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Enzymes of phospholipid metabolism: Localization in the cytoplasmic and outer membrane of the cell envelope of *Escherichia coli* and *Salmonella typhimurium*

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SUMMARY

The phospholipid biosynthetic enzymes of the cell envelope of *Escherichia coli* and *Salmonella typhimurium* had highest specific activities in the cytoplasmic membrane fraction and lowest specific activities in the outer membrane fraction obtained by isopycnic sucrose density gradient centrifugation of the total membrane fraction of lysozyme-EDTA spheroplasts. This finding suggests that the site of phospholipid synthesis is the cytoplasmic membrane. Several degradative enzymes of phospholipid metabolism had highest specific activities in the outer membrane fraction and lowest specific activities in the cytoplasmic membrane. These data suggest an intracellular separation of the degradative and biosynthetic enzymes of phospholipid metabolism.

The cell envelope of *Escherichia coli* is thought to contain all of the enzymes of phospholipid biosynthesis (*cf.* Fig. 1)[1-9]. In addition to the phospholipid biosynthetic enzymes, several enzymes catalyzing the degradation of phospholipids have also been localized in the cell envelope[9-12]. Studies concerning the phosphoglyceride content of *E. coli* under various conditions of growth have revealed that by and large both the amount and relative composition of log phase cells are unaffected by growth conditions[13]. Thus, the absolute content and composition of phospholipids appear to be regulated within the cell, but little is known about the mechanism of this regulatory process. Although it is recognized that the cell envelope houses the necessary machinery for the synthesis of the lipid components of the membrane, the mechanism of assembly of phospholipids and protein in the production of new membrane is unknown.

The cell envelope of *Salmonella typhimurium* and *E. coli* have been shown to be composed of three morphologically distinct layers[14,15]: a cytoplasmic membrane, a rigid peptidoglycan layer external to the cytoplasmic membrane, and a second

