerobic mechanism for plasmalogen biosynthesis.

MATERIALS AND METHODS

Cells and culture conditions

C. butyricum (ATCC6015) were grown, harvested, and washed as previously described (11). The membrane particles were prepared as follows. Cells were broken in a French pressure cell (11). The crude extract was centrifuged at 4500 *g* for 30 min to remove large debris and unbroken cells. For the preparation of the cytidyltransferase, the resulting supernatant was centrifuged at 150,000 *g* for 1 hr. The pellet was washed in 0.05 M KPO_4 (pH 7.2), 0.01 M 2-mercaptoethanol and centrifuged again at 150,000 *g* for 1 hr. The pellet was resuspended in 0.05 M KPO_4 (pH 7.2) and stored at −80°C. For the preparation of the phosphatidylserine synthetase and phosphatidylglycerolphosphate synthetase the 4500 *g* supernatant was centrifuged at 30,000 *g* for 1 hr and the supernatant was centrifuged at 150,000 *g*. The pellet from the high speed centrifugation was washed and stored as above (small particles).

Enzyme assays

Enzyme assay conditions are described in the "Results" section. The assays were performed either by a non-specific filter-disk assay (12, 13) or by extracting the assay products with butanol as described by Hajra (14) with some modifications to reduce the background. The incubation (0.1 ml) was terminated by adding 0.33 ml butanol and the mixture was stirred briefly with a vortex mixer. Then 0.43 ml of water was added and the mixture was stirred (vortex mixer) for 2 min. The resulting emulsion was centrifuged at 4400 rpm (Sorval GLC-1 centrifuge) for 10 min. The lower aqueous phase was removed, and the upper butanol phase was washed with 1 ml of water saturated with butanol. After centrifuging, the lower phase was removed and the wash procedure was repeated. After this wash the upper butanol layer was removed and the interface was washed two times with a minimal amount of butanol. The washings and the butanol phase were combined. This procedure was found to be superior to chloroform–methanol extraction for the isolation of such polar lipids as CDP-diglyceride. The experiment with [^{3}H]phosphatidic acid was done in an assay mixture with the following components: Tris-HCl (pH 8.0) 4 μmol; KCl, 20 μmol; 2-mercaptoethanol, 400 nmol; $MgCl_2$, 1 μmol; CTP, 75.9 nmol; [^{3}H]phosphatidic acid, 88.1 nmol (sp act 53.6 Ci/mol); small particles, 185 μg of protein. This assay (total volume 87 μl) was started by the addition of $MgCl_2$ and incubated for 10 min at 37°C, after which Triton X-100, 500 μg; L-serine, 100 nmol; and more small particles, 56 μg of protein were added (total final volume 100 μl). The assay was incubated for 10 min more, and the products were extracted with butanol and identified by thin-layer chromatography in solvent system (D). The [^{3}H]phosphatidic acid was diluted with phosphatidic acid derived from egg lecithin to give the above specific activity.

Except where noted otherwise the phosphatidic acid and CDP-diglyceride were commercial products derived from egg lecithin. The lipids were added to the assay as a chloroform solution, which was evaporated under N_2. They were suspended in the assay mixture by adding a glass bead and vortexing for 0.5 min.

Analytical procedures

Thin-layer chromatography was on silica gel HR (E. Merck, Darmstadt, Germany), which was slurried in either water (solvent systems A,B,C) or 0.01 M Na_2CO_3 (solvent system D). The solvent systems were (A) chloroform–methanol–acetic acid–water 50:30:8:4 (v/v); (B) chloroform–methanol–acetic acid 65:25:8 (v/v); (C) chloroform–methanol–7N NH_4OH 60:35:5 (v/v); and (D) chloroform–acetone–methanol–acetic acid–water 30:40:10:10:5 (v/v).

Lipid products were identified as follows. CDP-diglyceride cochromatographed with authentic standard in solvent systems A and D and it served as a substrate for phosphatidylglycerophosphate and phosphatidylserine formation (see below). Phosphatidylserine cochromatographed with an authentic standard in solvent systems C and D and served as a

substrate for the phosphatidylserine decarboxylase from *E. coli* (see below). Phosphatidylglycerol and phosphatidylethanolamine cochromatographed with authentic standards in solvent systems B, C, and D. Phosphatidylglycerophosphate cochromatographed with the products of the phosphatidylglycerophosphate synthetase assay from *E. coli* (15, 16). Lipids that were to be further analyzed were eluted with 4 ml of chloroform–methanol 1:2 (v/v) followed by 2 ml of solvent system C. Alkaline hydrolysis was done according to the method of Dittmer and Wells (17). Acetic acid hydrolysis was done as described previously (18). Protein was determined by the method of Lowry et al. (19).

MATERIALS

[^{3}H]Phosphatidic acid was prepared by growing an unsaturated fatty acid auxotroph of *E. coli*, strain K1060, on media supplemented with [^{3}H]oleic acid (20). Phospholipids were then extracted and fractionated on DEAE-cellulose according to Law and Essen (21). The phosphatidylethanolamine fraction was treated with phospholipase D and the resulting phosphatidic acid was separated from the original phosphatidylethanolamine by preparative thin-layer chromatography (solvent system D). The labeled phosphatidic acid prepared in this manner had a specific activity of 161 Ci/mol.

