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Enzymology, Genetics, and Regulation of Membrane Phospholipid Synthesis in *Escherichia coli*

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STRUCTURE AND FUNCTION OF THE GRAM-NEGATIVE CELL ENVELOPE

Gram-negative bacteria, like *Escherichia coli*, are enclosed by an envelope (91, 257, 260) consisting of two separate membranes (Fig. 1). The inner, or cytoplasmic, membrane is the primary permeability barrier of the cell, containing specific permeases and transport proteins (91, 257, 260). It is also the site of adenosine 5'-triphosphate (ATP) generation and of the biosynthesis of major membrane constituents, such as phospholipids (22, 259, 260, 313, 376). The outer membrane, which is exposed to the environment, carries the antigenic determinants of the bacterial surface (91, 257, 260). Certain of the proteins and the lipopolysaccharide of the outer membrane function as receptors for bacteriophages and colicins (90, 155, 260, 271, 348). The outer membrane provides the cell with a passive barrier to substances with molecular weights greater than several hundred (186, 223). This generally renders gram-negative bacteria more resistant to antibiotics than are gram-positive organisms, which lack this structure (37, 91, 105, 186, 347).

The membranes of *E. coli* are separated by a rigid peptidoglycan layer (Fig. 1), which imparts stability and shape to the cell and is the substrate for lysozyme (91, 257, 260). The periplasmic space, between the inner membrane and the peptidoglycan, contains numerous hydrolytic enzymes and various soluble binding proteins involved in transport and chemotaxis (2, 91, 257, 260). Flagella are anchored in the cytoplasmic membrane and extend through the outer parts of the envelope (2, 91, 257, 260).

Both membranes consist of approximately equal proportions of proteins and lipids, but the protein composition of each membrane is unique (259, 260, 313). Gel electrophoresis as well as direct purification reveal that there are a few major outer membrane proteins and nearly 50 additional minor species (260, 296, 310, 314, 315). Genetic studies have clarified the functions of many of these polypeptides. In general, they confer distinct selective advantages on the cell, but few, if any, are actually required for growth (138, 143, 203, 304, 322). The protein composition of the inner membrane is far more complex than that of the outer membrane (93, 310), and many of the inner membrane polypeptides are enzymes which catalyze essential biosynthetic reactions (260). For instance, the enzymes of phospholipid synthesis are inner membrane proteins (22, 376). Individually, they constitute less than 0.1% of the total cellular protein (103, 142). The inner membrane also contains various transport proteins and the energy-transducing adenosine triphosphatase (129, 260).

Lipopolysaccharide is a substance unique to gram-negative bacteria and is exclusively localized in the outer membrane (91, 219, 256). When injected into mammals, it causes endotoxic shock (91). The complete structure of lipopolysaccharide has not been determined, but it is known to consist of three portions, which are the outer (or antigenic) sugars, the core sugars, and lipid A (91, 186, 256). The core portion contains covalently linked phosphorylethanolamine moieties (186). Lipid A consists of glucosamine, fatty acids, and esterified phosphate (186). Like the flagella, the polysaccharide chains of this material project outward into the growth medium (Fig. 1). The sugar residues differ in various strains of *E. coli*, giving rise to the serological classes of O antigens (91, 186, 256).

The phospholipid compositions of the two membranes are relatively similar (259, 260). Per-

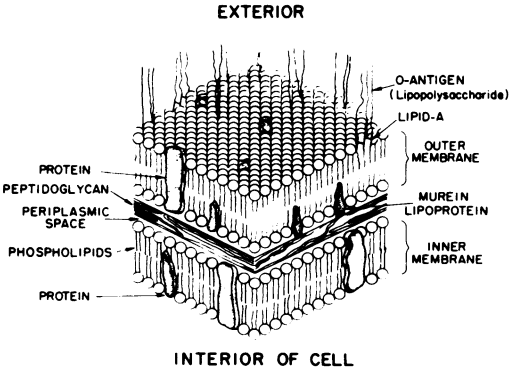


FIG. 1. *Cross section of the E. coli envelope as seen in the projection of Singer and Nicolson (336). The structure of the Salmonella typhimurium envelope is very similar (338).*

haps 60% of the total phospholipid of *E. coli* is recovered in the outer membrane, and the rest is in the inner membrane (193, 259, 260, 377). The phosphoglycerides are not generally found in the cytoplasm or in the growth medium (259). Phosphatidylethanolamine is the predominant species, whereas the remainder is composed of phosphatidylglycerol and cardiolipin (80, 111, 124, 298, 329). In general, *E. coli* and most related gram-negative bacteria do not contain phosphatidylcholine, phosphatidylinositol, sphingolipids, glycolipids, plasmalogens, or sterols, which are characteristic of eucaryotic systems (80, 111, 124, 287, 298, 329).

All components of the gram-negative envelope are synthesized on the inner membrane or in the cytoplasm (258, 259). They are subsequently translocated across the inner membrane and the peptidoglycan and then are assembled into the outer envelope structures (258, 260). The biochemistry and topology of these processes are not understood. Information is just beginning to accumulate regarding possible asymmetry in the distribution of membrane phospholipids, lipopolysaccharide, and proteins in *E. coli* (128, 160, 219, 293, 338, 362, 379).

The biogenesis of phospholipids is one of several inner membrane functions that must play an essential role in membrane growth. Enzymatic and genetic studies of this system should give some insight into the mechanisms of membrane assembly and into the factors that regulate the coordinated synthesis of the various membrane components.

This review will concentrate on the final steps of membrane phospholipid synthesis in *E. coli*, the organism used most extensively for studies of procaryotic lipid metabolism (75, 80, 111, 124, 298, 329). The conclusions drawn from this system are certainly valid for other gram-negative bacteria and probably also for most gram positives (104, 264). In the past 5 years, significant progress has been made in developing new methods for the purification to homogeneity of membrane enzymes (136). Genetic techniques for altering the fatty acid (75, 329) and polar headgroup (133, 274, 275) compositions in vivo are also available. These developments make it possible to consider certain models for the regulation of phospholipid biosynthesis and to determine which phosphoglycerides are most essential in membrane function.

MEMBRANE PHOSPHOLIPIDS OF *E. COLI*: AN OVERVIEW

Extraction and Composition

Phospholipids and membrane proteins each constitute about 3 to 9% of the dry weight of gram-negative bacteria (6, 87, 259, 260). The variability in estimates of *E. coli* lipid content (6, 87) probably reflects differences in extraction techniques and strains. When one strain, such as *E. coli* K-12, is grown on several different carbon sources and the extraction conditions are controlled, the lipid content does not vary appreciably (87).

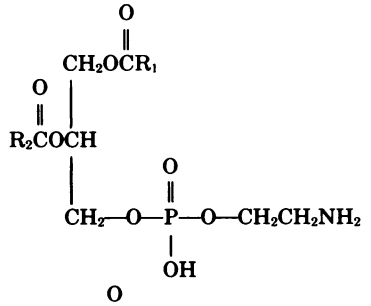
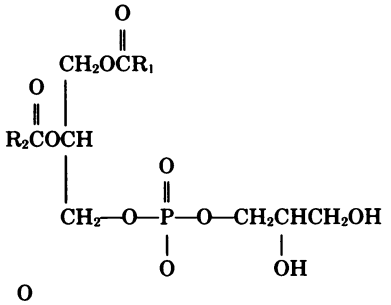
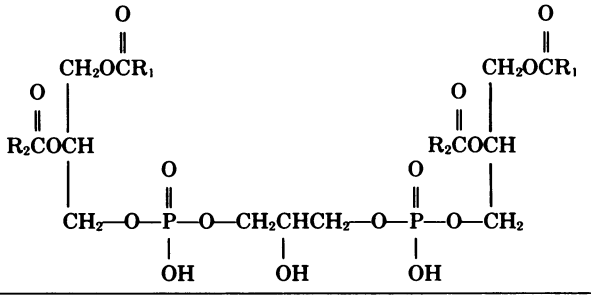
As reviewed in detail by Finnerty and Makula (111), the phospholipids are extracted with mixtures of chloroform and methanol (111). The Ames modification of the Bligh-Dyer procedure is frequently used (6). A few hydrophobic proteins are also recovered in such extracts (110). However, chloroform and methanol do not remove the wild-type lipopolysaccharide from the cell residue. Phenol-containing solvents are required for this purpose (36).

Microgram quantities of phospholipids do not partition reproducibly between phases of chloroform and aqueous methanol (51). They are recovered preferentially at the interface (C. R. H. Raetz, unpublished data), especially if the chloroform phase of the Bligh-Dyer extraction is washed with water or 2 M KCl (51, 161). This problem is avoided by including 1 to 2 mg of carrier phospholipid per ml of chloroform (51), which presumably saturates the interface, driving the bulk of the phospholipid into the chloroform. Omission of carrier in small-scale radiochemical lipid extractions can result in the loss of 70% (or more) of the phospholipid (Raetz, unpublished data).

Table 1 shows the structures of the major membrane phospholipids of *E. coli*. The fatty acid moieties are designated R_1 and R_2. Their structures and compositions are given in Table 2. As a rule, the 1 position of the *sn*-glycero-3-phosphate (*sn*-glycero-3-P) backbone is esterified with a saturated fatty acid, whereas the 2 position is unsaturated (80, 111, 124, 298, 329). Obviously, each of the major membrane phospholipids of Table 1 consists of several molecular species, differing in fatty acid distribution. Species bearing palmitic acid in the 1 position and palmitoleic or *cis*-vaccenic acid in the 2 position generally predominate (3, 198, 328).

The polar headgroup composition of wild-type strains is shown in Table 1. It is not altered dramatically by changes in growth conditions (80, 111, 287). The ratio of phosphatidylethanolamine to phosphatidylglycerol plus cardiolipin appears constant. In stationary cells, the level of cardiolipin tends to rise at the expense of phosphatidylglycerol (80, 111, 287). This occurs because the formation of cardiolipin from phosphatidylglycerol does not require metabolic energy, in contrast to the synthesis of phosphatidylglycerol itself (144, 285). Major changes in

TABLE 1. *Major membrane phospholipids of E. coli*

Name	Structure[a]	Fraction of total (%)[b]
Phosphatidylethanolamine		75–85
Phosphatidylglycerol		10–20
Cardiolipin		5–15

[a] Fatty acid compositions are shown in Table 2.
[b] See references (6, 51, 80, 133, 246, 269a, 275, 283, 287, 321).

polar headgroup composition are otherwise observed only in mutants defective in phospholipid biosynthesis or in the presence of certain chemicals (13, 133, 148, 232, 241, 274, 275, 385).