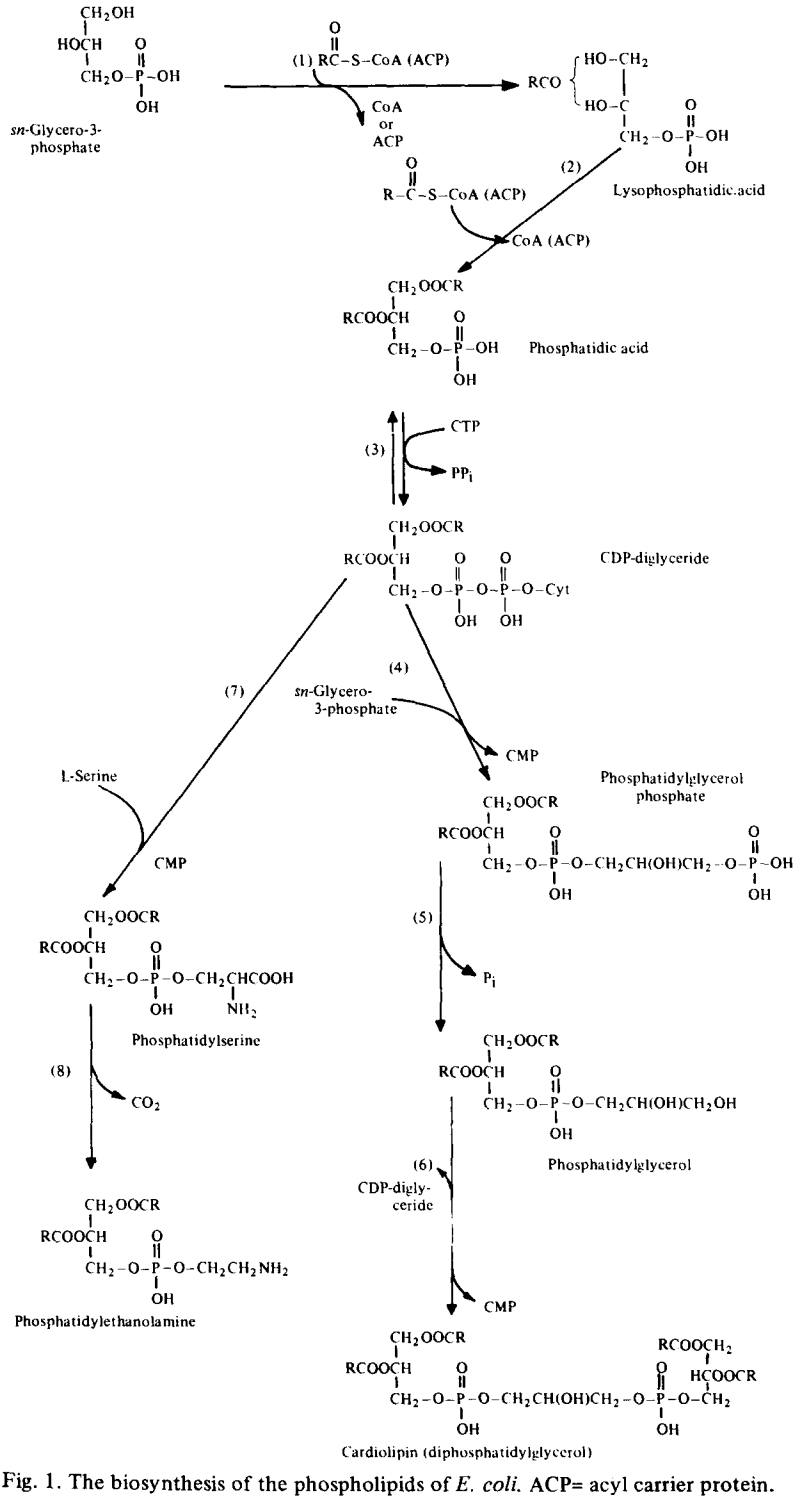

Fig. 1. The biosynthesis of the phospholipids of *E. coli*. ACP= acyl carrier protein.

membranous structure, the outer membrane, at the outer surface of the cell. Using a new procedure based on isopycnic sucrose density gradient centrifugation of the total membrane fraction obtained by lysis of lysozyme-EDTA spheroplasts,Osborn *et al.*[16] have succeeded in obtaining preparations of cytoplasmic and outer membranes of very high purity. The outer membrane contained 60% of the protein of the total membrane, 50% of the phospholipid and 90% of the lipopolysaccharide[16]. Preliminary analysis of the phospholipid compositions of inner and outer membranes showed significant quantitative differences in the relative distribution of the major phosphoglycerides[16]. Information concerning the site or sites of synthesis of phospholipids in the cell envelope is essential for understanding the regulation of phospholipid content, composition, biosynthesis and degradation and for understanding the mechanisms responsible for the biogenesis of new cytoplasmic and outer membranes. In this communication we present evidence that the cytoplasmic membrane contains the enzymes of phospholipid biosynthesis while the outer membrane contains several enzymic activities of phospholipid degradation.

Bacteria and media. Salmonella typhimurium strain G-30[16] and *Escherichia coli* strain PL-2[17] were grown as previously described.

Materials. Triton X-100, glycerol 3-phosphate, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), CTP, CDP-diglyceride, phosphatidic acid and phospholipase D were purchased from Sigma Chemical Company, St. Louis, Mo. [^{14}C] Glycerol 3-phosphate, [^{32}P] phosphoric acid, and [^{3}H] CTP were purchased from New England Nuclear. L-[^{14}C] Serine was the product of International Chemical and Nuclear Corp., Irvine, Calif. Palmityl-CoA and oleyl-CoA were purchased from P-L Biochemicals, Milwaukee, Wisc.

1-Acylglycerol 3-phosphate was prepared by treatment of 1-acylglycerol 3-phosphorylcholine (Cyclo Chemical, Los Angeles, Calif.) with phospholipase D as described previously[18]. [^{32}P] Phosphatidylglycerol was prepared from Bligh-Dyer extracts of *E. coli* cell pastes grown in the presence of [^{32}P] phosphate in low-phosphate medium 56[19] by preparative thin-layer chromatography on 500 μm Silica gel G Analtech Uniplates using chloroform–methanol–acetic acid (65: 25: 8, v/v/v) as the developing solvent[20]. [^{14}C] Phosphatidylserine was prepared enzymatically from CDP-diglyceride and L-[^{14}C] serine using essentially the conditions of Kanfer and Kennedy[2] except that 1 mM neutral hydroxylamine was added to inhibit the phosphatidylserine decarboxylase activity[21]. The [^{14}C] phosphatidylserine was extracted by the method of Bligh and Dyer[22] and purified using thin-layer chromatography as described above.