All other chemicals were purchased from commercial sources and were reagent grade or better. [U-^{14}C]*sn*-glycerol-3-phosphate (sp act 130 Ci/mol) and [9, 10-^{3}H(n)]oleic acid (sp act 5.66 Ci/mmol) were purchased from New England Nuclear (Boston, MA). L-[3-^{14}C]serine (sp act 49.6 Ci/mol) and [5-^{3}H]-CTP (sp act 24.7 Ci/mmol) were obtained from ICN Chemical and Radioisotope Division (Irvine, CA). L-[3-^{3}H]serine (sp act 19 Ci/mmol) was the product of Amersham Corporation (Arlington Heights, IL). L-[U-^{14}C]serine (sp act 156 Ci/mol) was bought from Schwartz/Mann (Orangeburg, NY).

Lipid standards and CDP-diglycerides were bought from Serdary Research Labs (London, Ontario, Canada). The CDP-diglycerides were analyzed by thin-layer chromatography. Any preparation showing significant impurities was purified on DEAE-cellulose (22) except that a step-wise gradient of ammonium-acetate (pH 7.4) was used. ATP, CTP, dCTP, UTP and GTP were bought from P-L Biochemicals (Milwaukee, WI). Glass-distilled solvents were the products of Burdick and Jackson Labs (Muskegan, MI); Miranol H2M and C2M were gifts from the Miranol Chemical Co. (Irvington, NJ).

RESULTS

CTP-phosphatidic acid cytidyltransferase

CDP-diglyceride, a key intermediate in both bacterial and mammalian phospholipid biosynthesis, has been described as an activated form of phosphatidic acid (23). As such it occupies a branch point in the formation of complex lipids. In *C. butyricum*, as in other bacteria, the CTP-phosphatidic acid cytidyltransferase is membrane-associated. The formation of CDP-diglyceride from [^{3}H]CTP and phosphatidic acid, catalyzed by a washed membrane preparation, was linear with respect to time and protein concentration. The pH optimum was near 7 with 14% activity remaining at pH 6, and 85% remaining at pH 9.5 (data not shown). The incorporation of labeled CTP into CDP-diglyceride was dependent on added phosphatidic acid, Mg^{2+}, and enzyme source. The optimal Mg^{2+} concentration was near 10 mM. Ca^{2+} and Mn^{2+} were not effective substitutes (data not shown). The monovalent cations K^+ and NH_4^+ (200 mM) were found to stimulate the reaction 70 to 80%, but Na^+ and Li^+ (200 mM) did not. K^+ was routinely added to the assays. Although Triton X-100 at low concentrations (0.05–0.1%) stimulated the reactions up to 50%, the effect was variable. At higher concentrations ($\geq$0.25%) Brij 35, Tweens 40 and 80 inhibited CDP-diglyceride formation, moderately, and Triton X-100, Miranol H2M, and C2M inhibited strongly. Therefore, routinely, no detergent was added. These inhibitory effects are unusual in that other microbial cytidyltransferases are greatly stimulated by detergents (4, 5, 23, 24). The enzyme exhibited saturation kinetics with regard to both substrates (**Fig. 1**). Hanes-Woolf plots (Fig. 1 inserts) yielded the apparent Km values of 0.35 mM for CTP and 0.72 mM for phosphatidic acid. These kinetics constants are similar to those reported for the enzyme in *E. coli* (23) even though detergent was added in those studies. The specific activity varied from 0.5–0.75 nmol/min/mg protein. Additionally, we found that dipalmitoyl phosphatidic acid was not a satisfactory substrate (data not shown). However, this may be due to differences in the physical state of the lipid at 37°C.

The nucleotide specificity of the enzyme was examined through competition experiments. With CTP at 0.5 mM, dCTP (1.3 mM) inhibited [^{3}H]CDP-diglyceride formations about 60% and ATP (1.4 mM) inhibited slightly. UTP and GTP (1.3 mM) produced no inhibition. The results shown in **Table 1** demonstrate that the CDP-diglyceride formed in situ was a good substrate for the formation of both phosphatidylglycerophosphate and phosphatidylserine.

Phosphatidylglycerophosphate synthetase

When CDP-diglyceride and [^{14}C]*sn*-glycerol-3-phosphate were incubated with a small membrane particle fraction from *C.butyricum* a mixture of phosphatidylglycerophosphate and phosphatidylglycerol was formed. The reaction was linear with respect to time and protein concentration in the experiments to be described. The pH optimum for the incorporation of [^{14}C]*sn*-glycerol-3-phosphate into butanol soluble lipid was pH 10; at pH 10.5 the rate was decreased to 60%. As the pH was decreased to 8.5 the rate decreased to 66% but it dropped drastically to 13% at pH 8. The proportion of phosphatidylglycerophosphate and phosphatidylglycerol in the reaction products was also pH-dependent. At pH 8 phosphatidylglycerol constituted 71% of the reaction products with phosphatidylglycerophosphate making up the rest. However, at pH 10 phosphatidylglycerol was only 1.5% of the reaction products. At pH 8.5, which was used routinely for the assays, phosphatidylglycerol constitutes 32% of the reaction products. These results were obtained with a membrane preparation that contained considerable phosphatidylglycerophosphate phosphatase activity. However, it should be noted that several preparations had very little of this activity even at pH 8.0. The formation of phosphatidylglycerophosphate was essentially completely dependent on added CDP-diglyceride, Mg^{2+} and Triton X-100 for activity (data not shown); however, the enzyme was active with either CDP-diglyceride or dCDP-diglyceride as the liponucleotide substrate (Fig. 2B, C). The apparent Km values were 0.03 mM for CDP-diglyceride, 0.025 mM for dCDP-diglyceride, and 0.055 mM for *sn*-glycero-3-phosphate (**Fig. 2A, B, C** inserts). We also examined the effect of the acyl-chain composition of CDP-diglyceride. **Table 2** shows that the dioleoyl species was the substrate giving the lowest apparent Km value. The enzyme

