MEMBRANE ASSEMBLY IN ESCHERICHIA COLI V. SUBCELLULAR LOCALIZATION OF PHOSPHOLIPID BIOSYNTHESIS

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The subcellular distribution of enzymes involved in lipid biosynthesis in *E. coli* K12 has been studied following various modes of cell disruption and fractionation of the subcellular components. Though most biosynthetic enzymes were found associated with the cytoplasmic membrane fraction regardless of the procédures of disruption or fractionation employed, the enzymes responsible for the synthesis of the major lipid of *E. coli* (phosphatidylethanolamine) and of its precursor (phosphatidylserine) had no distinct localization in extracts. These findings are discussed in the context of current models for the assembly of bacterial membranes.

Interest in the intracellular localization of phospholipid biosynthetic enzymes in gram negative bacteria has been stimulated by two general observations: 1. The cell envelopes of these organisms are composed of two distinct membranous layers, an inner (cytoplasmic) membrane and an outer membrane which is rich in lipopolysaccharide and localized external to the cell wall (1–5). 2. Interrelationships between lipid and membrane protein synthesis have been demonstrated for a number of membrane-associated enzyme systems (6–17). With respect to the first stated observation, Kennedy, Vagelos, and their associates had shown that most phospholipid biosynthetic activities co-sediment with the cell envelope (18–24). This raised the question as to whether the lipids in the inner and outer membranes are synthesized in one or both of these membranes. The solution to this question became approachable only recently with the advent of techniques for separating the various fractions of the cell envelope. The second stated observation suggests a mechanism which makes newly synthesized lipids accessible to newly synthesized proteins for their coordinated insertion into membrane.

The results of three studies on the localization of phospholipid biosynthetic enzymes in gram negative bacteria were published during the course of our investigation. Though the three studies are in agreement on most points, the localization of phosphatidylserine synthetase was left unresolved in two of them (25, 26), whereas this enzyme was reported to be localized in the ribosomal fraction in the third (27). Our data, however, did not indicate a ribosomal localization for this enzyme, nor were they in total agreement on the localization of yet another enzyme. Since different strains and/or cell disruption

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procedures were employed in the three aforementioned studies and in ours, it was necessary to critically examine the possible cause or causes of the dissimilarities in assignment of localization of these enzymes. We have therefore assessed the subcellular localization of lipid biosynthetic enzymes in cells of a single strain of *E. coli* disrupted by a variety of procedures, and have accounted for the recoveries of all enzymes studied in all the major subcellular fractions.

MATERIALS AND METHODS

Growth of Bacteria

The properties of *E. coli* K12 strain 30E, an unsaturated fatty acid auxotroph, have been described (3, 28). Growth was in Medium A (29) supplemented with 1% Difco Casamino acids, 5 μg/ml of thiamine·HCl, 0.5% Triton X-100 (Rohm and Haas), and 0.02% oleic acid. For labeling of cellular RNA, the culture medium contained 10 μmoles/liter of [6–^{3}H]–uracil at a specific activity of 10 μCi/μmole. Cultures of 500 ml were grown at 37°C in 2 liter flasks with rotary shaking. When the culture density reached 1×10^9 cells/ml, the cells were collected by a 10^5 g·min centrifugation and washed once by suspension in 0.01 M Tris·HCl buffer of pH 7.6 and centrifugation.

Preparation and Disruption of Spheroplasts

The methods employed for spheroplast formation and disruption by osmotic lysis and homogenization have been described (3, 30). For disruption by sonic irradiation, spheroplasts from a 500 ml culture were suspended in 10 ml of 0.5 M sucrose, and the suspension was diluted 10-fold with 0.01 Tris·HCl buffer of pH 7.6, containing 10 mM $MgCl_2$ and 100 mM KCl (TMK buffer). This suspension was sonicated in portions of 20 ml for three 1 min periods; the temperature did not rise about 10°C during sonication.

Fractionation of Disrupted Spheroplasts

Spheroplasts disrupted by sonication or homogenization were incubated with 40 μg per ml of DNase for 15 min at 30°. (Methods for experiments described in Fig. 1 and Tables I–IV.) The crude membrane fraction was then prepared by a 2.76×10^6 g·min sedimentation in the Spinco 42.1 rotor and processed further to separate the inner and outer membranes (3, 30).

The 2.76×10^6 g·min supernatant fraction was centrifuged for 18.6×10^6 g·min to sediment ribosomes. Ribosomes were further purified to remove membrane contamination. The 18.6×10^6 g·min pellet was suspended in 2.5 ml of TMK buffer, and the suspension layered on top of 2.5 ml of 65% (w/v) sucrose in the same buffer. During a 168×10^6 g·min centrifugation in the Spinco SW 50.1 rotor the ribosomes penetrated the 65% sucrose shelf and pelleted. The contaminating membranous material remained at the 65% sucrose shelf and was recovered and pooled with the membrane fraction (2.76×10^6 g·min pellet) prior to membrane purification and fractionation (Fig. 1).

Fractionation of Sonic Extracts of Intact Cells to Separate the Cell Envelope, Ribosomal and Supernatant Fractions

Cells from a 500 ml culture labeled with [^{3}H]–uracil were harvested and suspended

TABLE I. Localization of Phospholipid Biosynthetic Enzymes Derived from Spheroplasts Disrupted by Homogenization or Sonic Irradiation

	Total Extract		Supernatant fraction		Ribosomal fraction		Membrane fraction	
	Homo*	Sonic	Homog.	Sonic	Homog.	Sonic	Homog.	Sonic
Protein†	520.0 (100%)	257.0 (100%)	160.0 (53.1%)	165.0 (69.0%)	5.70 (1.8%)	0.54 (0.2%)	146.0 (46.9%)	74.0 (30.7%)
Acyl transferase**	74.2 (100%)	24.0 (100%)	2.40 (1.0%)	< 0.1 (< 0.1%)	4.59 (<0.1%)	< 0.1 (<0.1%)	170.0 (64.3%)	75.0 (89.0%)
CTP:PA transferase	4.23 (100%)	1.93 (100%)	< 0.1	< 0.1	<0.1	0.85 (0.1%)	14.4 (95.5%)	6.73 (100.0%)
PGP synthetase	62.4 (100%)	61.0 (100%)	0.64 (0.3%)	1.17 (1.2%)	6.42 (0.1%)	2.25 (0.1%)	115.0 (51.7%)	144.0 (67.7%)
PS decarboxylase	55.0 (100%)	23.8 (100%)	3.29 (1.8%)	9.77 (25.6%)	8.0 (0.2%)	52.5 (0.5%)	142.0 (72.3%)	52.4 (63.5%)
PS synthetase	33.0 (100%)	23.8 (100%)	29.6 (27.6%)	10.1 (26.4%)	46.0 (1.5%)	52.8 (0.5%)	89.5 (76.1%)	52.4 (63.5%)

*Cells of *E. coli* K12 strain 30E were converted to spheroplasts and disrupted by osmotic lysis aided by either gentle homogenization or sonic irradiation (see Methods section). The spheroplast membranes were recovered by centrifugation for 2.76×10^6 g·min and the ribosomes recovered from the supernatant fraction by centrifugation for 18.6×10^6 g·min. The ribosomes were further purified by centrifugation through a 65% sucrose shelf (see flow chart, Fig. 1). The subcellular fractions were then assayed for phospholipid biosynthetic activities.

