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Distribution of phospholipid-synthesizing enzymes in the wall and membrane subfractions of the envelope of *Escherichia coli*

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SUMMARY

The distribution of the enzymes of phospholipid synthesis in cell wall and membrane subfractions of the cell envelope of *Escherichia coli*, isolated by a procedure involving particle electrophoresis and sucrose gradient density centrifugation, has been studied.

The results show that, with the possible exception of CDP-diglyceride:L-serine phosphatidyltransferase all of the enzymes involved in the synthesis of phosphatidic acid, CDP-diglyceride, phosphatidylglycerol, and phosphatidylethanolamine, are localized in the inner cytoplasmic membrane.

A number of lines of evidence indicate that the cell envelope of *Escherichia coli* is composed of two membranous components, the cell wall and the cytoplasmic membrane[1]. Both the wall and the cytoplasmic membrane contain phospholipids[2]; detailed studies on the composition of each will be reported elsewhere[3]. This report deals with the enzymes involved in the synthesis of these phospholipids in *E. coli* 0111_{B4}. Although there has been speculation that the phospholipids might be synthesized by the inner membrane[4], no work to establish this has been reported. Therefore, this investigation was undertaken in order to define the site of their synthesis. In an accompanying paper by Bell *et al.*[5] similar studies on the localization of these enzymes in *Salmonella typhimurium* and *E. coli* are reported. Similar observations have also been made in *E. coli* K12 30E (personal communication, N. Machtiger and F. Fox).

A UDP-galactose epimerase-less strain of *E. coli* (0111_{B4}) was grown, harvested, and disrupted as previously described[6]. The crude envelope was fractionated by a modification[3] of the particle electrophoresis procedure of Heidrich *et al.*[7]. The wall and membrane fractions were further purified by sucrose gradient centrifugation, and the peak tubes of each fraction were combined and assayed for succinate dehydrogenase, and

for [^{14}C] galactose in lipopolysaccharide. Recovery of both membrane and wall fractions was about 20% based on the succinate dehydrogenase and [^{14}C] galactose content of the crude envelope. Control samples of the crude envelope were subjected to the same washing procedures as the purified fractions. Fractions were stored frozen.

The enzymatic reactions studied are outlined in Fig. 1. The radioactive products of acylation of *sn*-[^{14}C] glycerol 3-phosphate by palmityl-CoA were extracted from the incubation mixture as described by Van den Bosch and Vagelos[8]. The extracted lipids were chromatographed on Silica gel H thin-layer plates (0.4 mm thick) impregnated with sodium carbonate (1 mM) developed with chloroform–methanol–acetone–acetic acid–water (100:20:40:20:10, by vol.)[9] and on Silica gel H thin-layer plates (0.4 mm thick) impregnated with oxalic acid (250 mM) developed with chloroform–methanol–HCl (87:13:0.5, by vol.). Location of the radioactive lipids was determined by scraping 1-cm segments from the plates and counting them in a toluene–Triton scintillation solution[10].