The fatty acid composition shown in Table 2 is typical of cells growing at 37°C. As a rule, the C_{16} species predominate (75, 329). At lower temperatures, the unsaturated species are present in larger amounts, whereas the converse is true at higher temperatures (75, 204, 329). The cyclopropane derivatives (Table 2) of palmitoleic and *cis*-vaccenic acids are more abundant in stationary (and slowly growing) cells (75, 329). *E. coli* does not normally contain polyunsaturated fatty acids or species in which the double bond has the *trans* configuration (75, 329).

Although slight differences may exist (171, 173), the fatty acid compositions of isolated phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin are relatively similar (171). This contrasts with the fatty acid composition of lipopolysaccharide, which is strikingly enriched in lauric, myristic, and β-hydroxymyristic acids (186). Only one other structure in the membrane is known to contain esterified fatty acids. This is the unusual amino terminus of the murein lipoprotein (Fig. 2) of the outer membrane, first described by Braun (41, 130). The fatty acids esterified to the glycerol moiety of the lipoprotein (Fig. 2) are not very different from those of the total phosphoglyceride fraction (130), whereas the fatty acid linked to the terminal amino group is predominantly palmitate (130).

Formation of Common Precursors

Fatty acids. A soluble system of enzymes catalyzes the synthesis of fatty acids in *E. coli*

TABLE 2. *Fatty acids of E. coli*[a]

Name (length)	Structure	Fraction of total (%)
Major species		
Palmitic (16:0)	$CH_3(CH_2)_{14}COOH$	25–40
Palmitoleic (16:1)	$CH_3(CH_2)_5C{=}C(CH_2)_7COOH$ (H, H)	25–40
cis-Vaccenic (18:1)	$CH_3(CH_2)_5C{=}C(CH_2)_9COOH$ (H, H)	25–35
Minor species		
Lauric (12:0)	$CH_3(CH_2)_{10}COOH$	0–1
Myristic (14:0)	$CH_3(CH_2)_{12}COOH$	1–5
cis-9,10-Methylene-hexadecanoic (17:0)	$CH_3(CH_2)_5C(CH_2)C(H)H(CH_2)_7COOH$	1–20[b]
Stearic (18:0)	$CH_3(CH_2)_{16}COOH$	0–1
Lactobacillic (19:0)	$CH_3(CH_2)_5C(CH_2)C(H)H(CH_2)_9COOH$	1–20[b]
Unique to lipopolysaccharide		
3-D-Hydroxymyristic (14:0)	$CH_3(CH_2)_{10}CH(OH)CH_2COOH$	—[c]

[a] Typical composition of *E. coli* K-12 strains under ordinary conditions at 30 to 37°C (70, 75, 80, 81, 277, 332).

[b] Cyclopropane fatty acids accumulate in stationary phase at the expense of palmitoleic and *cis*-vaccenic acids.

[c] —, Not detected in the phospholipid fraction.

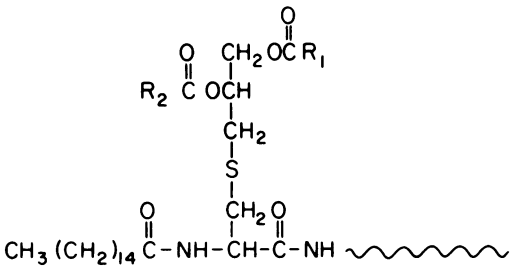


FIG. 2. *Covalent structure of the amino terminus of the outer membrane lipoprotein. The amino terminus is embedded in the outer membrane, whereas the carboxy terminus is covalently linked to the peptidoglycan (41).*

(34, 368). These reactions have been studied extensively by Vagelos and co-workers and are reviewed elsewhere in detail (34, 368). The fatty acid-synthesizing enzymes of *E. coli* do not form a complex, as is characteristic of eucaryotic systems (34, 368). A specific β,γ-hydroxydecanoyl acyl carrier protein dehydrase (Fig. 3) catalyzes the formation of the double bond at the C_{10} stage (34, 80, 368). Oxygen is not required, and *E. coli* cannot synthesize polyunsaturated fatty acids. The dehydrase is situated at a metabolic branch point (Fig. 3) leading either to the fully saturated palmitic acid or to the singly unsaturated palmitoleic and *cis*-vaccenic acids. Certain mutants exist which are defective in the elongation of palmitoleate to *cis*-vaccenate (15, 118).

The compound 3-decynoyl-*N*-acetyl cysteamine (163) specifically inhibits the dehydrase, preventing cell growth. This can be overcome by supplying exogenous unsaturated fatty acids. The dehydrase is also defective in mutants designated *fabA*, which consequently require unsaturated fatty acids for survival (73, 78). The antibiotic cerulenin blocks the elongation of all fatty acids by inhibiting β-ketoacyl-acyl carrier protein synthetase (86, 122).

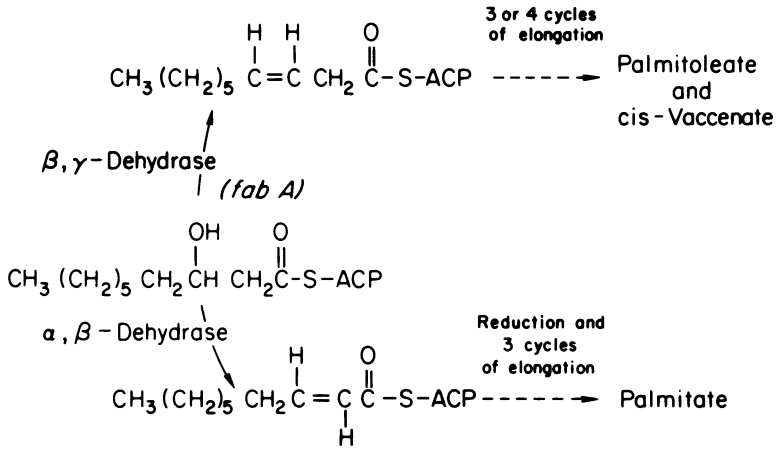


FIG. 3. *Branch point in fatty acid synthesis leading to either fully saturated or singly unsaturated species. The β,γ-hydroxydecanoyl dehydrase is inhibited by 3-decynoyl-N-acetyl cysteamine (163) and is defective in strains bearing the fabA mutation (73, 78).*

During stationary phase (and in certain slowly growing organisms), palmitoleate and *cis*-vaccenate are converted to their cyclopropane derivatives (Table 2). A membrane-bound enzyme utilizes *S*-adenosylmethionine to generate the cyclopropane ring (67, 183, 184). Mutants lacking this enzyme cannot make cyclopropane fatty acids in vivo but nevertheless appear to grow normally (349).

Fatty acids are elongated in vivo as thioester derivatives of acyl carrier protein (34, 368). Exogenously supplied fatty acids can be activated to form thioesters either with coenzyme A or with acyl carrier protein (262, 289, 290, 332). Two separate activating enzymes appear to be involved (289). In vitro, the fatty acyl derivatives of both coenzyme A and acyl carrier protein are substrates for the formation of phosphatidic acid, although their relative importance in vivo remains unknown (75, 329). Mutants defective in these enzymes and in acyl carrier protein itself would be valuable in resolving this question.

Various modifications of fatty acid composition are possible, since fatty acids, unlike intact phospholipids, are readily taken up from the growth medium by wild-type strains. The work of Silbert, Vagelos, Overath, and others (262, 329, 330, 332) has led to the development of mutants unable to synthesize (*fab*) and/or break down (*fad*) fatty acids. Double mutants (*fab fad*) are especially useful, since their fatty acid composition is determined largely by what is presented to the cell from the medium (330). In this way, extensive incorporation of abnormal fatty acids, such as polyunsaturated species, is possible, resulting in major perturbations of membrane fluidity (75, 329, 330, 332).

sn-Glycero-3-phosphate. Besides fatty acids, the other major precursor of all membrane phospholipids is *sn*-glycero-3-P. When cells are grown on glucose, this substance is normally formed from dihydroxyacetone phosphate by a specific dehydrogenase (176, 187) which utilizes either reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate with comparable efficiency (J. R. Edgar and R. M. Bell, manuscript in preparation) (Fig. 4). Mutants lacking this enzyme (designated *gpsA*) require exogenous *sn*-glycero-3-P for growth and are unable to generate any new membrane phospholipids in the absence of added *sn*-glycero-3-P (19, 71). As discussed more fully below (Genetic Modification of Membrane Lipid Synthesis), the *gpsA* mutants therefore provide a simple system for inhibiting total lipid synthesis and for studying the consequences on cell physiology.

sn-Glycero-3P can also be synthesized from glycerol and ATP (Fig. 4). However, glycerol kinase is not usually an effective route for the formation of *sn*-glycero-3-P when cells are grown on glucose, since the enzyme is inhibited by fructose-1,6-diphosphate (187, 391, 392). Glycerol kinase mutants can be isolated which are insensitive to this inhibition and are therefore able to generate *sn*-glycero-3-P from glycerol in the presence of glucose (19, 187, 391, 392). The *sn*-glycerol-3-P and acetate pools appear to be very small in *E. coli*, since there is no detectable lag in the radiochemical labeling of phospholipids by exogenous glycerol and acetate (185).