†Recoveries are expressed as the percentage of total extract protein or enzymic activity.

**All enzymic activities are expressed as nmoles product formed $(hr)^{-1}$ $(mg\ protein)^{-1}$.

TABLE II. Distribution of Phospholipid Biosynthetic Activities in Inner and Outer Membranes Where Localization is Well Defined

	Band I		Band II		Band III		Ratio I/III*	
	Homog.†	Sonic	Homog.	Sonic	Homog.	Sonic	Homog.	Sonic
Protein**	73.7 (23.7%)	41.7 (16.2%)	47.0 (15.5%)	17.7 (6.9%)	4.90 (1.6%)	8.02 (3.1%)	–	–
Acyl transferase ‡	270.0 (80.2%)	99.4 (65.9%)	16.8 (3.2%)	5.88 (1.7%)	19.1 (0.4%)	3.63 (0.5%)	14.2	27.3
CTP:PA Transferase	17.6 (61.8%)	9.00 (75.3%)	0.90 (2.0%)	4.10 (14.6%)	1.17 (0.3%)	<0.1 (<0.1%)	15.0	(>90.0)
PGP synthetase	123.0 (54.0%)	171.0 (67.2%)	55.30 (15.5%)	61.3 (10.2%)	40.8 (1.2%)	25.4 (1.9%)	4.8	6.7

*Ratio of specific activities.

†The spheroplast membrane fractions from Table I were further resolved by centrifugation on discontinuous sucrose density gradients as described in the Methods section. The three membrane fractions obtained, Band I (cytoplasmic membrane), Band II (an unresolved mixture of cytoplasmic and outer membrane), and Band III (outer membrane) were then assayed for enzymic activities.

**Recoveries are expressed as the percentage of total activity in the unfractionated spheroplast membranes (Table I).

‡All enzymic activities are expressed as nmoles of product formed $(hr)^{-1}$ $(mg\ protein)^{-1}$.

TABLE III. Distribution of Phospholipid Biosynthetic Activities in Inner and Outer Membranes Where Localization is Not Clearly Defined

	Band I		Band II		Band III		Ratio I/III*	
	Homog.†	Sonic	Homog.	Sonic	Homog.	Sonic	Homog.	Sonic
Protein**	73.7 (23.7%)	41.7 (16.2%)	47.0 (15.5%)	17.7 (6.9%)	4.90 (1.6%)	8.02 (3.1%)	–	–
PS decarboxylase‡	183.0 (65.3%)	63.0 (67.6%)	120.0 (27.3%)	55.6 (25.4%)	90.1 (2.1%)	12.5 (2.6%)	2.0	5.2
PS synthetase	94.8 (53.5%)	64.9 (69.9%)	90.1 (32.4%)	55.6 (25.5%)	110.0 (4.1%)	13.2 (2.7%)	0.87	4.9

*Ratio of specific activities.

†The spheroplast membrane fractions from Table I were further resolved by centrifugation on discontinuous sucrose density gradients as described in the Methods section. The three membrane fractions were then assayed for enzymic activities.

**Recoveries are expressed as the percentage of total activity in the unfractionated spheroplast membranes (Table I).

‡All enzymic activities are expressed as nmoles of product formed $(hr)^{-1}$ $(mg\ protein)^{-1}$.

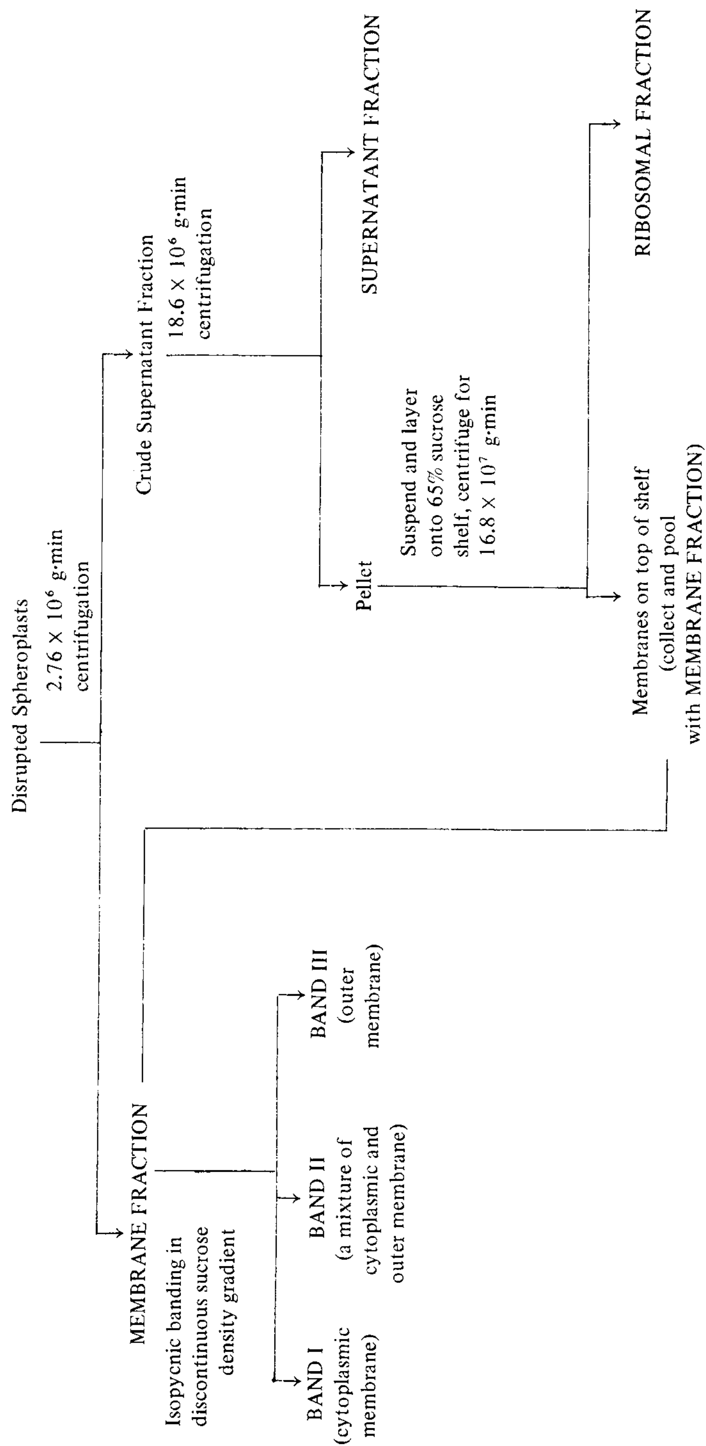
TABLE IV. Distribution of Acid Precipitable Radioactivity Derived from [^3H]-Uracil and Enzymic Activities in Subcellular Fractions Derived from Spheroplasts by Osmotic Lysis and Sonic Irradiation

	Acid precipitable radioactivity		Recovery of enzymic activities*		
	cpm/mg of protein	Total cpm	PGP synthetase	PS decarboxylase	PS synthetase
Ribosomal fraction	250,000	136,000	<0.1%	0.1%	0.3%
Membrane fraction	6,500	477,000	69.0%	64.0%	60.5%
Band I	5,300	222,000	47.0%	45.0%	41.0%
Band II	5,900	104,000	4.8%	10.0%	13.0%
Band III	2,300	18,000	1.4%	1.5%	2.0%

*Recovery is expressed as the percentage of total enzymic activity of the sonicated spheroplast preparation.