Counting procedures. All samples were counted in a Packard Tri Carb liquid scintillation spectrometer in Bray's[23] solution. Appropriate corrections were made for counting efficiencies.

Isolation of inner and outer membranes. Isolation of cytoplasmic and outer membranes was done as described previously[16]. The Salmonella membranes were separated into four discrete bands designated L_1, L_2, M, and H in order of increasing apparent buoyant density. The *E. coli* membranes were resolved into three components designated L, M, and H. The H ('heavy') band is the outer membrane which contains over 90% of the lipopolysaccharide[16]. L_1 and L_2 ('light') in the case of Salmonella and L for *E. coli* represents the cytoplasmic membrane(s). M ('middle') band represents unresolved

material. The relationship between L_1, L_2, M, and H has been discussed previously[16]. Membrane preparations were stored frozen at −78° or −15° in 0.02 M potassium phosphate buffer, pH 7.0, except for total membranes which were in 0.25 M sucrose, 3.3 mM Tris-HCl buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol.

Enzyme assays. Glycerol 3-phosphate dehydrogenase was assayed at 25° according to Lin *et al.*[24] by measuring the rate of reduction of the tetrazolium dye, MTT, to its formazan which absorbs at 550 nm. Glycerol 3-phosphate acyltransferase and 1-acylglycerol 3-phosphate acyltransferase were assayed spectrophotometrically at 25° as previously described[25]. CTP:phosphatidic acid cytidyltransferase was assayed using a filter disc method[26,27]. Whatman No. 3 filter discs (2.2 cm) were presoaked in 10% trichloroacetic acid, 1% sodium pyrophosphate and dried 1 h at room temperature or 5 min at 110°. The substrate concentrations were similar to those of Carter[5]. The reaction mixtures contained 10 μmoles potassium buffer, pH 6.5, 240 nmoles phosphatidic acid, 240 nmoles [5-^{3}H]CTP (10^{-2} μC/nmole), 10–100 μg membrane protein and 0.5 μmole $MgCl_2$ in a final volume of 60 μl. All components except $MgCl_2$ were premixed, and the reactions were initiated by addition of $MgCl_2$. The reaction mixtures were incubated for 10 min at 37°. Aliquots (50 μl) were then pipetted onto prewashed filter discs and allowed to dry for 10 min at room temperature. The discs were then washed 20 min in 10%, 5% and 1% trichloroacetic acid, each solution also containing 1% sodium pyrophosphate. Discs were finally dried 10 min at 110° and counted in Bray's solution. Glycerol 3-phosphate:CMP phosphatidyltransferase was assayed at 25° using essentially the conditions of Chang and Kennedy[3] by measuring the incorporation of [^{14}C]glycerol 3-phosphate into Bligh-Dyer[21] extractable lipid. Cardiolipin synthetase was assayed using the conditions of Stanacev *et al.*[9] by following the conversion of [^{32}P]phosphatidylglycerol to cardiolipin. Reaction mixtures, 200 μl, containing 20 nmoles of [^{32}P]phosphatidylglycerol and 50–100 μg protein, were incubated for 1 h at 37°. Lipids were extracted from the reaction mixture by the method of Bligh and Dyer[22], and chromatographed on Analtech Silica gel G Uniplates using the solvent system described above. The locations of the [^{32}P]phospholipids were determined by radioautography using Kodak No Screen X-Ray Film. The regions corresponding to [^{32}P]phosphatidylglycerol and [^{32}P]cardiolipin were scraped into scintillation vials and counted. As has been noted previously this enzyme was stimulated but not dependent upon CDP-diglyceride[9]. Phospholipase A_1 was assayed at 37° essentially as described by Scandella and Kornberg[10]. Phosphatidylserine decarboxylase was assayed at 37° using essentially the procedure of Kanfer and Kennedy[2]. L-Serine CMP:phosphatidyltransferase was assayed at 25° by measuring the incorporation of L-[^{14}C]serine into Bligh-Dyer[22] extractable lipid using essentially the conditions of Kennedy and Kanfer[2]. Each membrane fraction was assayed at two or more protein concentrations. Assays were linear with respect to time and quantity of protein employed. All specific activities are expressed as nmoles/min per mg protein.