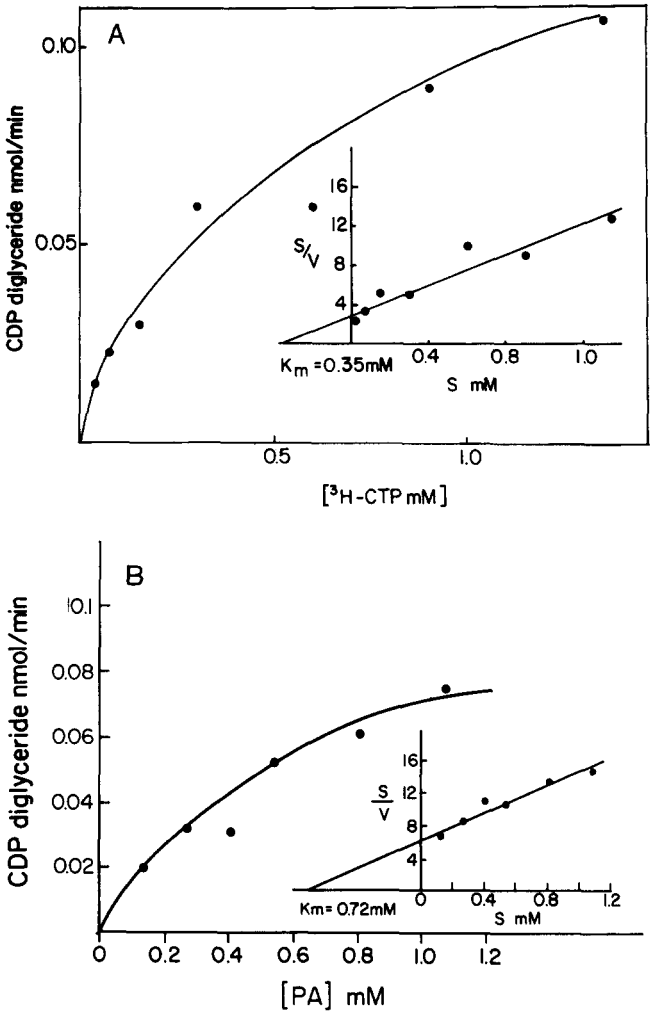

Fig. 1. Substrate dependence of CDP diglyceride formation. The assays were at 37°C for 7 min and contained in 0.1 ml: (A) Tris-maleate (pH 7.0), 50 mM; 2-mercaptoethanol, 5 mM; KCl, 200 mM; phosphatidic acid, 710 μM; particles, 110 μg protein; $MgCl_2$, 7.5 mM; [5-^{3}H]CTP (sp act 25 Ci/mol) as indicated. (B) Tris-maleate (pH 7.0); 50 mM; 2-mercaptoethanol, 5 mM; KCl, 200 mM; [5-^{3}H]CTP (sp act 25 Ci/mol), 70 μg of protein; Mg Cl_2, 7.5 mM; phosphatidic acid as indicated. Products were extracted with butanol as described in Materials and Methods.

TABLE 1. Coupled formation of phosphatidylglycerophosphate and phosphatidylserine

Assay Mixture[c]	NMol Product
Exp. A[a]	
Complete	0.399
−CTP	0.022
−PA	0.024
−particles	0.008
Exp. B[b]	
Complete	0.618
−CTP	0.062
−PA	0.083
−particles	0.043

[a] Coupled formation of phosphatidylglycerophosphate from CTP, phosphatidic acid, and [U-^{14}C]glycerol-3-phosphate. Assay contained in a final volume of 0.3 ml: Tris-HCl (pH 8.0), 30 μmol; KCl, 30 μmol; 2-mercaptoethanol, 1.5 μmol; CTP, 148.5 nmol; phosphatidic acid, 168 nmol; $MgCl_2$, 3 μmol; small particles, 547.5 μg of protein. Assay was incubated for 10 min at 37°C. Then the following was added: [U-^{14}C]-*sn*-glycerol-3-phosphate, (sp act 5 Ci/mol), 225 nmol; Triton X-100, 600 μg; $MgCl_2$, 7.5 μmol. This was incubated for an additional 15 min at 37°C. For product identification, see below.

[b] Coupled formation of phosphatidylserine from CTP, phosphatidic acid and L-[3-^{14}C]serine. Assay contained in a final volume of 0.3 ml: Tris-HCl (pH 8.0), 12.5 μmol; KCl, 50 μmol; 2-mercaptoethanol, 1.25 μmol; CTP, 225 nmol; phosphatidic acid, 220 nmol; small particles, 438 μg of protein; $MgCl_2$, 3 μmol. Assay was incubated for 10 min at 37°C. Then the following were added: L-[3-^{14}C]-serine (sp act 10 Ci/mol), 300 nmol; Triton X-100, 600 μg; small particles, 102 μg of protein. This was incubated for an additional 15 min at 37°C.