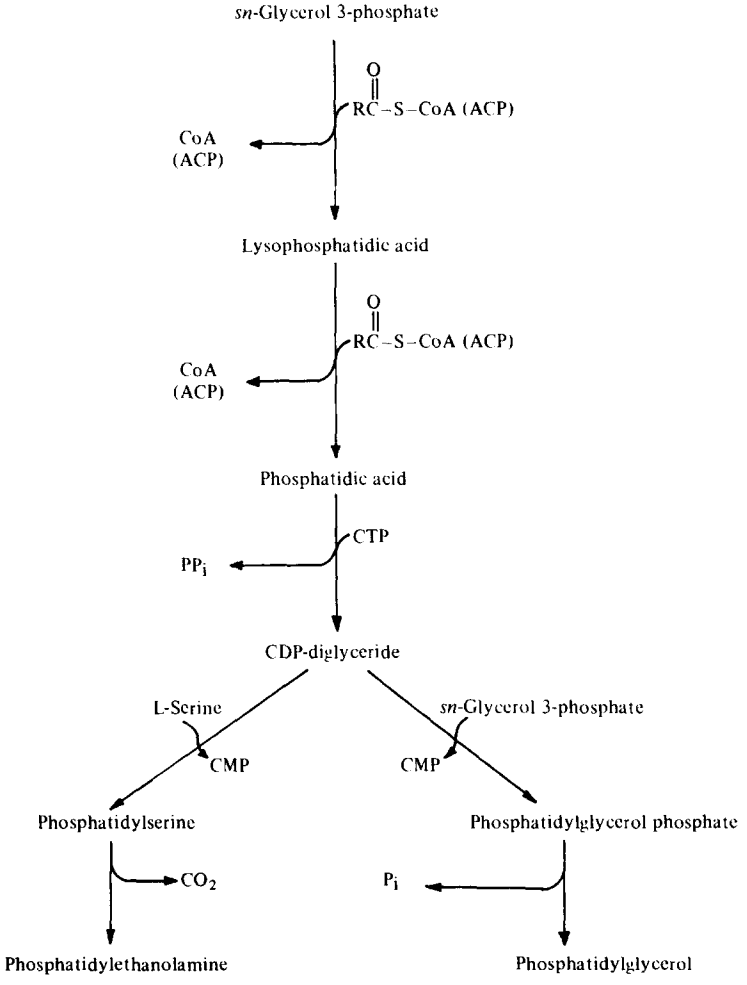

Fig. 1. Pathways of phospholipid synthesis in *E. coli*. ACP= acyl carrier protein.

Two major radioactive spots, corresponding in mobility to phosphatidic acid and lysophosphatidic acid, were observed for the whole envelope, and the membrane and wall subfractions. In all three fractions greater than 91% of the total radioactivity on the thin-layer plates was found in lysophosphatidic acid and phosphatidic acid. In addition to thin-layer chromatographic analysis the extracted radioactive lipids were further characterized, after recovery from thin-layer plates, by alkaline hydrolysis[11]. Both products yielded *sn*-[^{14}C]glycerol 3-phosphate as identified by paper chromatography[12]. Also, the lipid migrating with standard phosphatidic acid was treated with snake venom phospholipase A_2 and the product identified as lysophosphatidic acid by thin-layer chromatography as described above.

CTP:phosphatidic acid cytidyltransferase was assayed by the phosphatidic acid-dependent incorporation of [^{3}H]CTP into [^{3}H]CDP-diglyceride (dipalmityl)[13]. CDP-diglyceride:*sn*-glycerol 3-phosphate phosphatidyltransferase was measured by CDP-diglyceride-dependent incorporation of *sn*-[^{14}C]glycerol 3-phosphate into phosphatidylglycerol[14]. CDP-diglyceride:L-serine phosphatidyltransferase was assayed by the CDP-diglyceride-dependent incorporation of L-[^{14}C]serine into phosphatidyl[^{14}C]serine[15,16]. Phosphatidylserine decarboxylase was estimated using phosphatidyl[U-^{14}C]serine[15,16]. In all assays the radioactive lipid products were extracted from the incubation mixture with chloroform–methanol and counted as described[17]. The radioactive product of the decarboxylase, phosphatidyl[^{14}C]ethanolamine, was determined after separation from the substrate, phosphatidyl[^{14}C]serine, by thin-layer chromatography. The products of all of the above assays were identified by thin-layer chromatography and by paper electrophoresis of the products of mild alkaline hydrolysis[17].

Previous evidence suggested that, with the possible exception of CDP-diglyceride:L-serine phosphatidyltransferase, the enzymes of phospholipid synthesis in *E. coli* are particulate[13–16], and thus presumably part of the cell envelope. Because of the presence of phospholipids in both the wall and membrane, it was of interest to determine if both subfractions of the cell envelope contained the biosynthetic apparatus for synthesis of the major phospholipids.