Localization of enzyme activities: E. coli. The specific activities of the enzymes of phospholipid metabolism of *E. coli* in the membrane fractions isolated by isopycnic separation of lysozyme-EDTA spheroplasts are shown in Table I. Seven of the eight enzymes responsible for phospholipid biosynthesis (*cf.* Fig. 1) were assayed in these membrane preparations. The first enzyme of phospholipid biosynthesis, glycerol

3-phosphate acyltransferase, had the highest specific activity in the cytoplasmic membrane fraction (L), and lowest specific activity in the outer membrane fraction (H). The ratio of these specific activities (L/H) was 21.7. The 1-acylglycerol 3-phosphate acyltransferase, CTP:phosphatidic acid cytidyltransferase, glycerol 3-phosphate:CMP phosphatidyltransferase, cardiolipin synthetase, L-serine CMP:phosphatidyltransferase, and phosphatidylserine decarboxylase also had highest specific activities in the cytoplasmic membrane fraction and lowest specific activities in the outer membrane fraction. The L-serine CMP:phosphatidyltransferase showed the lowest specific activity of all the enzymes tested. Th is consistent with the observation that the activity is found predominantly in the soluble portion of cell[2]. The activity that remains bound to the cell envelope appears to be associated with the cytoplasmic membrane. The biosynthetic enzymes showes L/H ratios ranging from 8.58 to 21.7, and hence, appear to be associated with the cytoplasmic membrane. Since the outer membrane comprises about 60% and the cytoplasmic membrane about 40% of the cell envelope protein[16], calculation of the specific activity of the total membrane (unfractionated) can be made from the observed specific activities of cytoplasmic and outer membrane. Such a calculation for phosphatidylserine decarboxylase predicted a specific activity of 8.65 for total membrane while a value of 8.53 was observed. Similar calculations for the other enzymes showed generally good agreement between the calculated and observed values. Since the enzymatic activity of the total membranes could generally be predicted from the observed specific activities of cytoplasmic and outer membranes, the loss of activity in the separated cytoplasmic and outer membrane fractions was not a major problem. The only activity found to be associated with the outer membrane was phospholipase A_1. This finding is in agreement with the localization of this activity by Scandella and Kornberg[10]. Glycerol 3-phosphate dehydrogenase appears to be associated with the cytoplasmic membrane.

TABLE I

LOCALIZATION OF ENZYME ACTIVITIES: *E. COLI*

Membrane fractions were prepared from *E. coli* as previously described[16]. Four membrane fractions were assayed: total membranes, L, M, and H. L represents the cytoplasmic membrane, M unresolved material, and H the outer membrane. Enzyme assays were performed as described in the text. All specific activities are expressed as nmoles/min per mg protein.

Enzyme	*Reaction No. in Fig. 1*	*Specific activity*				
		Total membranes	*L*	*M*	*H*	*L/H*
Glycerol 3-phosphate acyltransferase	1		54.2	11.3	2.5	21.7
1-Acylglycerol 3-phosphate acyltransferase	2		100.0	16.4	4.8	20.8
CTP:phosphatidic acid cytidyltransferase	3	2.5	6.7	3.2	0.73	9.18
Glycerol 3-phosphate:CMP phosphatidyltransferase	4	23.7	27.2	5.50	2.17	12.5
Cardiolipin synthetase	6	3.07	7.35	2.00	0.53	13.9
Serine CMP:phosphatidyltransferase	7	0.123	0.223	0.106	0.026	8.58
Phosphatidylserine decarboxylase	8	8.53	20.2	4.47	0.97	20.8
Phospholipase A_1		1.93	0.28	5.16	8.88	0.032
Glycerol 3-phosphate dehydrogenase			380.0	70.8	40.5	9.38