[c] Both assay mixtures were extracted with butanol as described in Materials and Methods. Products were identified by TLC using solvent systems B (Exp. A) and C (Exp. B).

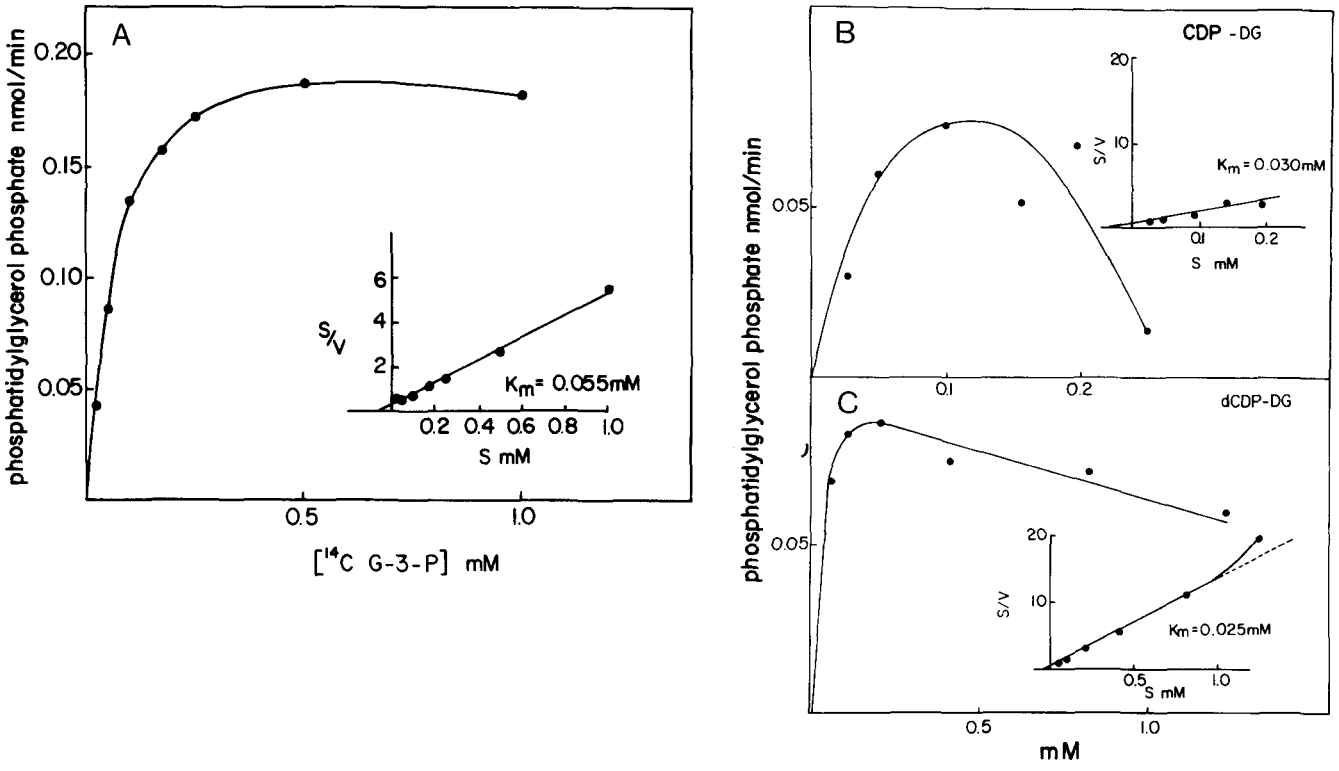

Fig. 2. Substrate dependence of phosphatidylglycerophosphate formation. The asays were at 37°C for 14 min and contained in 0.1 ml (A) Tris-HCl (pH 9.0), 100 mM; CDP-diglyceride, 100 μM; 2-mercaptoethanol, 5 mM; Triton X-100, 0.2%; $MgCl_2$, 25 mM; particles, 496 μg of protein; [U-^{14}C]-*sn*-glycerol[3] phosphate (sp act 5 Ci/mol), as indicated. (B) and (C) Tris HCl (pH 9.0) 100 mM; [U-^{14}C]-*sn*-glycerol-3-phosphate (sp act 5 Ci/mol), 500 μM; 2-mercaptoethanol, 5 mM; Triton X-100, 0.2%; $MgCl_2$, 25 mM; particles, 164 μg of protein; liponucleotide as indicated. The filter disk assay method was used in these experiments.

was found to have a specific activity of 0.9–1.0 nmol/min/mg protein.