In each of the enzyme systems studied, conditions were optimized so that the enzyme(s) was saturated with substrate, and product formation was linearly dependent on time. Using these conditions, product formation was determined as a function of enzyme concentration in order to determine the specific activity of the wall and membrane fractions of the envelope. In Fig. 2, this is illustrated in the case of the first steps in the overall sequence, the acylation of *sn*-glycerol 3-phosphate. It is evident that the enzymes involved in the acylation reaction are greatly enriched in the cytoplasmic membrane, the specific activity there being more than 40 times that of the cell wall. In the membrane the products were phosphatidic acid (61%) and lysophosphatidic acid (32%). Interestingly, although there was little acylation activity in the wall fraction, the product distribution was reversed; lysophosphatidic acid comprised 77% and phosphatidic acid 14% of the total product. The significance of this observation is not clear. However, it should be noted that although there is genetic evidence[18,19] indicating that two enzymes are involved in the overall formation of phosphatidic acid in *E. coli*, in the present case it is not known whether the lysophosphatidic acid produced is an intermediate in the formation of phosphatidic acid or a breakdown product arising from phospholipase A activity.

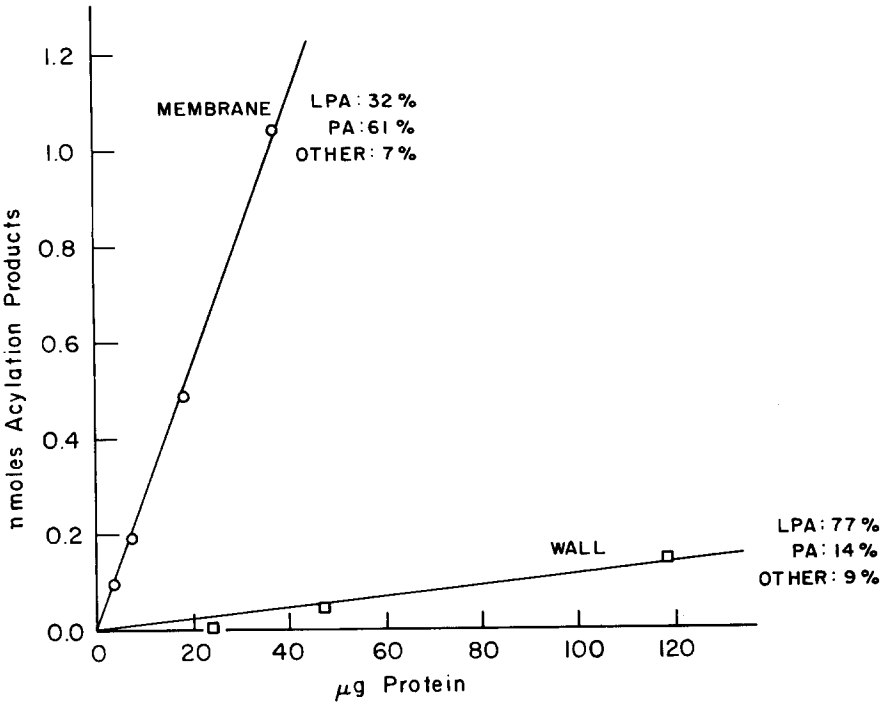

Fig. 2. Distribution of the enzymes of *sn*-[^{14}C]glycerol 3-phosphate acylation in envelope subfractions of *E. coli*. The conditions for acylation of *sn*-[^{14}C]glycerol 3-phosphate were as follows (final vol. 0.05 ml): 80 mM Tris–HCl, pH 8.5; 4 mM dithiothreitol; 6 mM $MgCl_2$; 0.08 mM palmityl-CoA; 0.43 mM *sn*-[^{14}C]glycerol 3-phosphate (spec. act. $2.2 \cdot 10^4$ disint./min per nmole); and varying quantities of cell wall and cytoplasmic membrane protein. Incubations were carried out for 10 min at 37°. The extracted radioactive lipids were analyzed by thin-layer chromatography as described in the text. LPA= lysophosphatidic acid; PA= phosphatidic acid.

Also, in this investigation the effect of the acyl thioester (*i.e.* saturated *vs.* unsaturated fatty acyl-CoA or acyl carrier protein esters) on product distribution has not been studied.