Cells labeled with [^3H]-uracil (Methods section) were converted into spheroplasts and disrupted by osmotic lysis aided by sonic irradiation. The spheroplast membranes were recovered by a 2.76×10^6 g·min centrifugation and the ribosomal fraction was then obtained from the 2.76×10^6 g·min supernatant fraction by an 18.6×10^6 g·min centrifugation. The ribosomal fraction was then further purified by centrifugation through a 65% sucrose shelf. The spheroplast membranes were further resolved by centrifugation through discontinuous sucrose gradients (see Methods section). Each of the subcellular fractions was assayed for acid precipitable radioactivity and enzymic activity.

FIGURE 1: Flow chart for Tables I, II, III and IV



in 10 ml of TMK buffer and sonicated as described above for spheroplasts. (Methods for experiments described in Figs. 2 and 3 and Table V.) Unbroken cells were removed by a 2.5×10^3 g·min centrifugation and the supernatant fraction was divided into three portions of 3 ml. Each of these was layered on top of a preformed 25 ml 30–15% (w/v) linear sucrose gradient which had been formed on top of a 5 ml 40% (w/v) sucrose cushion. The gradients were centrifuged at 28.0×10^6 g·min in the Spinco SW 25.1 rotor (31). The supernatant fraction (approximately 2 ml) was removed from the top of each gradient. The tubes were then punctured at the level of the cushion with a 22 gauge needle and the gradients were collected in fractions of 2 ml. Portions of each fraction were removed at this point for the determination of trichloroacetic acid precipitable radioactivity. The ribosomal fraction obtained by this procedure was contaminated with membranous material, and was further purified as described in the preceding section by a 168×10^6 g·min centrifugation through a 70% (w/v) sucrose shelf.

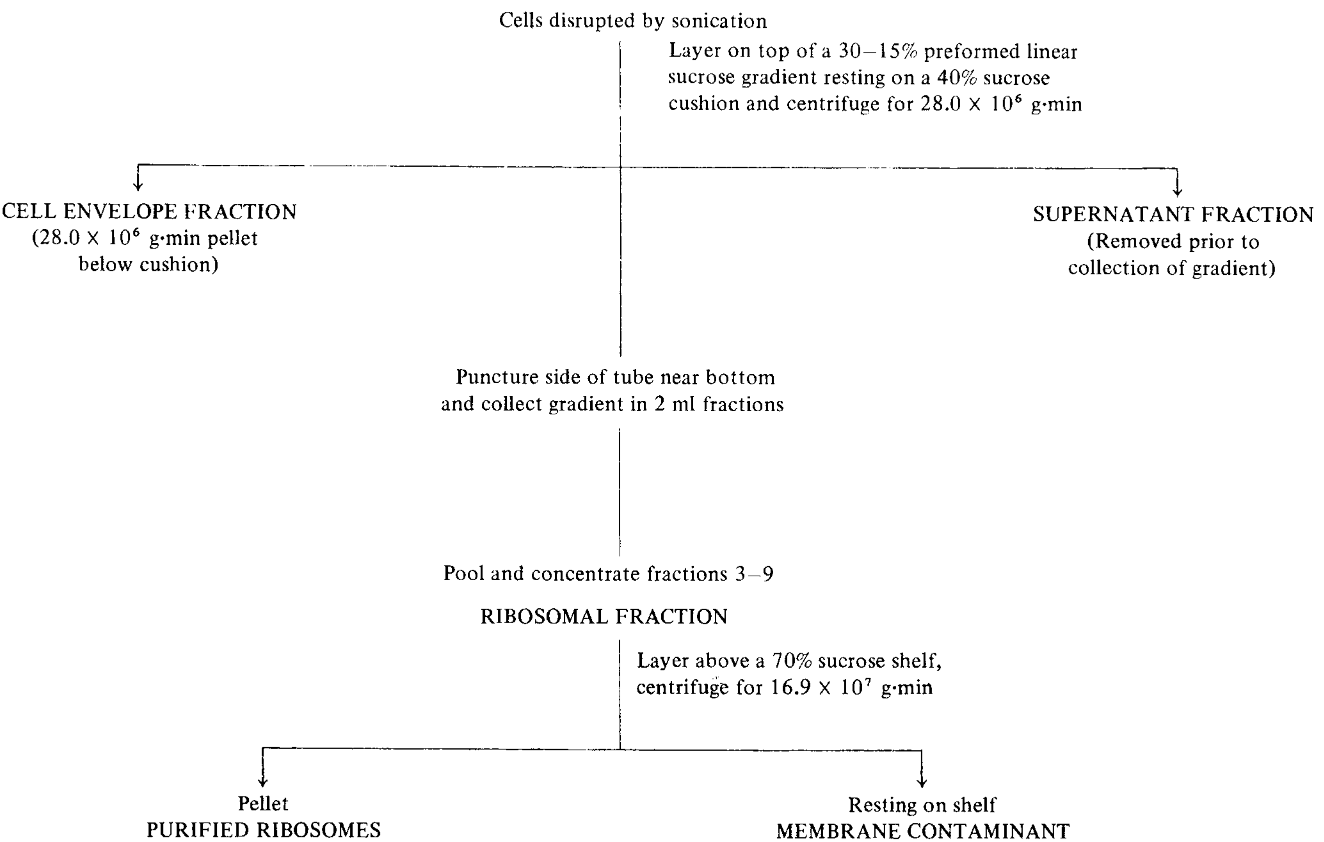
Fractionation of Sonic Extracts of Intact Cells to Separate the Ribosomal, Supernatant, Cytoplasmic Membrane and Outer Envelope Fractions