Phosphatidylserine synthetase

The formation of phosphatidylserine from CDP-diglyceride and L-[^{14}C]serine was catalyzed by the same membrane fraction that catalyzed the formation of phosphatidylglycerolphosphate and phosphatidylglycerol. Product formation was linear with respect to time and protein concentration. The addition of Triton X-100 (0.2%) stimulated approximately 4-fold and Mg^{2+} (10 mM) stimulated only slightly. The enzyme had a broad pH optimum between 7.5 and 9 (data not shown). Both CDP-diglyceride and dCDP-diglyceride were suitable substrates except that at higher concentrations dCDP-diglyceride was inhibitory, but CDP-diglyceride was not (**Fig. 3B, C**). Apparent Km values determined from Hanes-Woolf plots were 0.031 mM for CDP-diglyceride and 0.072 mM dCDP-diglyceride (Fig. 3B, C inserts). Here we also studied the effect of varying the acyl-chain composition of CDP-diglyceride. There was a 5-fold variation, with the dioleoyl species again giving the lowest apparent Km (**Table 2**). Serine had a Km value of 0.35 mM (Fig. 3A insert), which is significantly higher than that reported for a *Bacillus* (0.055 mM) (4) and slightly lower than that of *E. coli* (0.8 mM) (26).

A puzzling aspect of these experiments was the very limited amount of phosphatidylethanolamine formed

TABLE 2. Affinities of phosphatidylserine synthetase and phosphatidylglycerophosphate synthetase for different molecular species of CDP-diglyceride

Substrate Varied CDP-Diglyceride	Apparent Km (μM)	
	Phosphatidylserine[a] Synthetase	Phosphatidylglycerol-[b] Phosphate Synthetase
From egg lecithin	37	21
Dipalmitoyl	16	23
Dioleoyl	9	5
Dicaproyl	44	11

[a] Phosphatidylserine formation. The assay was incubated at 37°C for 2 min and contained in 0.1 ml: Tris-HCl (pH 8.5), 100 mM; L-[U-^{14}C]serine, (sp act 10 Ci/mol) 1 mM; Triton X-100, 0.2%; $MgCl_2$, 10 mM; small particles, 185 μg of protein.

[b] Phosphatidylglycerophosphate formation. The assay was incubated at 37°C for 4 min and contained Tris-HCl (pH 9.0), 100 mM; [U-^{14}C]-*sn*-glycerol-3-phosphate, (sp act 5 Ci/mol) 500 mM; 2-mercaptoethanol, 5 mM; Triton X-100, 0.2%; $MgCl_2$, 25 mM; small particles, 185 μg of protein. The filter-disk assay method was used.