Other precursors. Two other compounds are indispensable for the generation of membrane phospholipids. These are L-serine and cytidine 5'-triphosphate (CTP). As shown below, CTP is required for the synthesis of the common

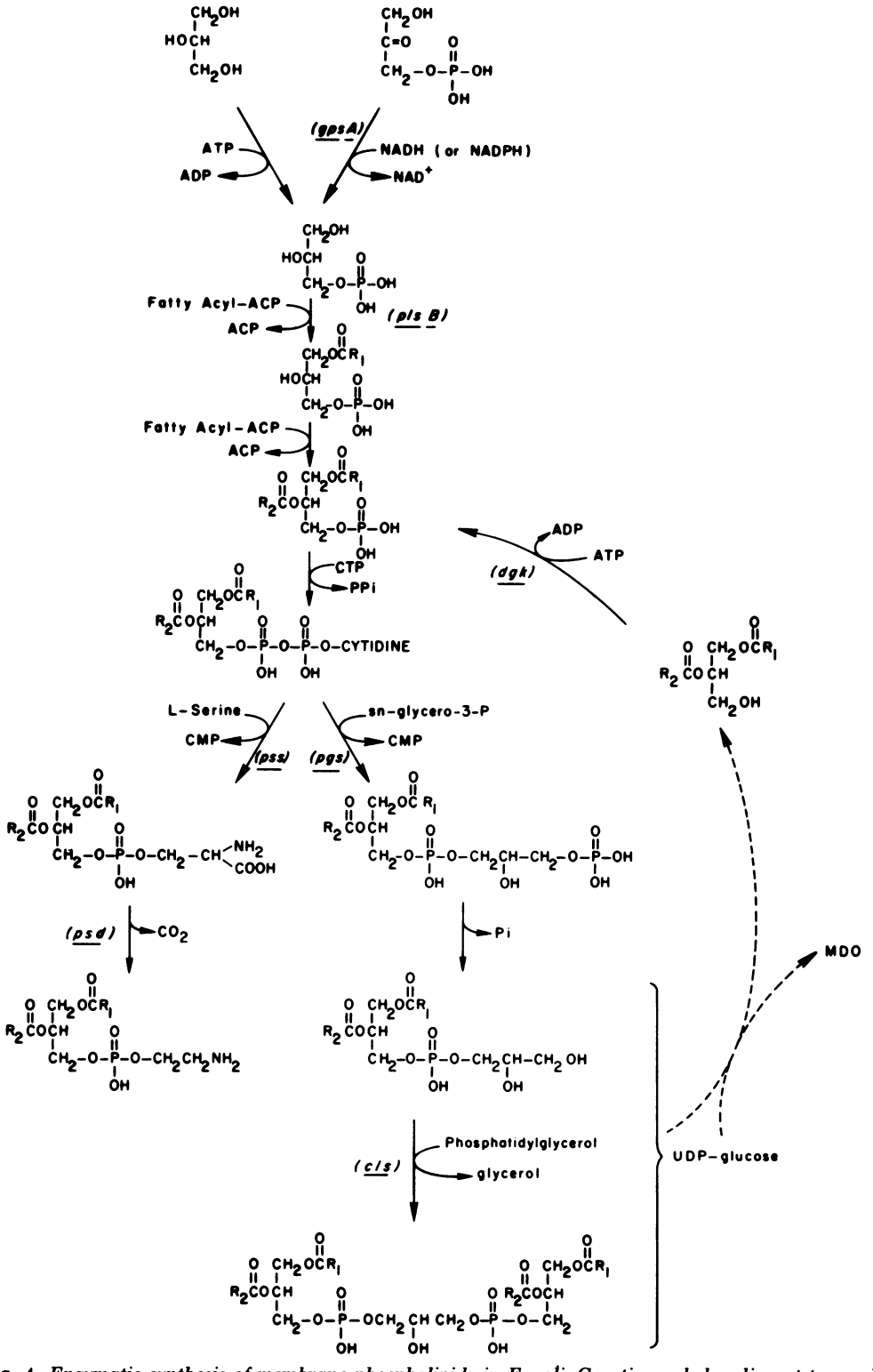


FIG. 4. *Enzymatic synthesis of membrane phospholipids in E. coli. Genetic symbols adjacent to specific enzymatic reactions indicate the existence of mutants. Reactions inferred solely on the basis of genetic studies, i.e., those leading to the MDO, are designated with dashed arrows.*

precursor cytidine 5'-diphosphate (CDP)-diglyceride (162, 280), whereas serine is needed for the generation of the polar headgroup of phosphatidylethanolamine (162, 280). The relationship of the in vivo levels of L-serine and CTP to the rate of phospholipid synthesis has not been studied adequately. For instance, it is not clear whether phosphatidylethanolamine or protein synthesis is the more sensitive to changes in the concentration of available L-serine. Questions like this could be resolved by growing serine-requiring mutants in serine-limited chemostat cultures. Similar studies could be carried out with cytidine auxotrophs (228) and might shed some light on regulation.

Phospholipid Biosynthesis

Before examining the details of each enzymatic reaction, it is useful to consider certain general features of the biosynthesis of phospholipids in *E. coli*. Figure 4 shows the major reactions and the structures of the metabolic intermediates leading to the formation of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. Reactions inferred from genetic studies, i.e., those responsible for the formation of the membrane-derived oligosaccharides (MDO), are indicated with dashed arrows.

Three independent experimental approaches have served to verify the scheme of Fig. 4. These are: (i) the classical demonstration primarily by Kennedy and co-workers of enzymes in crude cell extracts capable of catalyzing these reactions (47, 53, 144, 162, 164, 267); (ii) radioactive labeling studies demonstrating the existence and rapid turnover in vivo of intermediates, such as phosphatidic acid, CDP-diglyceride, and phosphatidylserine (54, 279, 280); and (iii) the isolation and lipid composition of mutants lacking certain of the enzymes of Fig. 4 (19, 133, 246, 269a, 274, 275, 283).

Beginning with the acylation of *sn*-glycero-3-P, all of the reactions of phospholipid synthesis (Fig. 4) occur on the cytoplasmic membrane (22, 376). This conclusion is based on the observation that key intermediates, such as phosphatidic acid, CDP-diglyceride, and phosphatidylserine, are membrane bound (172, 279, 359) and that the biosynthetic enzymes are recovered in the inner membrane fraction in cell extracts (22, 170, 376). The only exception is phosphatidylserine synthetase, which is tightly bound to ribosomes in extracts of most gram-negative bacteria (104, 152, 279), although it presumably acts on a membrane-bound substrate.

In the initial step (Fig. 4) *sn*-glycero-3-P is acylated in the 1 position with a saturated fatty acid (252, 292, 364). Very little lysophosphatidic acid accumulates in wild-type organisms, since this is rapidly acylated with an unsaturated fatty acid to form phosphatidic acid (252). Like the other intermediates of the pathway, phosphatidic acid turns over very rapidly and constitutes no more than 1% of the total membrane lipid (54, 280). The *sn*-glycero-3-P acylation step is a likely site for the regulation of fatty acid composition (334).

Phosphatidic acid is next converted to a liponucleotide derivative (47) by reacting with CTP (or deoxy-CTP [dCTP]). Both the ribo- and the deoxyriboliponucleotides are present in vivo and turn over with half-lives on the order of seconds (280, 359). There is some evidence that the formation of CDP-diglyceride (and dCDP-diglyceride) is rate limiting for the overall pathway (280). However, the specific function of the two liponucleotides of *E. coli* is unknown.

CDP-diglyceride is located at a biosynthetic branch point (Fig. 4). It can donate its phosphatidyl moiety either to the hydroxyl group of L-serine or to the hydroxyl group at the 1 position of *sn*-glycero-3-P (Fig. 4) (53, 162). Although both phosphatidyltransferase reactions are reversible, equilibrium lies far in the direction of the phosphodiester products (53, 142, 162, 181, 281). Presumably, the CDP-diglyceride branch point is the site at which the ratio of phosphatidylethanolamine to polyglycerophosphatides is regulated. Like the other intermediates of the pathway, phosphatidylserine and phosphatidylglycerophosphate do not accumulate under ordinary circumstances. The former is rapidly decarboxylated to form phosphatidylethanolamine (103, 162), whereas the latter is dephosphorylated, yielding phosphatidylglycerol (55). These reactions prevent reversals of phosphatidylserine and phosphatidylglycerophosphate syntheses in vivo.

Phosphatidylethanolamine is a stable end product under most circumstances (10, 11, 42, 161, 242, 321). In contrast, phosphatidylglycerol is further converted to cardiolipin in a reaction requiring no CDP-diglyceride and, hence, no metabolic energy (144). Approximately one-third of the total cellular phosphatidylglycerol pool turns over in one generation (242, 321). Cardiolipin turns over at about the same rate (321).

The turnover of phosphatidylglycerol and cardiolipin was recognized by Kanfer and Kennedy in 1963 (161). The biochemistry of this process is quite complex and is only now being elucidated. In 1973, Van Golde et al. (367) discovered a family of related, glucose-containing oligosaccharides to which *sn*-glycero-1-P, succinic acid,

and phosphorylethanolamine are covalently bound (165, 320, 367). This material (termed MDO) constitutes 0.5 to 1% of the dry weight of *E. coli* and is localized in the periplasmic space (165, 320, 321, 367). The time course of radiochemical labeling as well as the unusual stereochemistry suggest that the *sn*-glycero-1-P moiety is derived from the polar headgroups of the polyglycerophosphatides, most likely from phosphatidylglycerol (367). The formation of the MDO may account for at least 75% of the polyglycerophosphatide turnover that occurs in wild-type cells, since mutants unable to synthesize MDO show a dramatic reduction in the rate of polyglycerophosphatide turnover (320, 321).

An examination of the covalent structure of phosphatidylglycerol (Fig. 4) reveals that the transfer of the *sn*-glycero-1-P moiety to MDO (165) would generate *sn*-1,2-diglyceride as a by-product. In wild-type strains, this substance is converted back to phosphatidic acid by a membrane-bound diglyceride kinase (Fig. 4) (267, 283, 316). Although this "diglyceride cycle" (283) is only a minor route for phosphatidic acid synthesis, mutants lacking the kinase accumulate substantial amounts of diglyceride in vivo (283).

ENZYMOLOGY OF PHOSPHOLIPID SYNTHESIS

In the past few years, considerable progress has been made with the purification of the enzymes of phospholipid synthesis, although much remains to be done (Table 3). Phosphatidylserine synthetase (181), phosphatidylserine decarboxylase (103), and phosphatidylglycerophosphate synthetase (142) can be obtained in homogeneous form, whereas *sn*-glycero-3-P acyltransferase (339), CDP-diglyceride synthetase (K. Langley and E. P. Kennedy, personal communication), phosphatidylglycerophosphate phosphatase (55), and diglyceride kinase (316, 318) (see Fig. 4) have been partially purified (Table 3). The recent development of these purification schemes can be attributed to advances in the methodology for fractionating catalytically active membrane enzymes (136). Affinity chromatography has proven especially valuable in several instances (142, 182). Studies of the chemistry, regulation, and membrane insertion of the phospholipid enzymes should be possible in the coming years.

Synthesis of Phosphatidic Acid and Cytidine 5'-Diphosphate Diglyceride

Phosphatidic acid. *sn*-Glycero-3-P is acylated initially to form 1-acyl-*sn*-glycero-3-P (251, 339, 364) (Fig. 4). A separate enzyme subsequently acylates the 2 position, giving rise to phosphatidic acid (252, 253). Both activities are membrane bound in crude cell extracts (22, 252, 292, 364, 376). Acylation of the 2 position before the 1 position apparently does not occur to any significant extent (252), although the tendency of monoacyl-*sn*-glycero-3-P to undergo intramolecular fatty acyl migrations requires careful attention (205).