Schnaitman (4) has described a cell envelope fractionation technique which resolves the inner and outer membrane fractions where the latter fraction is obtained complexed with the cell wall (outer envelope fraction). This procedure was modified to enhance the resolution of the cytoplasmic membrane and outer envelope fractions from strain 30E. (Methods for experiments described in Fig. 4 and Table VI.) The cells were harvested and sonicated, and undisrupted cells were sedimented by a 2.5×10^6 g·min centrifugation in the Spinco 50 rotor. The pellet was suspended in 0.05 M Tris·HCl buffer of pH 7.4, containing 1 mM EDTA and washed twice by centrifugation (5.4×10^6 g·min) and suspension as above. The washed cell envelope fraction was suspended in 15% (w/v) sucrose, and 1.4 ml portions were layered on top of discontinuous gradients consisting of 3.4 ml of 70% (w/v) sucrose and 3.6 ml each of 64% and 59% (w/v) sucrose. All sucrose solutions were prepared in the Tris·EDTA buffer described above. The gradients were centrifuged for 77×10^6 g·min in the Spinco SW 41 rotor. Two bands were visible in the gradients, one at the 59–64% interface and the other at the 64–70% interface. The upper band corresponded to the cytoplasmic membrane fraction and the lower band to the outer envelope fraction. Ribosomes were recovered from the 5.4×10^6 g·min supernatant fraction by an 18.0×10^6 g·min centrifugation. The ribosomes were further purified by an 18.6×10^6 g·min centrifugation through a 65% sucrose shelf using the Spinco SW 50 rotor (Fig. 4).

Assays for Protein and Radioactivity

Protein was determined as described by Lowry et al. (32). For estimation of radioactivity by scintillation counting, aqueous samples were combined with water in a total volume of 0.75 ml, and 10 ml of the toluene/Triton X-100 [3:1 (v/v)] solution described by Patterson and Greene (33) was added. Radioactive lipids were transferred to vials as chloroform solutions, and the chloroform evaporated under a stream of nitrogen before addition of the scintillation cocktail. For the determination of radioactivity in RNA, the samples (in 5 ml of water containing 10 mg of bovine serum albumin) were mixed with an equal

FIGURE 2. Flow chart for Figure 3 and Table V



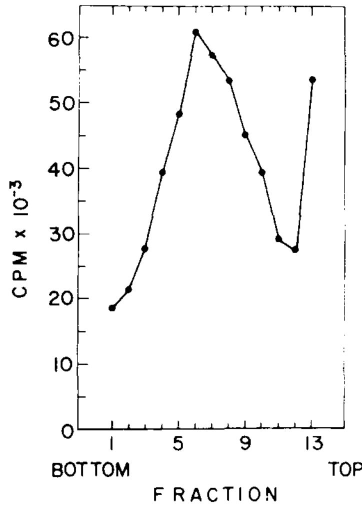


Fig. 3. Distribution of acid precipitable radioactivity derived from [^{3}H]-uracil in fractions collected from a 30–15% sucrose gradient. Sonicated whole cells labeled with [^{3}H]-uracil were layered on top of a preformed 25 ml 30–15% sucrose gradient resting on a 5 ml 40% sucrose cushion and centrifuged for 28.0 × 10^6 g·min. The supernatant fraction was first removed from the top of the gradient (see Methods section). The side of the tube was then punctured 2 mm above the cushion and fractions of 2 ml were collected and assayed for acid precipitable radioactivity. Fractions 3–9 were pooled, concentrated by sedimentation, and analyzed for enzymic activities (ribosomal fraction, Table V). The ribosomal fraction was then further purified (see Methods, Fig. 2) and the purified ribosomes were also assayed for enzymic activity and acid precipitable radioactivity.

TABLE V. Distribution of Enzymic Activities and of Acid Precipitable Radioactivity Derived from [^{3}H]-Uracil in Subcellular Fractions Recovered from a 30–15% Sucrose Gradient*

	Acid precipitable radioactivity (cpm)	Protein	SDH	PS synthetase	PS decarboxylase
Supernatant fraction	–	75.0 (42.6%)	0.15 (2.3%)	35.9 (26.7%)	8.47 (8.5%)
Cell envelope fraction (28.0 × 10^6 g·min pellet)	77,440	59.9 (34.0%)	4.99 (61.5%)	77.3 (45.9%)	76.8 (61.2%)
Ribosomal fraction (from gradient)	700,000	30.0 (18.0%)	4.95 (30.5)	92.1 (27.4%)	–
Purified ribosomes	560,000	8.2 (5.7%)	0.23 (0.2%)	38.8 (3.4%)	11.7 (1.3%)
Membrane contaminant	17,200	19.6 (12.0%)	9.09 (18.4%)	122.0 (21.8%)	112.0 (29.1%)

*Sonicated whole cells labeled with [^{3}H]-uracil were resolved into subcellular fractions as described in Figs. 2 and 3. All enzymic activities are expressed as nmoles of product formed $(hr)^{-1}$ (mg protein)$^{-1}$. Recoveries are expressed as the percent of total extract activity recovered in the various fractions.