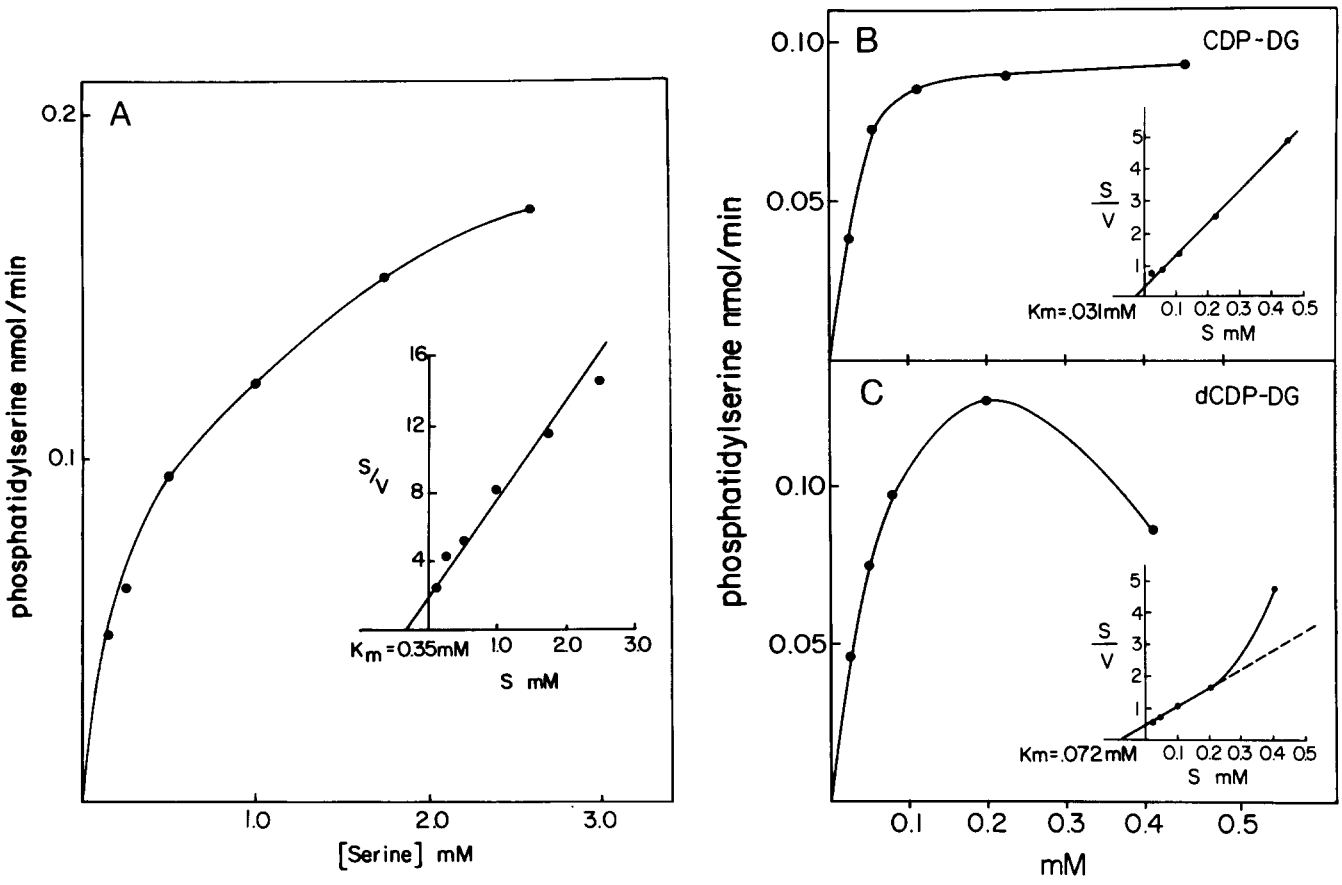

Fig. 3. Substrate dependence of phosphatidylserine formation. The assay contained in 0.1 ml: (A) Tris-HCl (pH 8.5), 100 mM; CDP-diglyceride, 112 μM; Triton X-100, 0.2%; $MgCl_2$, 10 mM; particles, 148 μg of protein; L-[3-^{14}C]serine (sp act 10 Ci/mol), as indicated. The assay was incubated at 37°C for 4 min. (B) and (C) Tris HCl (pH 8.5), 100 mM; L-[3-C^{14}]serine (sp act 10 Ci/mol), 1 mM; Triton X-100, 0.2%; $MgCl_2$, 10 mM; particles, 144 μg of protein; liponucleotide as indicated. The filter disk assay method was used in these experiments.

from phosphatidylserine in the in vitro assay whether membrane particles or unfractionated cell-free extract was used as the enzyme source (**Table 3**). This contrasts with extracts from both *E. coli* (26) and a bacillus (4) in which the presence of the enzyme was readily demonstrated. Dutt and Dowhan (6) also found decarboxylase activity in a number of other gram-negative microorganisms and in most cases the decarboxylase was more active than the synthetase. Furthermore, previous work showed that phosphatidylethanolamine in growing cells of *C. butyricum* is formed from added serine (27).

The first possible explanation considered was that either the phosphatidylserine formed was not biologically active or that an inhibitor of the decarboxylase was present. To test this we added to the assay mixture a preparation of *E. coli* membrane particles that had decarboxylase but very little synthetase activity. The results in Table 3 show 70% conversion of endogenously generated phosphatidylserine to phosphatidylethanolamine by the *E. coli* enzyme. These results indicate that the phosphatidylserine formed was biologically active and that there is no inhibitor present for the *E. coli* enzyme.

At this point it was important to determine

TABLE 3. Effect of added *E. coli* phosphatidylserine decarboxylase on phosphatidylethanolamine formation

C. butyricum Particles (148 μg)	*E. coli* PS Decarboxylase	PS	PE
		nmol	
+		0.91	0.035
	+ (40 μg)	0.013	0.021
+	+ (10 μg)	0.592	0.371
+	+ (20 μg)	0.415	0.484
+	+ (40 μg)	0.245	0.582

All assays were for 45 min at 37°C and contained in 0.1 ml. Tris-HCl (pH 8.5), 100 mM; CDP diglyceride, 0.98 mM; L-[^{3}H]-serine (sp act 50 Ci/mol), 1 mM; Triton X-100, 0.2%; $MgCl_2$, 10 mM. Particles were added as given (in μg of protein). Products were extracted with butanol as described in Materials and Methods and analyzed by TLC using solvent system C. PS, phosphatidylserine; PE, phosphatidylethanolamine.

whether any of the phosphatidylserine formed by the *C. butyricum* enzyme or the phosphatidylethanolamine formed by the addition of *E. coli* decarboxylase was present in the plasmalogen form. The work of Prins *et al.*, (28) with *Megasphaera elsdenii* suggested that phosphatidylserine is first converted to its plasmalogen form which is then decarboxylated to give the plasmalogen form of phosphatidylethanolamine. Characteristically alk-1-enyl ether bonds are alkali stable and acid labile. Therefore both lipid products formed in vitro were subjected to mild alkaline hydrolysis (17) and mild acid hydrolysis (18). Alkaline hydrolysis showed that less than 2% of the products were alkali stable, and acid hydrolysis produced virtually no breakdown. Plasmalogen content was also tested in another way. The phospholipids formed in our assay were extracted and chromatographed in one direction in solvent system C, then exposed to HCl fumes and chromatographed in the second dimension in the same solvent system (29). The HCl fumes in this procedure cleave the alk-1-enyl ether bonds of plasmalogens yielding a lysolipid which separates from the parent diacyl lipid in the second dimension on thin-layer chromatography. We observed 5 to 7% hydrolysis, but this was also observed with diacyl phospholipids isolated from *E. coli*. These experiments indicated that no or very little plasmalogen had been formed in vitro.

An alternative explanation for the lack of decarboxylase activity was that an unknown cofactor is required for phosphatidylethanolamine formation. Consequently, we added pyridoxal phosphate, thiamine pyrophosphate, KCl, and Na_2SO_4, but no phosphatidylethanolamine formation was observed. The substitution of Mn^{2+} for Mg^{2+}, as well as the omission of Mg^{2+}, had no effect, and adding Ca^{2+} in place of Mg^{2+} caused a 20% decrease in phosphatidylserine formation. The addition of 2-mercaptoethanol, dithiothreitol, or glutathione also had no effect. A recent report showed that the phosphatidylserine decarboxylase of *E. coli* contains bound pyruvate whose carbonyl function was required for activity (30). Carbonyl reacting compounds were shown to inactivate the enzyme (30). We therefore added either pyruvate or acetone, carbonyl sparing compounds, to the assay to test this possibility. Again, no phosphatidylethanolamine was formed.