The results of specific activity determinations on the crude envelope, the membrane, and the wall for the acylation steps, as well as for the subsequent reactions, are summarized in Table I. Clearly, the cell envelope of *E. coli* contains all of the enzymes necessary to synthesize phosphatidic acid from *sn*-glycerol 3-phosphate, and to convert phosphatidic acid to phosphatidylglycerol *via* CDP-diglyceride. In both the total envelope and its subfractions the major product (75%) upon addition of CDP-diglyceride and *sn*-[^{14}C]glycerol 3-phosphate was phosphatidylglycerol; accumulation of the intermediate, phosphatidylglycerol phosphate, was not detected.

More interesting, however, is the enzyme distribution in the individual subfractions of the envelope. As in the case of the acylation of *sn*-glycerol 3-phosphate, the levels of enzyme activities responsible for synthesis of CDP-diglyceride and phosphatidylglycerol were greatly enriched in the cytoplasmic membrane and, in both instances, the specific activity was increased at least 24-fold over that in the wall. Similarly, the activity of phosphatidylserine decarboxylase, the last enzyme in the sequence leading to

Biochim. Biophys. Acta, 249 (1971) 636–642

TABLE I

DISTRIBUTION OF ENZYMATIC ACTIVITIES FOR PHOSPHOLIPID SYNTHESIS IN ENVELOPE FRACTIONS

Specific activities expressed as nmoles product per mg protein per 20 min. RSA, specific activity relative to the unfractionated envelope.

	Glycerol 3-phosphate acylation[a]		*CDP-diglyceride synthesis*[b]		*Phosphatidylglycerol synthesis*[c]		*Phosphatidylserine decarboxylation*[d]		*Phosphatidylserine synthesis*[e]	
	Spec. act.	*RSA*	*Spec. act.*	*RSA*	*Spec. act.*	*RSA*	*Spec. act.*	*RSA*	*Spec. act.*	*RSA*
Envelope	11.1	1.00	13.5	1.00	98.8	1.00	17.1	1.00	10.85	1.00
Wall	1.5	0.14	2.2	0.16	8.4	0.09	6.7	0.39	1.94	0.18
Membrane	64.0	5.77	53.0	3.90	293.1	2.99	90.4	5.28	2.33	0.22
Membrane / Wall	42.5		24.0		34.7		13.5		1.2	

[a]The conditions for acylation of *sn*-[^{14}C]glycerol 3-phosphate were the same as in Fig. 1 except for *sn*-[^{14}C] glycerol 3-phosphate (0.33 mM); envelope protein (58 μg); membrane protein (19 μg); wall protein (117 μg). [b]Incubation system (final volume 0.4 ml) contained Tris-maleate (50 mM), pH 6.5; [^{3}H]CTP (2 mM), spec. act. $2.0 \cdot 10^4$ disint./min per nmole; $MgCl_2$ (10 mM); phosphatidic acid (2 mM); Cutscum (0.25%); and protein (20 μg). Incubation for 20 min at 37°. [c]Incubation system (final volume 0.2 ml) contained Tris-HCl (50 mM), pH 8.0; CDP-diglyceride (0.2 mM); $MgCl_2$ (12.5 mM); *sn*-[1,3-^{14}C]glycerol 3-phosphate (0.15 mM), spec. act. $1.8 \cdot 10^4$ disint./min per nmole; Cutscum (0.25%); and protein (20 μg). Incubation for 20 min at 37°. [d]Incubation system (final volume 0.2 ml) contained Tris-maleate (50 mM), pH 7.0; $MgCl_2$ (20 mM); Na_2SO_4 (100 mM); phosphatidylserine (5 μM), spec. act. $3.3 \cdot 10^4$ disint./min per nmole; and protein (15 μg). Incubation for 7 min at 37°. [e]Incubation system (final volume 0.2 ml) contained Tris-HCl (50 mM), pH 8.0; CDP-diglyceride (0.25 mM); L-[^{14}C]serine (0.25 mM), spec. act. $1.7 \cdot 10^5$ disint./min per nmole; Na_2SO_4 (100 mM); Cutscum (0.25%); and protein (15 μg). Incubation for 60 min at 37°.

phosphatidylethanolamine synthesis, is localized in the membrane. This enzyme was assayed on a separate preparation of lower overall activity and this may account for the lower difference in activity (13-fold) in these subfractions. It is likely that the low level of activity of all of these biosynthetic enzymes detected in the wall fraction is due to contamination by membrane, since the succinate dehydrogenase content of the wall suggests that it may contain 5% of the recovered cytoplasmic membrane[3].