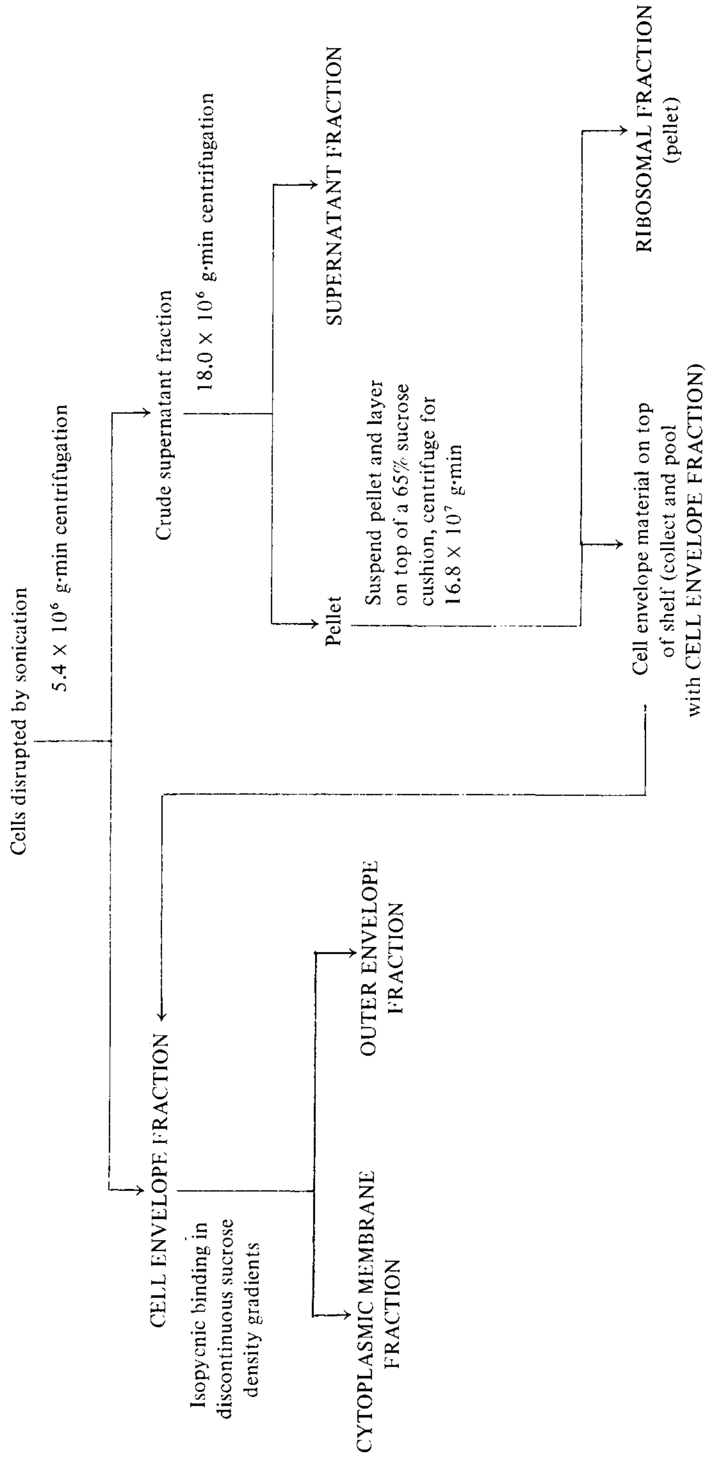


FIGURE 4: Flow chart for Table VI

TABLE VI. Distribution of Enzymic Activities and of Acid Precipitable Radioactivity (from [^{3}H]-Uracil) in Subcellular Fractions Derived from Intact Cells Disrupted by Sonication*

	Acid precipitable radioactivity (cpm)	Protein	SDH	PS synthetase	PS decarboxylase	Phospholipase A_1
Disrupted cells	–	152.0 (100%)	3.43 (100%)	31.9 (100%)	31.8 (100%)	6.7 (100%)
Supernatant fraction	165,000	80.0 (53.0%)	0.19 (0.5%)	33.6 (47.6%)	7.90 (11.2%)	–
Ribosomal fraction	153,000	1.9 (1.3%)	0.2 (< 0.1%)	37.8 (0.1%)	1.5 (< 0.1%)	–
Cell envelope fraction (5.4 × 10^6 g·min pellet)	504,000	67.2 (44.5%)	4.79 (95.5%)	50.0 (48.5%)	87.7 (88.9%)	15.1 (99.5%)
Cytoplasmic membrane fraction	10,000	40.7 (26.8%)	5.06 (95.3%)	30.0 (39.2%)	53.0 (69.0%)	0.7 (2.7%)
Outer envelope fraction	8,500	7.7 (5.1%)	0.93 (2.5%)	46.5 (10.8%)	82.0 (19.5%)	128.0 (95.2%)

*Cells of *E. coli* K12 strain 30E, labeled with [^{3}H]-uracil, were disrupted by sonic irradiation and processed as described in Fig. 4 and the Methods section. The various subcellular fractions were then assayed for acid precipitable radioactivity and for enzymic activities. All enzymic activities are expressed as nmoles of product formed $(hr)^{-1}$ $(mg\ protein)^{-1}$. Recoveries are expressed as the percent of total extract activity recovered in the various fractions.

volume of 10% trichloroacetic acid. After 30 min incubation at 0 to 4°C, the acid precipitable material was collected by a 4.0×10^5 g·min centrifugation and washed three times by suspension in 5 ml portions of 5% trichloracetic acid. Each precipitate was then incubated with 0.4 ml of 0.3 M NaOH for 18 hr at 37°C. The alkaline digested material was neutralized with 0.3 ml of 0.3 M acetic acid and a 0.5 ml portion removed for determination of radioactivity.

Enzymatic Assays

All enzymes except for phospholipase A_1 were assayed under conditions where a linear relationship exists between protein concentration and activity, and all assays were at the temperatures specified in the original methods cited below. Succinate dehydrogenase was assayed by the procedure of Slater and Bonner (34). The methods employed for the assay of phospholipid biosynthetic enzymes are: acyl transferase, Ray et al. (23); CTP:phosphatidic acid transferase, Carter (22); phosphatidylglycerol phosphate synthetase, Chang and Kennedy (21); phosphatidylserine decarboxylase, Patterson and Lennarz (35); and phosphatidylserin synthetase, Kanfer and Kennedy (19), where Triton X-100 (0.1% w/v) was used in place of cutscum (0.1% w/v). The substrate for the PS decarboxylase assay, 3-phosphatidyl [^{3}H]-serine, was synthesized as described by Patterson and Lennarz (35). Phospholipase A_1 was assayed as described by Scandella and Kornberg (36) at a protein concentration of 1 mg/ml of assay mixture with the following modifications: Following the addition of chloroform and water to the single phase methanol-water mixture, the lower phase was removed and evaporated to dryness under nitrogen. The dried material was dissolved in 50 μl of chlorofom and applied to a thin layer chromatography plate (silica gel G 20 X 20 cm, 250 μm thick layer, Analtech, Inc., Newark, Del.). The plate was developed in hexane:diethyl ether:acetic acid [60:40:1 (v/v/v)]. The spot corresponding to free fatty acid was scraped from the plate and suspended in 10 ml of scintillation cocktail, and the radioactivity determined. To prepare the substrate for the phospholipase A_1 assay, [^{3}H]-acyl phosphatidylethanolamine, cultures of strain 30E were grown as described under the Growth of Bacteria, except that 0.02% linoleic acid was added as the essential fatty acid, and 1.5 mmoles per liter of [2-^{3}H]-Na acetate was added as a precursor for synthesis of radioactive lipid. The cells were collected and washed, and the cell paste was lyophilized. The dry cells were extracted with chloroform:methanol as described by Vorbeck and Marinetti (37), and the extract washed with 2 M KCl to remove nonlipid radioactivity (18). The neutral lipids were separated from phospholipids by silicic acid column chromatography. The labeled phosphatidylethanolamine was then recovered from the phospholipid extract by DEAE-cellulose column chromatography as described by Law and Essen (38). The resulting lipid was chromatographically homogeneous, and was devoid of contaminating radioactive phosphatidylserine, phosphatidylglycerol, or cardiolipin.

Chemicals

Palmityl coenzyme A and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., St. Louis, Mo. Dipalmitoyl-CDP-diglyceride was the generous gift of E. P. Kennedy. Oleic and linoleic acids were purchased

from the Hormel Institute, Austin, Minn. DNase and lysozyme were purchased from Worthington Biochemical Corp., Freehold, N.J.; phosphatidic acid from Pierce Chemical Co., Rockford, Ill.; and phosphatidylserine from Applied Sciences Laboratories, State College, Pa. The following radioisotopes were purchased from New England Nuclear Corp., Boston, Mass., and were diluted to the specific activities indicated: cytidine-[5-^{3}H]-5′-triphosphate, tetrasodium salt (10^{6} cpm/nmole); L-[U-^{14}C]-glycerol-1-phosphate, disodium salt (2.5×10^{6} cpm/μmole); L-[G-^{3}H]-serine (6.7×10^{6} cpm/μmole); [6-^{3}H]-uracil (10 μCi/μmole); and [2-^{3}H]-sodium acetate (2.0 mCi/nmole). The substrates for phosphatidylserine decarboxylase and phospholipase A_1 assays prepared as described above were used at specific activities of 10^{5} cpm/μmole ([^{3}H]-phosphatidylserine) and 6.8×10^{5} cpm/μmole ([^{3}H]-phosphatidylethanolamine), respectively. All other chemicals were of the highest purity commercially available.