These experiments left open the possibility that phosphatidylethanolamine could be formed by an exchange of the base portion of phosphatidylserine with an ethanolamine-containing compound to give phosphatidylethanolamine. Since in the above experiments the label was in the serine portion of the lipid, such an exchange of bases would not be detected. To rule out this possibility, we employed a coupled assay in which [^{3}H]phosphatidic acid labeled in the acyl chains was incorporated into [^{3}H]phosphatidylserine. This experiment (see Materials and Methods) assumed that the label remained in the acyl-chain portion. This resulted in the formation of 0.55 nmol of phosphatidylserine, but no phosphatidylethanolamine was formed. The products were identified by thin-layer chromatography using solvent system D. The phosphatidylserine formed in this assay was also subjected to mild acid hydrolysis to test for plasmalogen formation. No plasmalogen was detected.

The negative results of these experiments prompted us to consider the anaerobic nature of this organism. It was conceivable that either the decarboxylase was oxygen-sensitive or that it was only active with the plasmalogen form of phosphatidylserine, whose formation was oxygen-sensitive. Therefore we investigated the effect of anaerobic and reducing conditions during cell breakage on phosphatidylethanolamine formation. The resulting cell-free extracts were also assayed anaerobically under N_2. The reducing conditions were brought about by addition to the cell breakage buffer of *a*) 10 mM Na pyrophosphate and 5 mM ferrous ammonium sulfate (31), *b*) 3 mM 2,3-dimercaptopropanol and 1 mM ferrous sulfate (32), *c*) 10 mM dithionite, or *d*) 10 mM Na pyruvate. Cell disruptions were carried out under N_2. These modifications did not result in phosphatidylethanolamine formation.

Localization of phospholipid biosynthetic enzymes

Attempts to define the cellular distribution of the phospholipid biosynthetic enzymes in gram-negative bacteria (6, 33) showed that, with the exception of phosphatidylserine synthetase, all the enzymes are membrane-associated. The phosphatidylserine synthetase is apparently soluble but binds tightly to the ribosomes (6, 33). This anomolous behavior was detected by sucrose density gradient centrifugation. When the sucrose gradients are 2mM in Mg^{2+}, the ribosomes are present as a single 70S species, and the synthetase gives a single peak that co-sediments with the 70S species. At a Mg^{2+} concentration of 0.1 mM, the ribosomes are present as 30S and 50S species and the synthetase also appears as two fractions which co-sediment with the two ribosomal species. We therefore examined the cellular localization of both the phosphatidylglycerophosphate and the phosphatidylserine synthetases by sucrose density gradient centrifugation. As shown in **Fig. 4**, at both the high and low Mg^{2+} concentration both enzymes co-sediment with the particles near the bottom of the

gradient. Some activity is found in other areas of the gradient and this may be due to small membrane fragments not sufficiently dense to sediment to the bottom of the gradient.

DISCUSSION

These studies show that enzyme activities are present in *C. butyricum* membranes that catalyze several of the phospholipid biosynthetic reactions described by Kennedy and co-workers in *E. coli* (for reference, see 2). Some important differences, though, should be noted. One of these concerns the intracellular localization of the phospholipid biosynthetic enzymes.Work done with *E. coli* (33) and other gram-negative bacteria (6) indicated that while most of these enzymes are membrane-bound, phosphatidylserine synthetase was soluble or readily detached from the membranes, and was very strongly adsorbed onto the ribosomes. The results of this study indicate that in *C. butyricum* both the phosphatidylserine and phosphatidylglycerophosphate synthetases are not soluble, and cosediment on sucrose density gradients with membrane particles. This is, to our knowledge, the first such study of a gram-positive anaerobic bacterium. Therefore, it is not clear whether this divergent behavior is characteristic of either gram-positive or of anaerobic bacteria. Patterson and Lennarz (4) found that in a gram-positive *Bacillus* species, phosphatidylserine synthetase seemed to be associated with the membrane fraction of the cell. Dutt and Dowhan (6) cite preliminary studies on the localization of PS synthetase in *B. licheniformis*, where it also appears to be associated with the membrane fraction. These results suggest a possible difference in this enzyme between gram-negative and gram-positive bacteria.

While the localization of phosphatidylserine synthetase appears to be different in *C. butyricum* than in *E. coli*, the liponucleotide requirements seem to be similar. That is both CDP-diglyceride and dCDP-diglyceride appear to be equally effective substrates for both phosphatidylserine and phosphatidylglycerophosphate synthesis.