In marked contrast to these findings, the specific activity of the central enzyme involved in phosphatidylethanolamine synthesis, CDP-diglyceride:L-serine phosphatidyltransferase, was low in both purified wall and membrane subfractions, as compared to the crude envelope. Kanfer and Kennedy[20] earlier noted the apparent soluble nature of this enzyme in extracts prepared by sonication. Under our conditions of envelope preparation the recovery of this enzyme in the crude envelope fraction was highly variable. Clearly, this enzyme is not tightly associated with the cytoplasmic membrane, unlike the other enzymes of phospholipid synthesis. Recent evidence indicates that this enzyme is associated with the ribosomal fraction (personal communication, C.H. Raetz and E.P. Kennedy).

With regard to phosphatidylglycerol synthesis in the envelope of *E. coli*, it is clear that all of the enzymes necessary to synthesize it from *sn*-glycerol 3-phosphate are localized in the inner, cytoplasmic membrane. Since phosphatidylglycerol is found in the wall as well as in the membrane[3], and undergoes relatively rapid turnover in intact cells[20], a number of interesting questions arise concerning the transport of phosphatidylglycerol from its site of synthesis in the membrane to the wall and the mechanism of its turnover. Hopefully, further experiments with these subfractions of the cell envelope, as well as *in vivo* studies, will provide answers to these questions.

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REFERENCES

1 M.J. Osborn, *Annu. Rev. Biochem.*, 38 (1970) 501.
2 C.A. Schnaitman, *J. Bacteriol.*, 104 (1970) 890.
3 D.A. White, W.J. Lennarz and C.A. Schnaitman, *J. Bacteriol.*, in the press.
4 L. Rothfield, D. Romeo and A. Hinckley, *Fed. Proc.*, 30 (1971) 1033.
5 R.M. Bell, R.D. Mavis, M.J. Osborn and P.R. Vagelos, *Biochim. Biophys. Acta*, 249 (1971) 628.
6 C.A. Schnaitman, *J. Bacteriol.*, 104 (1970) 882.
7 H.G. Heidrich, R. Stahn and K. Hannig, *J. Cell Biol.*, 46 (1970) 137.
8 H. Van den Bosch and P.R. Vagelos, *Biochim. Biophys. Acta*, 218 (1970) 233.
9 A.K. Hajra and B.W. Agranoff, *J. Biol. Chem.*, 243 (1968) 1617.
10 R.M. Gould and W.J. Lennarz, *J. Bacteriol.*, 104 (1970) 1135.
11 R.M.C. Dawson, *Biochem. J.*, 75 (1960) 45.
12 J.T. Kiyasu, R.A. Pieringer, H. Paulus and E.P. Kennedy, *J. Biol. Chem.*, 238 (1963) 2293.
13 J.R. Carter, *J. Lipid Res.*, 9 (1968) 748.
14 Y.-Y. Chang and E.P. Kennedy, *J. Lipid Res.*, 8 (1967) 447.
15 J. Kanfer and E.P. Kennedy, *J. Biol. Chem.*, 237 (1962) PC 270.

16 J. Kanfer and E.P. Kennedy, *J. Biol. Chem.*, 239 (1964) 1720.
17 P.H. Patterson and W.J. Lennarz, *J. Biol. Chem.*, 246 (1971) 1062.
18 T.K. Ray, J.E. Cronan, R.D. Mavis and P.R. Vagelos, *J. Biol. Chem.*, 245 (1970) 6442.
19 K. Hechemy and H. Goldfine, *Biochem. Biophys. Res. Commun.*, 42 (1971) 245.
20 J. Kanfer and E.P. Kennedy, *J. Biol. Chem.*, 238 (1963) 2919.

Biochim. Biophys. Acta, 249 (1971) 636–642