The first acyltransferase preferentially utilizes palmitoyl coenzyme A, but it also incorporates unsaturated fatty acids in the 1 position if presented with enough substrate (252, 253, 364). The second acyltransferase, which is a separate enzyme (20), converts 1-acyl-*sn*-glycero-3-P to phosphatidic acid and has the converse specificity, since unsaturated fatty acyl coenzyme A is the better substrate (252, 253). Obviously, this pattern of fatty acid specificity observed in vitro partly accounts for the fatty acid distribution observed in the membrane phospholipids (see above), and the acyltransferases are probably also involved in the temperature-dependent regulation of fatty acid composition (334). Extracts of *E. coli* do not catalyze the rapid conversion of 2-acyl-*sn*-glycero-3-P to phosphatidic acid (252), nor is dihydroxyacetone phosphate a substrate for the first acyltransferase (364).

Because phospholipid enzymes are generally assayed in the presence of detergent under nonphysiological conditions, it is very difficult to correlate in vitro activity with in vivo function. When the kinetic properties of the *sn*-glycero-3-P acyltransferase are quantitated, the fatty acid specificity of the acyltransferase is also affected by the concentration of *sn*-glycero-3-P, the temperature, and the ratio of saturated to unsaturated fatty acids presented to the enzyme (175, 252, 253, 334). The K_m for *sn*-glycero-3-P is on the order of 50 to 500 μM (19, 175, 252, 339, 364). The true fatty acyl coenzyme A concentration is difficult to determine, since this material is a detergent, forms micelles, and tends to be inhibitory at high concentrations (175). For this reason, it may be advantageous to reexamine the specificities of both acyltransferase in vitro, using the fatty acyl acyl carrier protein as the substrate (288).

Earlier studies with a mutant (*plsA*) thought to be temperature sensitive in the acylation of *sn*-glycero-3-P (77) led to the conclusion that a single enzyme is capable of acylating either the 1 or the 2 position of *sn*-glycero-3-P (292). Depending on the position being acylated, the enzyme was thought to utilize a saturated or an unsaturated fatty acid preferentially (292). This model seems quite improbable in view of the specificity observed with the wild-type enzyme (252, 253, 339) and is unlikely a priori on ster-

TABLE 3. *Purification and properties of E. coli enzymes involved in biosynthesis of membrane phospholipids*

Enzyme	Best available purification[a]	Subunit mol wt	Known activators or cofactors	References
sn-Glycero-3-P acyltransferase	30-fold	Not known	Mg^{2+}, mercaptoethanol, phospholipids	19, 339, 364
Monoacyl-*sn*-glycero-3-P acyltransferase	None	Not known	Mg^{2+}	20, 252, 364
CDP-diglyceride synthetase	100-fold	Not known	Triton X-100[b], Mg^{2+}, K^+	47; E. P. Kennedy, personal communication; C. R. H. Raetz, unpublished data
Phosphatidylserine synthetase	5,000-fold[c]	54,000[d]	Triton X-100, high ionic strength	181, 281
Phosphatidylserine decarboxylase	3,600-fold[c]	36,000[d]	Triton X-100, covalently bound pyruvate	103, 311
Phosphatidylglycerophosphate synthetase	6,000-fold[c]	24,000[d]	Triton X-100, Mg^{2+}	53, 142
Phosphatidylglycerophosphate phosphatase	10-fold	Not known	Triton X-100, Mg^{2+}	55
Cardiolipin synthetase	None	Not known	Triton X-100, Mg^{2+}	144, 343
Diglyceride kinase	600-fold	Not known	Triton X-100, Mg^{2+}, cardiolipin and other phospholipids	316, 318

[a] Relative to crude cell extract.
[b] Triton X-100 is octylphenoxy polyethoxyethanol.
[c] Available in near-homogeneous form.
[d] Native molecular weights are not known.

eochemical grounds. Recently, Snider and Kennedy have partially purified the *sn*-glycero-3-P acyltransferase (339) and have shown that it synthesizes only 1-acyl-*sn*-glycero-3-P (339), further supporting the reaction sequence shown in Fig. 4 (252, 253). However, more detailed studies of acyltransferase specificity under various conditions that rigorously exclude acyl group migration (205) would be desirable.

It is unfortunate that neither of the acyltransferases is available in a homogeneous form. Since they represent the initial steps of phospholipid synthesis, they are probable sites of metabolic control. The first acyltransferase (Fig. 4) has been purified about 30-fold (339), but no reports on the purification of the second acyltransferase have appeared. Recent advances in gene cloning techniques (62, 282) permit the construction of strains that overproduce the *sn*-glycero-3-P acyltransferase by about 10- to 15-fold (R. M. Bell, personal communication; M. D. Snider and E. P. Kennedy, personal communication), which should facilitate purification.

The acyltransferases, unlike the other enzymes of Fig. 4, are not activated in the presence of nonionic detergents, such as Triton X-100 (53, 162). The *sn*-glycero-3-P acyltransferase of *E. coli* is somewhat unusual in that it appears to be inactivated totally upon extraction from the membrane with detergents (206, 207, 339). However, activity can be reconstituted if the detergent concentration is lowered by dilution in the presence of *E. coli* phospholipids (339). Sucrose gradient analysis of the reconstituted system indicates that a large amount of lipid is bound to the enzyme (339). The *sn*-glycero-3-P acyltransferase preparations described by Kito and co-workers appear to be most active in the presence of phosphatidylglycerol (153, 154).

Inhibitors of the first acyltransferase include ATP (175), *cis*-9,10-methylenehexadecanoic acid (169), phenethyl alcohol (233), and guanosine tetraphosphate (209). Perturbation of the fatty acid composition (207) or treatment of membranes with phospholipase C also causes major changes in *sn*-glycero-3-P acyltransferase activity (206). Some of these effects are not observed when fatty acyl acyl carrier protein is used as the substrate (192). The physiological significance of these phenomena is not known.

1-Acyl-*sn*-glycero-3-phosphorylethanolamine can be acylated to form phosphatidylethanola-

mine in crude extracts (272). The subcellular localization and cofactor requirements of this activity suggest that it is distinct from the second acyltransferase involved in phosphatidic acid synthesis (369).

Cytidine 5'-diphosphate diglyceride. Phosphatidic acid reacts with CTP (or dCTP) to form the corresponding liponucleotide (Fig. 4). Cytidine triphosphate:phosphatidic acid cytidylyltransferase (CDP-diglyceride synthetase), originally discovered in animal tissues (48), is present in all bacteria examined so far (22, 47, 197, 264, 376). In *E. coli*, it is associated with the cytoplasmic membrane (22, 376). Radioactive labeling studies show that there is a 10-fold excess of phosphatidic acid over the liponucleotides in vivo, and both are metabolized rapidly (280). About 10^4 liponucleotide molecules are present in each cell.

Extraction of the CDP-diglyceride synthetase from the membrane is achieved with digitonin, and further partial purification is performed on diethylaminoethyl-cellulose in the presence of this detergent (Langley and Kennedy, personal communication). The extracted enzyme does not require the addition of lipids for optimal activity. In contrast to the subsequent enzymes in the pathway (Fig. 4), CDP-diglyceride synthetase specifically requires phosphatidic acid esterified with unsaturated fatty acids and does not utilize dipalmitoyl phosphatidic acid (47). This may reflect a requirement for a fluid physical state under the in vitro assay conditions that are generally used (47, 48). Reversal of the reaction can be demonstrated by incubating CDP-diglyceride with inorganic pyrophosphate (47, 48). Under appropriate conditions, isolated membrane preparations can be made to generate CDP-diglyceride in situ (172). The enzyme requires divalent cations and potassium ions for maximal activity (47).

Although not purified to homogeneity, the results of a partial purification strongly suggest that the same enzyme synthesizes CDP-diglyceride and dCDP-diglyceride at comparable rates (Langley and Kennedy, personal communication). Other nucleotides (ATP, uridine 5'-triphosphate [UTP], and guanosine 5'-triphosphate) are not substrates (47). The chloroform-soluble nucleotides of *E. coli* contain no bases other than cytosine (280, 359). The mechanism of CDP-diglyceride synthesis shown in Fig. 4 is further supported by radioactive labeling of the liponucleotides in vivo with *sn*-glycero-3-[^{32}P]-phosphate and [5-^{3}H]cytosine (280).

About 60 to 80% of the liponucleotide fraction of *E. coli* is CDP-diglyceride, whereas the rest is the deoxy derivative (280, 359). Yeast cells contain no dCDP-diglyceride (317), and the yeast liponucleotide synthetase does not react with dCTP in vitro (G. Getz, personal communication). The synthesis of dCDP-diglyceride has been detected in the nuclear fraction of HeLa cells (386), but the significance of the two forms of liponucleotide has not been determined in any system. Both compounds are substrates for the phosphatidyltransferases of *E. coli* (142, 181, 280).

A specific hydrolase (see below) which converts CDP-diglyceride to phosphatidic acid and cytidine 5'-monophosphate (CMP) (276, 278) is present in the membrane, but this enzyme has no affinity for dCDP-diglyceride (276). Since the hydrolase can interfere with the assay of the CDP-diglyceride synthetase, it is advisable to use dCTP as the substrate in crude extracts. The regulation of the CDP-diglyceride synthetase has not been investigated in any procaryotic system.

Phosphatidylserine and Phosphatidylethanolamine

Phosphatidylserine synthetase. CDP-diglyceride:L-serine *O*-phosphatidyltransferase (phosphatidylserine synthetase) catalyzes the de novo synthesis of phosphatidylserine from CDP-diglyceride and L-serine (162). High levels of phosphatidylserine synthetase are found in extracts of all gram-negative bacteria, whereas lower levels are characteristic of gram-positive extracts (104, 162, 264). Extensive biochemical (162, 181, 281) and genetic (246, 248, 275) studies have shown that phosphatidylserine synthetase is the major source of phosphatidylserine (and hence phosphatidylethanolamine) in gram negatives. Eucaryotic systems, which generally lack phosphatidylserine synthetase, make phosphatidylserine by a headgroup exchange reaction involving L-serine and phosphatidylethanolamine (38).

Phosphatidylserine synthetase was discovered by Kanfer and Kennedy (162), who noted that the enzyme was not bound to the membrane. Detailed analyses of the subcellular localization revealed that much of the enzyme is tightly associated with ribosomes in crude extracts (279), whereas only 10 to 30% is cytoplasmic (152, 279). A report by Machtiger and Fox (199) failed to substantiate the ribosomal localization of the synthetase, but their recovery of ribosomal proteins was two orders of magnitude lower than that expected for *E. coli* (177, 199). The ribosomal association also explains the low specific activity of the synthetase in purified membrane fragments when compared with other lipid enzymes (22, 376). That portion of the

phosphatidylserine synthetase activity recovered with membranes can be accounted for by ribosomal contamination (104).