RESULTS

In order to determine the intracellular distribution of the phospholipid biosynthetic enzymes in *E. coli* K12 strain 30E, spheroplasts were disrupted by osmotic lysis aided by either homogenization or sonication. The phospholipid biosynthetic activities of the supernatant, ribosomal, and membrane fractions, as well as those of the cytoplasmic and outer membrane fractions prepared from the membrane fraction by isopycnic banding in sucrose gradients, were measured and compared in order to determine whether methods of cell disruption have a significant effect upon the subsequent fractionation of these activities. Our studies also sought to ascertain if phosphatidylserine synthetase is specifically associated with the ribosomal fraction in this strain of *E. coli* and to determine the distribution of phospholipid biosynthetic activities among the subcellular fractions which result from the fractionation of intact cells disrupted by sonication.

Enzyme Localization in Spheroplasts

The localization of phospholipid biosynthetic enzymes in the major fractions of homogenized and sonicated spheroplast preparations is summarized in Table I. The recovery of membrane protein is reduced and that of soluble protein increased when spheroplasts are disrupted by sonication, compared with disruption by homogenization. All of the enzymes studied with the exception of phosphatidylglycerol phosphate (PGP)* are partially inactivated by sonication since the specific activities are reduced in sonciated preparations.

Acyl transferase, CTP:phosphatidic acid (PA) transferase, and PGP synthetase appear in the membrane fraction following homogenization or sonication of spheroplasts. These enzymes are apparently tightly bound to the membrane since sonication does not result in the release of singificant levels of enzymic acticities to the supernatant or ribosomal fractions.

Phosphatidylserine decarboxylase appears to be less tightly bound to membrane. This enzyme is found in the membrane fraction of homogenized prepara-

*See Fig. 6 for abbreviations.

tions, but sonication results in its appearance in other cellular fractions, primarily the supernatant fraction.

Although there is significant PS synthetase activity in the ribosomal fraction, less than 1.5% of the total activity is recovered there. This observation differs from that of Raetz and Kennedy (27), who reported that the PS synthetase is associated with the ribosomes of *E. coli* B. We have conducted further experiments regarding the localization of PS synthetase and these are detailed below.

Localization in Membrane Fractions

Having ascertained the localization of the phospholipid biosynthetic enzymes, we next determined their distribution among the membrane fractions which result from isopycnic banding of discontinuous sucrose density gradients. The procedure of Fox et al. (3) gives rise to three membrane fractions. Band I corresponds to the inner or cytoplasmic membrane and Band III corresponds to the outer membrane of the *E. coli* cell envelope. Band II represents a mixture of inner and outer membrane material which we and other groups of investigators have been unable to resolve (2, 3, 5, 25).

Acyl transferase, CTP:PA transferase, and PGP synthetase are associated with the inner membrane fraction of both homogenized and sonicated preparations (Table II). Sonication appears to improve the resolution of Bands I and III since the ratios of the specific activities of these enzymes in Band I and III is greater in the sonicated preparation. This enhanced resolution in sonicated preparations indicates that redistribution of these enzymes among the inner and outer membranes does not occur as the result of sonication. Sonication may in fact break down points of adhesion between inner and outer membranes and enhance their separation.

The localization of PS decarboxylase is not so clear as that of the three enzymes discussed previously (Table III). In homogenized preparations, PS decarboxylase is associated with the membrane fraction and the bulk of this is on the inner membrane. Sonication results in the appearance of nearly one-third of the activity of PS decarboxylase in the supernatant fraction, but most of the recovered activity of this enzyme is associated with the inner membrane fraction. PS synthetase is not exclusively associated with the membrane fraction (Table I), and has been suggested by other investigators to reside in the ribosomal fraction (27). The association of this enzyme with membrane must be weak, since even gentle homogenization results in its appearance in the supernatant fraction.

Further Characterization of the PS Synthetase: Ribosome Distribution in Spheroplasts

Raetz and Kennedy (27) have reported that PS synthetase is associated with the ribosomes of *E. coli* B. Since our data indicated that this enzyme might not be ribosomal in *E. coli* K12, and that it is rather loosely bound to the membrane of this organism, we undertook a series of further experiments in an attempt to clarify its localization.

Spheroplasts were prepared from cells grown in the presence of [^{3}H]-uracil for four generations. These spheroplasts were fractionated and the mem-

branes further separated by isopycnic centrifugation. The ribosomal and membrane fractions and Bands I, II, and III were then analyzed for acid precipitable radioactivity and for PGP synthetase, PS decarboxylase, and PS synthetase activities (Table IV). There was three to four times as much radioactivity in the membrane as in the ribosomal fraction of this preparation, although the ribosomal fraction has a greater specific activity. However, the membrane associated PS synthetase activity is 200-fold greater than that of the ribosomal fraction. This supports our contention that the PS synthetase is not specifically associated with the ribosomes of *E. coli* K12, although we cannot rule out the possibility that the ribosomes to which the enzyme might be attached might be more strongly associated with membrane.

Ribosome and Enzyme Distribution on Linear Sucrose Gradients

It is conceivable that the large quantity of membrane bound ribosomes observed in the spheroplast preparation resulted from the formation of lysozyme mediated membrane-ribosome complexes, as have been described by Patterson et al. (39). To determine if there is significant binding of ribosome associated PS synthetase activity to membranes in the absence of lysozyme, we employed intact cells labeled with [^{3}H]-uracil. These cells were disrupted by sonic irradiation in the presence of 10 mM magnesium chloride to prevent ribosome dissociation and the cell lysates were layered onto 30–15% sucrose gradients on a 40% sucrose cushion. After centrifugation, fractions were collected and analyzed for radioactivity (Fig. 2). As shown in Fig. 3, the ribosomes sedimented as a broad band centered in the middle of the gradient. The fractions containing the bulk of the ribosomes were pooled, concentrated, and analyzed for radioactivity, succinate dehydrogenase (SDH; a cytoplasmic membrane marker enzyme), PGP synthetase, PS decarboxylase, and PS synthetase (Table V). The ribosomal fraction so isolated was contaminated by membrane fragments, as indicated by the high level of SDH activity in this fraction. The ribosomal fraction was freed of the membrane contaminant by centrifugation through a 70% sucrose shelf. The majority of the ribosomal associated SDH activity recovered from the gradient appeared in the membranous material which did not penetrate the sucrose shelf.