Since CDP-diglyceride is at a branch point of phospholipid synthesis, it represents a possible control point at which a cell may influence the physical nature of its membrane phospholipids by manipulating their fatty-acyl chain composition. Rat liver microsomes have recently been reported to contain an enzyme that can acylate CDP-monoglyceride, and this has been proposed as a possible explanation for the difference in acyl-chain composition between phosphatidic acid and CDP-diglyceride found in some mammalian tissues (34, 35). We, therefore, varied the acyl-chain composition of the CDP-diglyceride to see if one molecular species would be a better substrate for either the phosphatidylserine synthetase or the phosphatidylglycerophosphate synthetase. Our results showed the dioleoyl molecular species to have a lower Km than the other molecular species with both the phosphatidylserine and the phosphatidylglycerophosphate synthetases. However, the different molecular

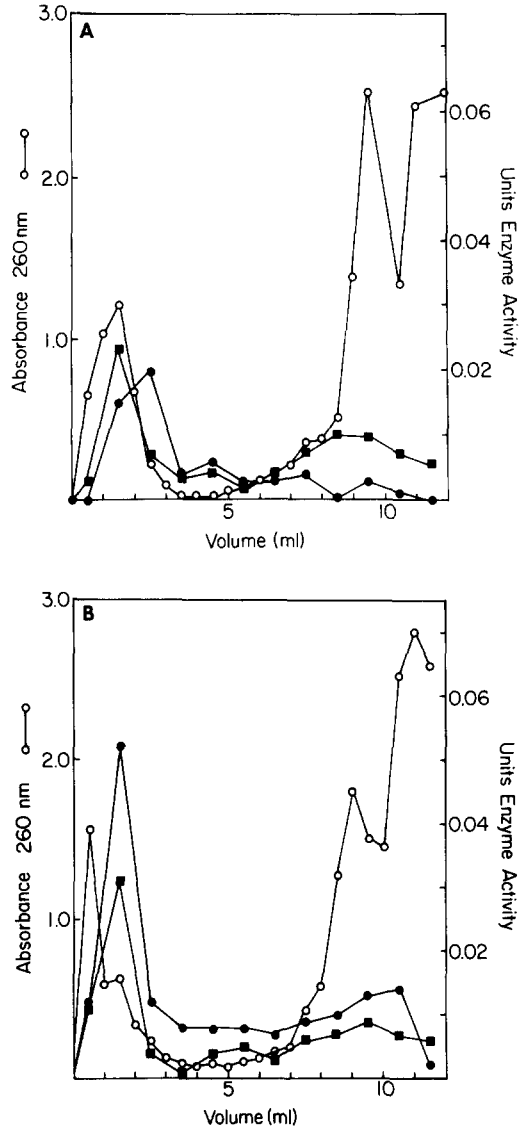

Fig. 4. Sedimentation profile of phosphatidylserine synthetase and phosphatidylglycerolphosphate synthetase on sucrose density gradient centrifugation. This was done according to Raetz and Kennedy (33). Runs were at 200,000 *g* for 90 min and 0.5-ml fractions were collected. (A) High magnesium buffer (10 mM Tris HCl (pH 8.0) and 2 mM $MgCl_2$). (B) Low magnesium buffer (10 mM Tris HCl (pH 8.0) and 0.1 mM $MgCl_2$). Cell-free extract (65 mg of protein) was layered on each gradient. (○ —— ○) Abs. 260 nm; (● —— ●) PGP synthetase nmoles per 14 min; (■ —— ■) PS synthetase nmoles per 7 min.

species gave similar V_{max} values (data not shown). This effect may simply be due to the physical nature of the dioleoyl species when present as a dispersion in water.

An important aspect of our study that needs to be examined further is the virtual absence in both cell-free extracts and membrane particles of phosphatidylserine decarboxylase activity. In most bacteria the decarboxylase is very active in cell-free extracts (6) and since few microorganisms have phosphatidylserine in their membranes it is probably very active in vivo, converting all the phosphatidylserine to phosphatidylethanolamine. In *C. butyricum* cells, phosphatidylserine is present in only trace amounts compared to phosphatidylethanolamine and phosphatidylmonomethylethanolamine; presumably, therefore, the decarboxylase activity is not rate-limiting (27, 36). An alternative to the presence of decarboxylase activity would be to postulate an enzyme capable of exchanging bases with ethanolamine-containing compounds. This possibility seems unlikely in view of the experiment in which phosphatidylserine was labeled in the diglyceride portion by using [^{3}H]phosphatidic acid as its precursor. If a base exchange had occurred, the label would presumably be found in any phosphatidylethanolamine made in this fashion.

Another important negative observation was that none of the phospholipids formed in vitro had significant plasmalogen content. Not only was this true of the phosphatidylserine formed but also the phosphatidylethanolamine made in the presence of *E. coli* decarboxylase. Van Golde et al. (37), have shown that *E. coli* decarboxylase is active with the plasmalogen form of phosphatidylserine.

We have recently examined phospholipid biosynthesis in other anaerobes. Particulate membrane preparations of *M. elsdenii* and *Veillonella parvulla* (both plasmalogen-containing anaerobes) in the presence of CDP-diglyceride and L-[^{14}C]serine yielded primarily phosphatidylserine and very little phosphatidylethanolamine.[4] However, membrane preparations of *Desulfovibrio vulgaris*, an anaerobe lacking plasmalogens (38),[5] under the same assay conditions yielded both phosphatidylserine and phosphatidylethanolamine. Thus, there may be something unique about the phospholipid biosynthetic pathways of anaerobic bacteria possessing plasmalogens. Clearly more work must be done on phospholipid biosynthesis in anaerobes, both those containing and those lacking plasmalogens.

[4] Silber, P., R. P. Borie, and E. Mikowski. Unpublished results.
[5] Johnston, N. C., and H. Goldfine. Unpublished results.

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