The physiological significance of the ribosomal association of the synthetase is uncertain (279). Synthetase activity is recovered on both ribosomal subunits (279), and the enzyme is not a major ribosomal protein (181). Studies with superproducers (282) (see below) demonstrate that ribosomes have the capacity to bind a large amount of additional synthetase (282). The enzyme can be separated from the ribonucleic acid (RNA) in the presence of 5 M NaCl and polyethylene glycol (281) without a substantial loss or increase of activity (181, 281). It can also be resolved from ribosomes by 1 M KCl or 1 M NH_4Cl in the presence of 10 to 20% sucrose (W. Dowhan, personal communication) or by isopycnic centrifugation in CsCl (281; see Addendum in Proof).

More significant than the ribosomal association is the virtual absence of phosphatidylserine synthetase in the membrane fraction (22, 376). Apparently, the synthetase is not as insoluble as the others shown in Fig. 4, and it may be a peripheral membrane protein (181). The enzyme has been purified 5,000-fold, to homogeneity (181). Two critical steps in this procedure are the extraction of the enzyme from the ribosomes by aqueous polymer partitioning in the presence of 5 M NaCl (181, 281) and subsequent adsorption and substrate-specific elution with CDP-diglyceride from phosphocellulose (181). There are approximately 800 phosphatidylserine synthetase subunits per cell, and the apparent molecular weight of a subunit is 54,000, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (181). This contrasts with 150,000 ribosomal subunits (177, 181). The molecular weight of the native enzyme is not known. Recent advances in gene cloning techniques permit the overproduction of the synthetase in *E. coli*, greatly simplifying purification (282). Mutants defective in phosphatidylserine synthetase lack not only the ribosomal enzyme (275), but also the small amounts of activity recovered with the membranes or in the cytoplasm (248, 275).

The homogeneous phosphatidylserine synthetase not only transfers the phosphatidyl unit from CDP-diglyceride to L-serine, but also exchanges free CMP with the CMP moiety of CDP-diglyceride and L-serine with phosphatidylserine (181, 281). The former occurs in the absence of serine, and the latter occurs in the absence of CMP (181, 281). Reversal of phosphatidylserine synthesis can be demonstrated by incubating phosphatidylserine and CMP with the enzyme, but the equilibrium favors the phosphodiester product (181, 281). The enzyme also hydrolyzes CDP-diglyceride or phosphatidylserine at a slow rate to form phosphatidic acid (181, 281). All the partial reactions of phosphatidylserine synthetase can be demonstrated with isotopic techniques, and they are consistent with a phosphatidyl-enzyme intermediate (Fig. 5).

It is conceivable that the phosphatidyl moiety is covalently bound to the enzyme during catalysis (Fig. 5). A seryl residue of the enzyme or a CMP cofactor is a potential site for such an attachment. A covalent phosphatidyl-enzyme intermediate would also provide a mechanism for transient association of the enzyme with the membrane (181). As yet, however, there is no direct chemical evidence for such an intermediate. The partial reactions catalyzed by the synthetase (Fig. 5) resemble base exchange reactions, which are found primarily in microsomal membranes of eucaryotic cells (31, 32, 284).

CDP-diglyceride and dCDP-diglyceride are the best substrates for phosphatidylserine synthetase, although some activity is detected with UDP-diglyceride and ADP-diglyceride (181, 281). The specificity of the CMP exchange reaction is similar (181, 281). Phosphonic acid analogs of CDP-diglyceride are also effective substrates (361). The enzyme does not require added divalent metal ions (162, 181, 281).

Triton X-100 dramatically enhances the rate of the reaction, although concentrations greater than 0.2% are somewhat inhibitory (162, 181, 281). Liponucleotides containing unsaturated fatty acids are nearly identical to saturated liponucleotides, both with respect to their reaction rate and with respect to Triton X-100 stimulation (181). Using the approach of Dennis and co-workers (92, 95), Larson and Dowhan have proposed that the enzyme acts on mixed micelles of Triton X-100 and CDP-diglyceride (181). A convenient spectrophotometric assay has been developed for the pure enzyme, which depends on the release of CMP (46). As with the other enzyme of Fig. 4, however, it is difficult to determine whether the detergent stimulation repre-

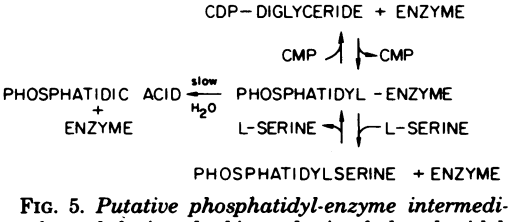


FIG. 5. *Putative phosphatidyl-enzyme intermediate formed during the biosynthesis of phosphatidylserine.*

sents an effect on the structure of the substrate or a direct interaction with the enzyme. A physiological substitute for Triton X-100 has not been found in living cells (see Addendum in Proof).

L-Serine is the best phosphatidyl acceptor which has been identified (162, 181, 281). The K_m for L-serine is 0.1 to 1 mM and can be influenced by the addition of lipids (150, 162, 181, 281), including CDP-diglyceride. D-Serine, threonine, homoserine, serylglycine, ethanolamine, α-methylserine, and serine methylester have little, if any, activity in the millimolar range (162, 181, 281). The pure enzyme catalyzes a slow transfer of the phosphatidyl moiety to water (181, 281) (Fig. 5). Larson and Dowhan have detected an even slower transfer to substances like glycerol and *sn*-glycero-3-P when present in molar quantities (181).

Lampen and co-workers have reported that phosphatidylserine is the covalent amino terminus of certain membrane proteins of *Bacillus licheniformis* (4, 387–389). The membrane penicillinase has been examined in considerable detail (387–389). The mechanism by which this terminal structure is synthesized remains to be established. Perhaps the seryl residue at the amino terminus of the penicillinase is modified post-translationally by reacting with CDP-diglyceride, or perhaps phosphatidylseryl transfer RNA initiates the synthesis of some membrane proteins. The role of phosphatidylserine synthetase (or related enzymes) in the formation of these interesting amino termini deserves further study. However, covalently bound phosphatidylserine has not been identified in the membrane proteins of *E. coli*.

Since phosphatidylserine synthetase is indispensable for the growth of *E. coli* (275), inhibitors of the enzyme might be novel antibiotics. No specific compounds of this kind have been discovered, but certain substances, such as levorphanol, phenethyl alcohol, and ethanol, change the lipid composition of *E. coli* as if the synthetase were inhibited (148, 241, 385).

Phosphatidylserine decarboxylase. Very little phosphatidylserine is present in wild-type strains of *E. coli*, since it is rapidly converted to phosphatidylethanolamine by a membrane-bound decarboxylase (162, 279). Treatment of wild-type organisms with hydroxylamine (279) or growth of phosphatidylserine decarboxylase mutants at nonpermissive temperatures (133) results in the accumulation of large amounts of phosphatidylserine. This shows that the decarboxylase represents the major biosynthetic route to phosphatidylethanolamine in vivo.

The decarboxylase of *E. coli* can be extracted from the inner membrane with Triton X-100 and purified to homogeneity by a combination of ion-exchange chromatography, gel filtration, and density gradient centrifugation (103). As with most membrane enzymes, Triton X-100 must be included in all solutions to prevent aggregation (103). Tyhach and Kennedy have prepared antibodies to the homogeneous enzyme and have developed a rapid purification for the enzyme, using immunoaffinity chromatography (R. J. Tyhach and E. P. Kennedy, personal communication).

The homogeneous decarboxylase (purified 3,600-fold) has a subunit molecular weight of about 36,000, but the native molecular weight has not been determined (103). As in the case of the phosphatidylserine and phosphatidylglycerophosphate synthetases, there are about 10^3 subunits per bacterium (103). The homogeneous decarboxylase contains no carbohydrate, and its amino acid composition is not exceptionally hydrophobic (103). The structure of its membrane attachment site has not been determined.

Inhibition of the decarboxylase by hydroxylamine and related compounds (103, 268, 311) suggests the presence of an essential aldehyde or ketone group at the active site. Satre and Kennedy have recently demonstrated that pyruvate is the bound cofactor, rather than pyridoxal phosphate (311). The existence of a covalently bound pyruvate moiety indicates that posttranslation modification of the decarboxylase must occur. Pyruvate has also been identified as a cofactor of several other decarboxylases, such as *S*-adenosylmethionine decarboxylase and histidine decarboxylase (294, 378).

The substrate specificity of phosphatidylserine decarboxylase has not been examined very thoroughly. The enzyme acts neither on free serine nor on glycerophosphorylserine (103, 162). In the homogeneous state, it has an absolute requirement for Triton X-100 but does not exhibit a divalent metal requirement (103). It does not attack phosphatidylserine in liposomes (in the absence of detergent) or in the intact membranes of erythrocytes (370). In the presence of Triton, the fatty acid composition is not critical (103), but short-chain fatty acids have not been examined. High concentrations of Triton inhibit the enzyme (103), possibly because of surface dilution of the phosphatidylserine in the mixed micelles (370, 371). A direct effect of Triton on the enzyme rather than on the substrate cannot be excluded, since sucrose gradient centrifugation shows that the enzyme binds sufficient detergent to alter its buoyant density (103) relative to soluble, globular proteins of similar size (103).

Phosphatidylglycerol and Cardiolipin

Phosphatidylglycerophosphate synthe-

tase. CDP-diglyceride:*sn*-glycero-3-P phosphatidyltransferase (phosphatidylglycerophosphate synthetase) catalyzes the formation of phosphatidylglycerophosphate from CDP-diglyceride (or dCDP-diglyceride) and *sn*-glycero-3-P (Fig. 4) (53, 142, 162, 280). The reaction is analogous to the enzymatic synthesis of phosphatidylserine and results in the release of CMP (53, 142). Phosphatidylglycerophosphate synthetases occur in all procaryotic and eucaryotic systems examined so far (53, 111, 164, 182, 264, 363).

The phosphatidylglycerophosphate synthetase of *E. coli* has been extracted from membranes and purified to homogeneity (53, 142). The enzyme appears to bind a large amount of detergent (142). It cannot be purified by the general methods used for the phosphatidylserine decarboxylase (142). Affinity chromatography on CDP-diglyceride-modified Sepharose (142, 182) affords the only major purification step. The apparent subunit molecular weight is 24,000, as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (142). Triton X-100 and Mg^{2+} greatly stimulate the enzymatic activity (53, 142).