With this procedure, in contrast to the procedure employed in the experiment described in Table IV, we observed a marked reduction of membrane associated radioactivity and a large increase in ribosomes not associated with membrane. These ribosomes contained only a small quantity of PS synthetase activity. Twenty-seven percent of the total PS synthetase activity was soluble and 68% was membrane bound. Over 90% of the total PS decarboxylase activity and all of the PGP synthetase also appeared in the membrane fraction. Thus a large reduction in membrane associated ribosomes was achieved without materially affecting the distribution of PS synthetase.

Distribution in Membrane Fractions from Whole Cells

In light of the results of our study on ribosome distribution in sonicated intact cells, we employed our modification of the procedure of Schnaitman (4) to prepare cytoplasmic membrane and cell wall enriched fractions (Fig. 4 and Table VI). The ratio of specific activities of SDH in the cytoplasmic membrane

to outer envelope fraction is greater than 5:1 and we recovered more than 95% of the total SDH activity in the cytoplasmic membrane fraction. This represents a greater than two-fold increase in the ratio of SDH activities over that achieved by Schnaitman, who recovered only about 70% of the total SDH activity in the cytoplasmic membrane fraction. Electron micrographs (Fig. 5) illustrate that the two fractions which we obtain by our procedure are relatively homogenous.

We have studied the distribution of SDH, PS synthetase, PS decarboxylase, PGP synthetase, and phospholipase A_1 [an outer membrane marker enzyme (36)] among these fractions (Table VI). A large quantity of acid precipitable radioactivity sediments with the cell envelope fraction. This appears to be adventitious binding of radioactivie material to membranes and is not specifically associated with the membranes since it is not recovered in the purified envelope fractions. In the two envelope subfractions we recovered 95% of membrane bound PS synthetase activity, but only 4% of the radioactivity which initially cosedimented with the membranes. All of the PGP synthetase and SDH and nearly 90% of the PS decarboxylase appear in the cell envelope fraction, and the bulk of these enzyme activities are recovered in the cytoplasmic membrane fraction. Phospholipase A_1 activity appears in the cell envelope fraction, and the majority of this is associated with the outer envelope fraction. Nearly equal quantities of PS synthetase are found in the supernatant and cell envelope fractions. The portion of PS synthetase which is envelope associated appears primarily in the cytoplasmic membrane fraction, but the specific activity of this enzyme is actually higher in the outer envelope fraction. These observations indicate that PS synthetase is readily released from membranes since it was recovered in all fractions derived either from sonicated intact cells or from spheroplasts disrupted by sonication or homogenization.

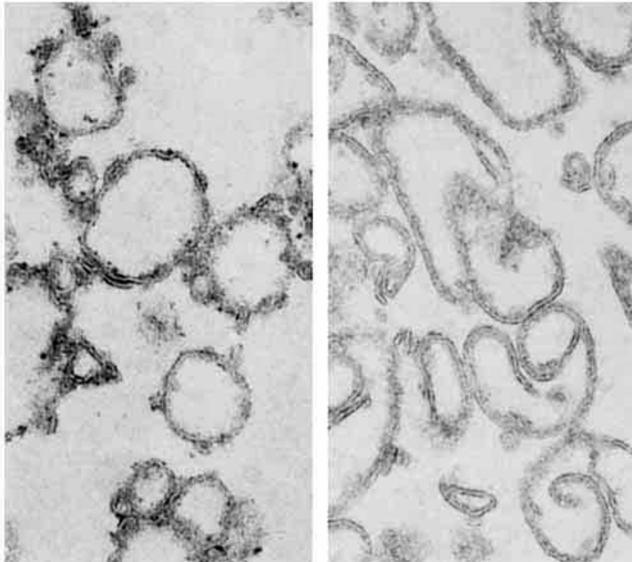


Fig. 5. Electron micrographs of cytoplasmic membrane and outer envelope fractions derived from sonicated cells. Sonicated intact cells were processed as described in the Methods section and Fig. 4 to recover the cell envelope fraction which was then fractionated by isopycnic banding in discontinuous sucrose gradients to produce the cytoplasmic membrane (left, magnification 125,000) and outer envelope (right, magnification 120,000) fractions.

DISCUSSION

Studies similar to ours have been undertaken by White et al. (26) and Bell et al. (25), who reported only on the enzymatic activity recovered with the cell envelope. A thorough analysis of all subcellular fractions is necessary, however, in light of the observation by Raetz and Kennedy (27) that PS synthetase is largely ribosome associated in extracts of *E. coli* B.

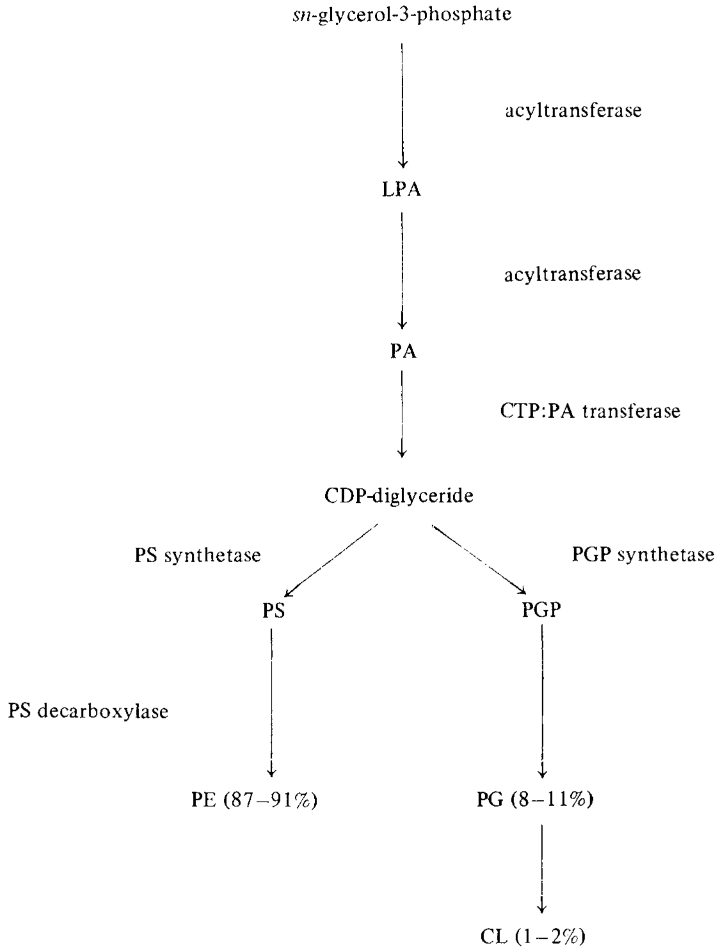
Our data indicate that CTP:PA transferase, PGP synthetase, and enzymes that catalyze the acylation of sn-glycerol-3-phosphate are firmly associated with the cytoplasmic (inner) membrane of *E. coli* K12. (See Fig. 6 for a schematic representation of the pathways of phospholipid synthesis in *E. coli.*) The bulk of these activities fractionated with the inner membrane following all modes of cellular disruption and all procedures used for resolution of the subcellular fractions. Only minor activity was associated with the supernatant, ribosomal, or outer membrane fractions. We therefore think it likely that steps leading to the synthesis of CDP-diglyceride and phosphatidylgylcerol phosphate occur in or on the inner membrane. These conclusions agree with those of White et al. (26) and Bell et al. (25). We are also in agreement with Bell et al. (25) who observed that phospholipase A_1 activity was associated with the outer membrane fraction.