Localization of enzyme activities: S. typhimurium. The specific activities of the enzymes of phospholipid metabolism in membrane fractions of *S. typhimurium* are shown in Table II. Two cytoplasmic membrane fractions, L_1 and L_2, were assayed. The biosynthetic enzymes had highest specific activities in the cytoplasmic membrane fractions, L_1 and L_2, lowest specific activities in the outer membrane fraction, H. The L_1/H and L_2/H ratios for the biosynthetic enzymes varied from 6.48 to 16.6. Assays of CTP:phosphatidic acid cytidyltransferase in the Salmonella membranes revealed much lower specific activities than those observed for the *E. coli* membranes, but yielded qualitatively similar results. Therefore, the biosynthetic enzymes appeared to be localized in the cytoplasmic membrane, a pattern similar to that found with the membranes of *E. coli.*

TABLE II

LOCALIZATION OF ENZYME ACTIVITIES: *S. TYPHIMURIUM*

Membrane fractions were prepared from *S. typhimurium* using conditions described previously[16]. Two cytoplasmic membrane fractions L_1 and L_2 were obtained. Other conditions are as described in Table I or in the text.

Enzyme	*Reaction No. in Fig. 1*	*Specific activity*						
		Total membranes	L_1	L_2	M	H	L_1/H	L_2/H
Glycerol 3-phosphate acyltransferase	1		18.2	12.0	3.8	1.2	15.2	10.0
1-Acylglycerol 3-phosphate acyltransferase	2		121.0	129.0	25.0	11.1	10.9	11.6
Glycerol 3-phosphate:CMP phosphatidyltransferase	4	16.9	30.8	28.4	5.68	1.85	16.6	15.4
Cardiolipin synthetase	6	2.73	3.95	5.02	0.75	0.61	6.48	8.23
Phosphatidylserine decarboxylase	8	5.07	12.7	12.6	5.00	1.10	11.5	11.5
Phospholipase A_1		2.91	0.40	0.39	3.46	6.13	0.065	0.064
Phospholipase A_1 + lysophospholipase*		1.48	0.23	0.43	3.31	3.31	0.069	0.130
Glycerol 3-phosphate dehydrogenase			199.0	133.0	45.0	27.4	7.30	4.85

*Data previously presented[16].

The phospholipase A_1 specific activity was highest in the outer membrane fraction, H, and lowest in the cytoplasmic membrane fractions, L_1 and L_2. Similarly, an activity which probably represents the combined action of phospholipase A_1 and lysophospholipase had highest specific activity in the outer membrane and lowest specific activity in the cytoplasmic membrane fractions, L_1 and L_2 [16]. Therefore, the outer membrane appeared to house the degradative enzyme activities of phospholipid metabolism. Preliminary evidence suggests that the lysophosphatidic acid phosphatase activity discovered by Van den Bosch and Vagelos[8] is also associated with the outer membrane fraction (R. Fall and R.M. Bell, unpublished observations).

These findings indicate that the phospholipid biosynthetic enzymes are associated with the cytoplasmic membrane, since the specific activities of these enzymes in the outer membrane fraction can generally be accounted for by the known contamination of the outer membrane with the cytoplasmic membrane[16]. The phos-

pholipid degradative enzymes appear to be associated with the outer membrane since the specific activities of the degradative enzymes in the cytoplasmic membrane can be accounted for by the known contamination of the cytoplasmic membrane with outer membrane[16]. Therefore, these findings suggest that the enzymes of phospholipid biosynthesis and degradation may be neatly compartmentalized within the cell. Such separation of degradative and biosynthetic activities may represent an important means of control of phospholipid metabolism.

Since these results strongly suggest that the cytoplasmic membrane is the site of phospholipid synthesis, the phospholipids of the outer membrane are probably not synthesized *in situ*. Therefore, some mechanism must function to translocate the phospholipid synthesized in the cytoplasmic membrane to the outer membrane where it may be assembled into the membrane matrix. The finding that the cytoplasmic and outer membrane have different phospholipid compositions suggests that these differences may result from the specificity of the degradative enzymes associated with the outer membrane or may reflect the specificity of a possible translocation process.

The localization of the phospholipid biosynthetic enzymes of *E. coli* in the cytoplasmic membrane of the cell envelope is in agreement with similar studies on the localization of these enzymes by White *et al.*[28] in an accompanying paper.

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