Alternate substrates for the enzyme include ADP-diglyceride and UDP-diglyceride (280). L-Serine, glycerol, *myo*-inositol, *sn*-glycero-2-P, and *sn*-glycero-1-P cannot substitute for *sn*-glycero-3-P (53, 142). However, 3,4-dihydroxybutyl-1-phosphonic acid, an analog of *sn*-glycero-3-P, is an effective substrate (58). The phosphatidylglycerophosphate analog synthesized in this manner cannot be dephosphorylated by phosphtidylglycerophosphate phosphatase (360). Consequently, if cells are grown in the presence of 3,4-dihydroxybutyl-1-phosphonate, this abnormal lipid accumulates in vivo and growth is inhibited (360). Studies with radioactivity labeled 3,4-dihydroxybutyl-1-phosphonic acid indicate that this material has other metabolic fates besides its reaction with CDP-diglyceride (58, 324–326, 360). Therefore, it has not been used to isolate mutants altered in phosphatidylglycerophosphate synthetase, since the inhibition of cell growth has multiple causes.

The mechanism of phosphatidylglycerophosphate synthetase may differ from that of phosphatidylserine synthetase, since it does not catalyze the exchange of CMP with CDP-diglyceride or the exchange of *sn*-glycero-3-P with phosphatidylglycerophosphate (53, 142, 281). However, reversal of the reaction can be demonstrated by incubating phosphatidylglycerophosphate with CMP (53, 142). Thus, the exchange of *sn*-glycero-3-P with phosphatidylglycerophosphate is detected only when CMP is also present (58, 142), and exchange of CMP with CDP-diglyceride is observed in the presence of *sn*-glycero-3-P (142). These observations are compatible with a sequential reaction mechanism (142). Unlike phosphatidylserine synthetase (181, 281), the phosphatidylglycerophosphate synthetase does not catalyze the slow hydrolysis of CDP-diglyceride (142).

In crude extracts from certain strains of *E. coli*, some formation of phosphatidylglycerol has been reported which cannot be attributed to the phosphatidylglycerophosphate synthetase (196). The enzymology and stoichiometry of this reaction(s) have not been characterized. It is possible that phosphatidylglycerol could arise by the reversal of cardiolipin synthesis, described below (144). There are no satisfactory genetic studies of the function of phosphatidylglycerophosphate synthetase in *E. coli* (274).

Phosphatidylglycerophosphate phosphatase. Like phosphatidylserine, phosphatidylglycerophosphate does not accumulate in growing cells, presumably because it is rapidly dephosphorylated to give phosphatidylglycerol (55). The exact level of phosphatidylglycerophosphate in wild-type *E. coli* is not known (80), since it has never been isolated from living cells.

The phosphatase is a particulate enzyme which requires Mg^{2+} and Triton X-100 for activity (55). Its precise localization within the cell envelope is not known. The enzyme can be resolved from the phosphatidylglycerophosphate synthetase and from the periplasmic alkaline phosphatase, but it has not been purified to homogeneity (55). The enzyme has no affinity for *sn*-glycero-3-P and little, if any, for phosphatidic acid (55).

Cardiolipin synthetase. The enzymatic synthesis of cardiolipin occurs in extracts of various bacteria, including *E. coli* (97, 111, 124, 144, 327, 343). The membrane-bound enzyme responsible for cardiolipin synthesis has not been extracted or purified. Early work by Stanacev et al. (343) suggested that cardiolipin might be formed from phosphatidylglycerol according to the following equation:

$$\text{phosphatidylglycerol} + \text{CDP-diglyceride} \rightarrow \text{cardiolipin} + \text{CMP} \quad (1)$$

This scheme was supported by the observations that radioactive phosphatidylglycerol was converted into cardiolipin in vitro (343) and that CDP-diglyceride stimulated this reaction threefold (144, 343).

Further studies of this system proved to be incompatible with the above reaction (97, 144, 285, 327). Rampini et al. (285) observed that inhibition of energy generation, which blocks the formation of CDP-diglyceride, does not inhibit the synthesis of cardiolipin in vivo. Since phos-

phatidylglycerol levels dropped as cardiolipin accumulated, these workers proposed the following reaction:

2 phosphatidylglycerol → cardiolipin + glycerol (2)

This mechanism was also compatible with the work of Lusk and Kennedy (194), who demonstrated that free glycerol was released from phosphatidylglycerol in radiochemical labeling experiments with living cells.

Further evidence for equation 2 was obtained by De Siervo and Salton (97), who demonstrated conversion of phosphatidylglycerol into cardiolipin in extracts of *Micrococcus lysodeikticus* in the absence of added CDP-diglyceride. However, an exchange reaction between the radioactive phosphatidylglycerol and the endogenous cardiolipin present in the crude enzyme preparations used could not be excluded.

Definitive evidence for equation 2 was obtained independently by Short and White with *Staphylococcus aureus* (327) and Hirschberg and Kennedy with *E. coli* (144). Both laboratories found that the phosphatidyl moiety of CDP-diglyceride was not incorporated into cardiolipin in vitro (144, 327), although the formation of cardiolipin from phosphatidylglycerol was stimulated 3- to 10-fold by the liponucleotide (144). Glycerol release was also demonstrated (144, 327). When phosphatidylglycerol doubly labeled with ^{32}P and ^{3}H in the glycerol headgroup was used as the substrate, the ratio of ^{32}P to ^{3}H was two times greater in cardiolipin than in phosphatidylglycerol (144). This was compatible with equation 2 but not with equation 1. In analogy to its role in cardiolipin synthesis, phosphatidylglycerol has recently been shown to serve as a donor of phosphatidyl moieties in the synthesis of phosphatidyl monoglucosyl diacylglycerol in extracts of *Pseudomonas diminuta* (323).

Eucaryotic systems differ from those of procaryotes in their mechanism of cardiolipin biosynthesis. In mitochondria, equation 1 appears to be the primary route for the formation of cardiolipin (363).

Phospholipid Turnover and Membrane-Derived Oligosaccharides

Phosphatidylglycerol and cardiolipin are further metabolized in exponentially growing cells, whereas phosphatidylethanolamine is relatively stable (7, 10, 11, 42, 84, 85, 161, 173, 210, 242, 321, 390). In some strains, phosphatidylglycerol turnover occurs during the cell division cycle (242). However, polyglycerophosphatide turnover can be inhibited almost completely under certain circumstances without any adverse effect on growth or division (321).

Although recognized as early as 1963 (161), the enzymology of polyglycerophosphatide turnover still has not been elucidated. Considerable progress in this direction has been made by Van Golde et al., who discovered a family of water-soluble oligosaccharides containing glycerophosphate moieties derived primarily from the polar headgroups of phosphatidylglycerol and/or cardiolipin (367). The average molecular weight of these MDO is about 2,000 (367). The sugar consists exclusively of glucose (8 to 10 residues per molecule), and genetic studies indicate that UDP-glucose is required for biosynthesis (165, 320, 367). The MDO is localized in the periplasmic space and accounts for about 1% of the dry weight of the cell (165, 367). It can be subfractionated by ion-exchange chromatography (165, 367). The components so obtained vary in their content of glycerophosphate residues, which are linked as phosphodiesters to the 6 position of glucose (165). Some species also contain esterified succinate residues and phosphorylethanolamine moieties, which further account for the charge heterogeneity of MDO (165, 367; H. Schulman, Ph.D. thesis, Harvard University, Cambridge, Mass., 1976). Preliminary studies indicate that there is some branching of the carbohydrate backbone, which appears to be similar in all MDO species (E. P. Kennedy, personal communication). The absolute configuration is not known for any of the subfractions, and this material does not contain esterified fatty acids (165, 367; Schulman, Ph.D. thesis).

Three different lines of experimental evidence suggest that the glycerophosphate residues attached to the MDO arise from the polyglycerophosphatides and account for much of their turnover. (i) Pulse labeling of the polyglycerophosphatides with *sn*-[2-^{3}H]glycero-3-[^{32}P]phosphate followed by a chase with nonradioactive *sn*-glycero-3-P reveals that the radioactivity in the MDO increases while that of the polyglycerophosphatides decreases and that the ratio of ^{3}H to ^{32}P is conserved in the MDO (367). (ii) The glycerophosphate moiety of MDO is linked to the 6 position of glucose as a phosphodiester (165). The stereochemistry is *sn*-glycero-1-P, which is unusual (165). This fragment might logically arise by the transfer of the glycerophosphate headgroup of phosphatidylglycerol (or possibly cardiolipin) to the oligosaccharides (165, 283). An additional product of such a reaction would be *sn*-1,2-diglyceride (283). (iii) MDO synthesis can be blocked by genetic methods (320, 321). This does not prevent cell growth (320, 321). For instance, strains carrying the *galU* mutation cannot make UDP-glucose and therefore contain no MDO (320). Mutants of

this kind show very little turnover of their polyglycerophosphatide fraction (321).

The number of enzymatic steps between the polyglycerophosphatides, UDP-glucose, and MDO has not been determined, and these reactions consequently are designated by dashed arrows in Fig. 4. The origin of the succinate ester and the phosphorylethanolamine moieties of MDO is also uncertain (Schulman, Ph.D. thesis), and the function of the MDO is unknown. Perhaps it is involved in some transport system not essential for growth in broth culture. It is probably not involved in the regulation of fatty acid synthesis (34), since it appears to be localized in the periplasmic space and is not essential for growth.

Metabolites of phosphatidylglycerol, such as acylphosphatidylglycerol and bisphosphatidic acid, occur in small amounts in the lipid fraction of *E. coli* and *Salmonella typhimurium* (24, 59, 60, 231, 255). The polar headgroups of phosphatidylglycerol and of acylphosphatidylglycerol appear to be precursors for the glycerol residue attached via a thioether linkage to the amino-terminal cysteine of the murein lipoprotein (Fig. 2) (57). The enzymology of these reactions is currently under investigation (60, 231). Since MDO formation and cardiolipin synthesis account for about 75% of the polyglycerophosphatide turnover observed in vivo (321), these other interconversions of the polyglycerophosphatides must be quantitatively minor pathways.

Polyglycerophosphatide turnover is faster in cells adapted to growth on media of low osmolarity (221, 222). Some investigators have also observed phosphatidylethanolamine turnover under these conditions (221), which otherwise do not alter the polar headgroup composition (198, 221). The synthesis of MDO was not examined in these studies. A small amount of phosphatidylethanolamine turnover might be expected if the phosphorylethanolamine moiety of MDO were derived from phosphatidylethanolamine (321).

Diglyceride Kinase

The inner membrane of *E. coli* contains a kinase (Fig. 4) which converts *sn*-1,2-diglyceride to phosphatidic acid (267, 316, 318, 353, 375) according to the following equation:

sn-1,2-diglyceride + ATP

→ phosphatidic acid + ADP

The role of the kinase in phosphatidic acid synthesis has been questioned (316, 318), since the acylation of *sn*-glycero-3-P is the major de novo route to phosphatidic acid in vivo (19, 364). Very little diglyceride is actually present in the lipid fraction of *E. coli* (approximately 0.2 to 0.5% of the total), and this material does not appear to turn over rapidly in radiochemical labeling experiments (54, 283). In contrast, true de novo intermediates, such as phosphatidic acid and CDP-diglyceride, turn over with half-lives on the order of seconds (54, 280).