In contrast to the observations of Bell et al. (25) and White et al. (26) we find that neither PS synthetase nor PS decarboxylase have a distinct subcellular localization in extracts. Both enzymes exhibited substantial activity in the supernatant and outer membrane fractions, and the distribution of these two activities changed when the procedure for cell disruption was altered. In cells disrupted by sonic irradiation, nearly 50% of PS synthetase activity was found associated with the supernatant fraction, and the specific activities of both PS synthetase and PS decarboxylase were higher in the outer envelope fraction (a complex of cell wall and outer membrane) than in the inner membrane fraction (Table VI). Neither enzyme had a significant ribosomal complement of activity. This is in contrast to the findings of Raetz and Kennedy (27) who observed that the bulk of PS synthetase activity was ribosome associated in *E. coli* B. However, we place greater emphasis on the fact that in both of these studies, a substantial portion of PS synthetase activity was largely *not* associated with a membrane fraction.

One of the greatest enigmas in membrane biochemistry is the process by which the so-called "integral" (40, 41) or "intrinsic" (42) proteins migrate from their ribosomal points of origin to their distinctive sites of localization in membranes. It is reasonable to surmise that proteins which penetrate into or through the hydrocarbon portion of a membrane lipid bilayer have considerable surface area comprised largely of nonpolar amino acid side chains. There thus arises a question as to how a protein with a high proportion of nonpolar surface area is translocated from its site of synthesis to its predestined site of deposition. Further questions arise upon considering the nature of a folding process which would favor the acquisition of a protein conformation consisting in large part of nonpolar surface regions. We can envisage at least four possible solutions to these problems: 1. "Apolar"* proteins can fold to form and then maintain native

*In this discussion we shall refer to proteins which have substantial surface area comprised of amino acids with nonpolar side chains as apolar proteins.

FIGURE 6. Flow scheme for phospholipid biosynthesis in *E. coli* beginning with the acylation of *sn*-glycerol-3-phosphate



Abbreviations are: LPA, lysophosphatidic acid; PA, phosphatidic acid; CDP-diglyceride, cytidine diphosphate diglyceride; PS, phosphatidylserine; PE, phosphatidylethanolamine; PGP, phosphatidylglycerol phosphate; PG, phosphatidylglycerol; CL, cardiolipin. The numbers in parentheses give the steady state values for lipid phosphorus in the cell envelope phospholipids of strain 30E harvested in exponential phase (Machtiger, Tsukagoshi and Fox, unpublished data).

structure in an aqueous environment, but partition preferentially into a nonaqueous milieu. 2. Apolar proteins initially fold to form largely polar surfaces, but undergo conformational change with consequent acquistion of apolar surface characteristics upon insertion into a hydrocarbon environment. 3. Apolar proteins are formed on membrane associated ribosomes, and the ribosomes are so situated as to provide an environment compatible with folding which gives rise to apolar protein surfaces. 4. Apolar proteins can be formed on ribosomes which are not membrane associated. Proper folding arises as the result of a cooperative interaction between lipids and protein during folding, and the lipids are made available by translocation from their site (sites) of synthesis to the ribosomes. The protein is then translocated from ribosome to membrane as a lipoprotein complex. All four alternatives provide a basis for specification of a given membrane site of deposition by a given protein. Multiple membranous structures are present during some stage of the cellular life span in all eucaryotic and some procaryotic cells, and each of these membranous structures has its own complement of proteins (4, 43). It is logical to postulate that such specification arises from interactions between newly formed proteins and proteins already present in membranes, the latter in a sense acting as "template" for specifying deposition of the former.

Our studies bring nothing to bear on the first two alternatives. However, we would tend to reject the first alternative on thermodynamic grounds alone for those proteins with a high proportion of nonpolar surface area. The second alternative is certainly plausible, but we know of no data which provide support or grounds for rejection. The third and fourth alternatives are two sides of the same coin. In the third alternative, the site of protein synthesis is brought to a source of lipids, and in the fourth alternative, lipids are translocated to the site(s) of protein synthesis. With respect to the third alternative, much attention has been brought to bear on the possible significance of membrane association of ribosomes. It has been proposed that in eucaryotes, the membrane associated ribosomes are the sites of synthesis of membrane proteins (44–47) and free ribosomes are the sites of synthesis of soluble proteins (48–51). [For a recent treatment of the subject, see Borgese et al., (52).] Lodish (53), on the other hand, has demonstrated that membrane-free reticulocyte polyribosomes produced the same globin and nonglobin proteins as does the intact cell. In procaryotes, and more specifically in *E. coli* the possible role of membrane-ribosome association has been the subject of inconclusive debate for some time. [For recent reviews on the subject see Patterson et al. (39) and Scharff et al. (54)]. In the case of eucaryotic cells, electron microscopy gives splendid evidence for specific association of ribosomes with the endoplasmic reticulum. To the best of our knowledge, there are no electron micrographs of whole cells which indicate a bias in the direction of membrane associated over unassociated ribosomes in procaryotes. Like Patterson et al. (39), we observed a significant fraction of membrane associated ribosomes in extracts only when the cells had been previously treated with lysozyme, and in this case the ribosomes were associated equally with both the inner and outer membranes. On genetic grounds, it appears unlikely that membrane associated ribosomes in procaryotes (should they exist and have distinct functional qualities) could function exclusively in the translation of membrane proteins. This would require that different ribosomal species function in the translation of polycistronic messages that have cistrons for both soluble and membrane proteins, e.g., the

lactose operon. Thus while the third alternative remains an attractive hypothesis, there is little evidence to support it, especially in the case of procaryotic cells.

In light of evidence presented here and by Raetz and Kennedy (27), we entertain the hypothesis that lipids are translocated from their sites of synthesis to the site of protein synthesis. In eucaryotes, this could be accomplished by the lipid translocating proteins initially identified by Wirtz and Zilversmit (55). [For a review of phospholipid exchange see McMurray and Magee (56).] In procaryotes, and more specifically in *E. coli*, the translocation might actually be catalyzed by lipid biosynthetic enzymes, e.g., PS synthetase. Though we have no definite evidence for this type of function for lipid biosynthetic enzymes in *E. coli*, this hypothesis provides an appealing explanation for data which indicate that certain proteins are inserted into membrane with lipids synthesized concomitantly (10, 11, 13, 14, 16).

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