Schneider and Kennedy (316, 318) have extracted the enzyme from the membrane with Triton X-100 and have purified it 600-fold in the presence of detergent. The same enzyme catalyzes not only the phosphorylation of *sn*-1,2-diglyceride, but also those of monoglyceride, ceramide, and certain other diacylglycerol-like molecules (316, 318) but not that of *sn*-2,3-diglyceride (267). Besides *sn*-1,2-diglyceride, however, there are no other substrates for the kinase in the lipid fraction of *E. coli* (318). Purine nucleotides are much better phosphate donors than pyrimidine nucleotides (267, 318), and cardiolipin stimulates the reaction in vitro (316, 318). The diglyceride kinase has a catalytic specificity that is distinctly different than that of the C_{55} isoprenoid alcohol kinase described by Sanderman and Strominger (306) which is involved in peptidoglycan synthesis. Additional protein factors appear to influence the activity of the kinase in crude extracts under certain circumstances (352, 354), but their function is not known.

Genetic studies (reviewed in more detail below) clearly demonstrate that the kinase represents a minor pathway for phosphatidic acid synthesis (283) and may function to salvage diglyceride molecules that arise during the biosynthesis of MDO (Fig. 4). This diglyceride cycle is not incompatible with earlier metabolic and genetic studies (19, 54). Additional sources of diglyceride in vivo might include phosphatidic acid phosphatase and phospholipase C, both of which have been detected in crude cell extracts (251, 273, 364). In any event, there is a striking accumulation of diglyceride in the membranes of mutants defective in the kinase (up to 10% of the total lipid), suggesting that *sn*-1,2-diglyceride is the true substrate for the enzyme in vivo (283).

Phospholipid Breakdown

Extracts of *E. coli* contain at least nine distinct enzymes capable of degrading phospholipid molecules (Table 4). Some of these proteins have been purified to homogeneity, whereas others have not been extensively characterized (5, 8, 27–29, 66, 98–100, 102, 114, 225, 230, 251, 273, 276, 281, 312, 364). The functions of these catabolic enzymes are uncertain. They are probably not responsible for the major portion of the

TABLE 4. *Enzymes that catalyze phospholipid breakdown in E. coli*[a]

Enzyme	Location	Known substrates	Bonds cleaved	Purified (reference)	Other properties
Lipases					
Phospholipase A	Outer membrane	PE, PG, CL, lyso derivatives	A_1, A_2, L_1, L_2	Yes (230, 312)	Detergent and heat resistant; requires Ca^{2+}
Phospholipase A	Cytoplasm	PG	A_1 or A_2 (or both)	No (102)	Detergent and heat sensitive; requires Ca^{2+}
Lysophospholipase	Inner membrane	Lyso-PE	L_2	No (5)	Heat sensitive; no Ca^{2+} required
Lysophospholipase	Cytoplasm	Lyso-PE, lyso-PG, monoglyceride	L_1, L_2	Yes (100)	Heat sensitive; no Ca^{2+} required
Phospholipase C	Unknown	PE		No (251, 273)	
Phospholipase D	Cytoplasm	CL		No (66)	Mg^{2+} and possibly ATP are required
Phospholipase D	Cytoplasm	PS		Yes (281)	Associated with phosphatidylserine synthetase
Other hydrolases					
Phosphatidic acid phosphatase	Membrane	Phosphatidic acid		No (364)	Separable from other phosphatases
CDP-diglyceride hydrolase	Inner membrane	CDP-diglyceride	Pyrophosphate bond	Yes (276)	Highly specific; no Ca^{2+} required

[a] Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PS, phosphatidylserine; A_1, phospholipase A_1; A_2, phospholipase A_2; L_1, lysophospholipase L_1; L_2, lysophospholipase L_2.

turnover of phosphatidylglycerol and cardiolipin in vivo, since this results primarily from the synthesis of MDO (321).

Detergent-resistant phospholipase A of the outer membrane. The best characterized phospholipase of *E. coli* is the phospholipase A of the outer membrane (22, 376), which is unusual because of its resistance to inactivation by heat and ionic detergents (Table 4). Scandella and Kornberg first solubilized the enzyme from *E. coli* B with sodium dodecyl sulfate and purified it 5,000-fold to homogeneity, in the presence of this detergent (312). More recently, Nishijima et al. described a similar purification for *E. coli* K-12 (230).

Both laboratories found that the enzyme contains a single subunit, with a molecular weight of approximately 28,000 (230, 312). The enzyme requires Ca^{2+} but does not appear to have any special cofactors (230, 312). The three major phospholipids of *E. coli*, as well as phosphatidylcholine and lysophospholipids, are utilized effectively as substrates (230, 312).

Scandella and Kornberg reported that the enzyme was primarily a phospholipase A_1 with an associated lysophospholipase, L_1 (312). However, Nishijima et al. also observed phospholipase A_2 and lysophospholipase L_2 activities in their homogeneous preparations (230). Apparently, isomerization of the lysolipids did not occur under their conditions. The discrepancy between these observations is explained by the finding that the phospholipase A_2 and lysophospholipase L_2 activities are preferentially inhibited by 0.05% Triton X-100 (230), which was included in the assays described by Scandella and Kornberg (312). The capacity of the same outer membrane phospholipase to cleave fatty acids esterified in either the 1 or the 2 position can also be demonstrated by genetic methods (98).

The function of this and other lipases in *E. coli* remains unknown. Mutants lacking the outer membrane phospholipase have no obvious defects in growth, and the turnover of their polyglycerophosphatides occurs at normal rates (1, 101, 243). The detergent-resistant phospholipase mediates the release of free fatty acids observed during T4 and λ infection (43, 82, 83, 158, 227, 303, 366), but this hydrolysis of phos-

pholipids is not essential in the life cycle of these bacteriophages (303). The possibility must also be considered that the phospholipase is important during adaptation of the bacterium to changes in growth conditions, for instance, in modulating the fatty acid composition. Recent work by Nishijima et al. suggests that the enzyme may play a biosynthetic role, since lysophosphatidylethanolamine can be used as a source of fatty acid for the acylation of the headgroup of phosphatidylglycerol (231). More work is clearly needed to elucidate the roles of the phospholipases in vivo.

Detergent-sensitive phospholipase A. Detergent-sensitive phospholipase A is localized in the cytoplasm and acts preferentially on phosphatidylglycerol (5, 102). It is uncertain whether the enzyme also has a lysophospholipase activity associated with it. The enzyme has not been purified, and its specificity with regard to the 1 and 2 positions is not known.

Lysophospholipases. In addition to the two phospholipase A activities described above, there are at least two lysophospholipase activities present in extracts of *E. coli* (5, 100, 102). A lysophospholipase L_2 which does not require calcium is localized in the inner membrane (5). This has not been purified. A separate cytoplasmic lysophospholipase has been purified 1,500-fold, to homogeneity (100). This enzyme hydrolyzes 1-acylglycerophosphorylethanolamine, 2-acylglycerophosphorylethanolamine and 1-acylglycerophosphoryl-glycerol, but not diacyl phospholipids (100). It also hydrolyzes 1-acylglycerol (100). In addition to these lipases, extracts of *E. coli* contain a phosphodiesterase which cleaves glycerophosphorylethanolamine to yield *sn*-glycero-3-P and ethanolamine (5). Thus, enzymes are available which catalyze the complete breakdown of phosphatidylethanolamine (5).

Phospholipases C and D. Neither phospholipase C nor phospholipase D activity is present in a large amount in cell-free extracts (25, 64–66, 251, 273). Evidence for phospholipase C is circumstantial, since it is based on the observation that phosphorylethanolamine is released from membranes in crude extracts (251, 273). Proulx and co-workers have presented preliminary evidence for a soluble, cardiolipin-specific phospholipase D, but the combined action of several enzymes cannot be excluded under the conditions employed (25, 64–66). Phosphatidylserine is slowly hydrolyzed by homogeneous phosphatidylserine synthetase (Fig. 5) to form phosphatidic acid and serine (181, 281), but this reaction is quantitatively insignificant.

Other specific hydrolytic enzymes. (i) Phosphatidic acid phosphatase. Phosphatidic acid phosphatase was first observed by van den Bosch and Vagelos (364). It is distinct from alkaline phosphatase and phosphatidylglycerophosphate phosphatase (55), but it has not been purified or characterized. It may represent a minor source of diglyceride in vivo.

(ii) Cytidine 5′-diphosphate diglyceride hydrolase. CDP-diglyceride hydrolase splits CDP-diglyceride to form phosphatidic acid and CMP (276, 278). It is associated with the cytoplasmic membrane and is very specific for liponucleotides (276, 278). Water-soluble pyrophosphates, such as nicotinamide adenine dinucleotide, ATP, and CDP-choline are not substrates (276, 278). The deoxy derivative of CDP-diglyceride, which is found in *E. coli*, is not hydrolyzed (276), but the analog, CDP-ceramide (which is not found in vivo), is rapidly cleaved (317). The hydrolase has been purified 1,000-fold, using a procedure identical to that employed for phosphatidylserine decarboxylase (276).

In view of its striking specificity, a regulatory function might be envisaged for the enzyme (276, 278), especially if the liponucleotide levels are rate limiting for total phospholipid synthesis (280). Perhaps the ratio of ribo- to deoxyribo-liponucleotide has some significance in the regulation of phospholipid composition. Alternatively, the hydrolase may have a biosynthetic function, since the phosphatidylserine synthetase (181, 281) also has a small amount of CDP-diglyceride hydrolase activity in the absence of serine (Fig. 5). These questions might be answered by studies of mutants lacking the CDP-diglyceride hydrolase. Extracts of gram-negative bacteria other than *E. coli* also catalyze CDP-diglyceride hydrolysis (104).

The enzyme is very useful for the specific degradation of liponucleotides isolated from natural sources (132, 355). Its inability to hydrolyze dCDP-diglyceride must be taken into account in such studies.

GENETIC MODIFICATION OF MEMBRANE LIPID SYNTHESIS

Until recently, genetic studies of membrane lipid synthesis were limited to the isolation of glycerol, *sn*-glycero-3-P, and fatty acid auxotrophs (330). These substances are taken up rapidly by *E. coli* (329). Fatty acid auxotrophs have been especially useful for altering the hydrocarbon moieties of the membrane lipids (75, 329). Glycerol and *sn*-glycero-3-P auxotrophs permit the selective inhibition of total lipid synthesis (19, 329). Starvation of such mutants for their required supplements results in the rapid cessation of growth (19, 75, 137, 329). This indi-

cates that *E. coli* does not contain a large reserve of unused phospholipids.

The isolation of mutants in the later stages of polar headgroup synthesis (Fig. 4) presents a difficult experimental problem. In contrast to deoxyribonucleic acid (DNA), RNA, and protein synthesis, specific inhibitors of the phospholipid enzymes, which would greatly facilitate mutant selection, have not been found. Wild-type strains of gram-negative bacteria do not take up intact phospholipids from the growth medium (156, 157), and therefore auxotrophs have not been sought. However, it may still be feasible to isolate phospholipid auxotrophs. Jones and Osborn (156, 157) have discovered that *S. typhimurium* can take up exogenous phospholipid vesicles to a limited extent, provided the recipient is a "deep rough" mutant defective in lipopolysaccharide. McIntyre and Bell have observed that deep rough mutants of *E. coli* can incorporate even larger amounts of exogenous lysophospholipids, such as 1-oleoyl-*sn*-glycero-3-P, although extensive degradation may also occur (201).

Because of these restrictions, most existing phospholipid mutants have been isolated by the "brute force" screening of colonies derived from mutagen-treated cultures (133, 269a, 274, 275, 283). The feasibility of this approach had been demonstrated previously for mutants in the enzymes of DNA replication (45, 94). The recent development of rapid autoradiographic screening procedures for identifying specific enzyme defects in bacterial colonies makes the brute force approach very attractive and yields mutants that are biochemically defined (274, 275, 283). Considerable information concerning the properties of phospholipid mutants is now accumulating (Table 5). The following discussion will emphasize mutants in the acylation of *sn*-glycero-3-P and beyond (Fig. 4). Properties of fatty acid auxotrophs and mutants in fatty acid degradation have been reviewed elsewhere (75, 329).

sn-Glycero-3-Phosphate Auxotrophs and Mutants in Phosphatidic Acid Synthesis

The possibility of isolating mutants in the enzymes that acylate *sn*-glycero-3-P was first explored by Cronan et al. (77). These workers exposed cells to *sn*-[2-^{3}H]glycero-3-P of high specific radioactivity, which was selectively incorporated into membrane lipids (77). Wild-type organisms were killed by this procedure, and mutants unable to make lipids were sought among the survivors (77). Many of these strains were temperature sensitive for growth (77). A subsequent study by Godson (121) revealed that the survivors of such a "suicide" selection could be sorted into at least 13 distinct complementation groups.

Early investigations of one such mutation, termed *plsA*, by Cronan et al. (77, 121) suggested that the temperature sensitivity for growth was caused by a defective *sn*-glycero-3-P acyltransferase. A preferential inhibition of phospholipid synthesis was reported at elevated temperatures (77). The location of the *plsA* gene and its associated phenotypic traits were determined (76,

TABLE 5. *Properties of existing mutants defective in phospholipid biosynthesis*[a]

Gene (reference)	Enzyme	Composition[b]	Phenotype
gpsA (19, 71)	Biosynthetic *sn*-glycero-3-P dehydrogenase	↓Total lipid	*sn*-Glycero-3-P auxotroph
plsB (19, 71, 200)	*sn*-Glycero-3-P acyltransferase	↓Total lipid	*sn*-Glycero-3-P auxotroph
pss (248, 274, 275)	Phosphatidylserine synthetase	↓PE, ↑PG, ↑CL	Temperature-sensitive
psd (133, 134)	Phosphatidylserine decarboxylase	↓PE, ↑PS, normal PG and CL	Temperature-sensitive
pgs (274, 275)	Phosphatidylglycerophosphate synthetase	↑PE, ↓PG	See footnote *a*
cls (269a)	Cardiolipin synthetase	Normal PE, ↑PG, ↓CL	None
dgk (283)	Diglyceride kinase	Normal phospholipids, ↑diglyceride	Osmotic fragility

[a] Other mutations. Strains defective in the detergent-resistant phospholipase (*pldA*) have no apparent phenotype (1). Existing strains which are defective in the phosphatidylglycerophosphate synthetase in vitro (*pgs*) still make about half the normal amount of phosphatidylglycerol in vivo and have no obvious phenotype (274).

[b] ↑, Increased; ↓, decreased; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

79, 119, 224). The additional mutants isolated by Godson (121) were not studied biochemically.

Further work, by Glaser et al. (119), revealed that the *plsA* mutants were actually defective in all macromolecular synthesis above 37°C. This was traced to a lesion in ATP synthesis caused by an alteration of the adenylate kinase in these strains (120). Almost all of the phenotypic properties of *plsA* reported earlier (76, 77, 79, 119) were explained by these findings. Ray et al. (291) described a selective inhibition of phospholipid synthesis in *plsA* mutants at 35°C, but they did not determine the ATP level under their conditions.

Several further lines of evidence argue that the *plsA* gene is not involved in phospholipid metabolism. (i) Snider and Kennedy (339) could not reproduce the thermal instability of the acyltransferase in extracts of the *plsA* mutants originally reported by Cronan et al. (77). They (339) questioned the reliability of the enzyme assays used in the earlier work (77). (ii) In 1974, Bell (19) identified a new class of mutants (*plsB*) defective in the same acyltransferase. These mutations mapped at a different site, far from *plsA* (72, 76). The conclusions of Bell were confirmed by Snider and Kennedy (339). Thus, a variety of enzymatic and genetic criteria now indicate that *plsB* is the structural gene for the acyltransferase, a role previously assumed for *plsA*.

Despite the problems encountered in characterizing the *plsA* mutation and the risk of obtaining strains defective in ATP synthesis, mutant selections based on radiation suicide are frequently successful (212). For instance, the acetate suicide procedure described by Cronan and co-workers (131) permits the isolation of mutants in saturated fatty acid biosynthesis. Therefore, the collection of *sn*-[2-^{3}H]glycero-3-P suicide survivors isolated by Godson (121) should be carefully reexamined for defects in phospholipid metabolism.

A different approach was used to isolate strains carrying the *plsB* mutation (19). These were found among a large number of *sn*-glycero-3-P auxotrophs enriched from a mutagen-treated culture by penicillin selection (19). In addition to acyltransferase mutants (*plsB*), some of these auxotrophs were defective in biosynthetic *sn*-glycero-3-P dehydrogenase (*gpsA*) and in transport of inorganic phosphate (19, 342). Mutants in the later stages of phospholipid synthesis (for instance, in phosphatidylglycerophosphate synthetase) were not obtained. The genetic loci of *plsB*, *gpsA* (71, 72), and all other known mutations in phospholipid biosynthesis (Table 5) are shown in Fig. 6. Most of these are probably the structural genes for the biosynthetic enzymes. No operons have been identified.

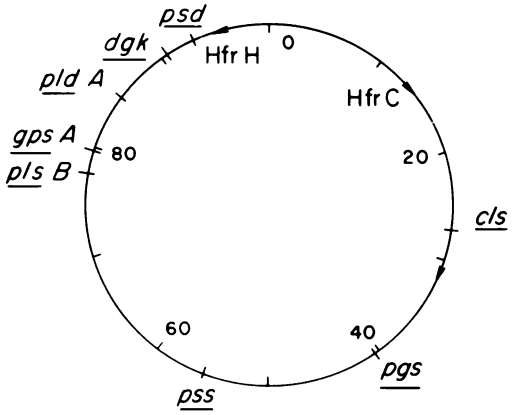


FIG. 6. *Genetic locations of mutations responsible for specific defects in the enzymatic synthesis of membrane phospholipids. Most of these appear to be structural genes.*

Existing acyltransferase mutants (*plsB*) are not temperature sensitive for growth (19), although such mutations could probably be isolated. The mutant enzyme of strain BB-26 has a 10-fold lower affinity for *sn*-glycero-3-P than has the wild type (19, 20). To make phospholipids and grow normally, these strains must be supplemented with exogenous *sn*-glycero-3-P (19). When this substance is withheld, the endogenous rate of phospholipid synthesis drops by 90% or more (19). Several *sn*-glycero-3-P auxotrophs isolated earlier by Kito, Pizer, and co-workers were not characterized in as much detail, but they may also be defective in the *plsB* gene (174, 269).

Like *plsB*, mutants defective in the biosynthetic *sn*-glycero-3-P dehydrogenase (*gpsA*) rapidly stop making phospholipids when *sn*-glycero-3-P is removed from the medium (19). In both cases, growth and macromolecular synthesis continue for about one generation (19, 202). The cells do not form filaments and do not lose viability over the course of a few hours. Phospholipid synthesis resumes immediately after *sn*-glycero-3-P is added back (202). Although total phospholipid synthesis is inhibited, most membrane proteins continue to be made, and consequently the buoyant density of the membrane increases (200, 202). These properties of strains carrying the *plsB* and *gpsA* mutations resemble those of glycerol auxotrophs of gram-positive bacteria isolated by Mindich (216).

Revertants of *plsB* able to grow in the absence of *sn*-glycero-3-P fall into two classes (21). Some appear to be true revertants in that the K_m of the acyltransferase for *sn*-glycero-3-P returns towards normal, whereas others are still completely defective (21). The latter class of revert-

ants no longer requires exogenous *sn*-glycero-3-P, because the endogenous level of *sn*-glycero-3-P is much higher than that in the wild-type (21). This bypasses the *plsB* mutation (21). The increased level of endogenous *sn*-glycero-3-P is the consequence of a mutation in the biosynthetic *sn*-glycero-3-P dehydrogenase, which has lost its normal feedback inhibition by *sn*-glycero-3-P (21). This has been examined in considerable detail by purification of the feedback-insensitive dehydrogenase to homogeneity (Edgar and Bell, manuscript in preparation).

The *plsB* mutants offer several advantages over other types of mutants for studies of membrane biogenesis. (i) During *sn*-glycero-3-P starvation, endogenous *sn*-glycero-3-P is still generated, though at levels that do not permit lipid synthesis. This allows other metabolic processes to continue which might require *sn*-glycero-3-P. (ii) *sn*-Glycero-3-P starvation is reversible. Cells remain at the same temperature, and the mutant enzyme is not inactivated. (iii) Since *plsB* mutants are blocked in the first step of Fig. 4, potentially toxic intermediates cannot accumulate. For these reasons, Bell and co-workers have performed most of their studies on membrane biogenesis with mutants defective in the *plsB* gene (19, 200, 202). Efforts are also underway to use strains bearing the *plsB* and *gpsA* mutations as probes of the fusion of phospholipids with bacteria, since growth in the absence of *sn*-glycero-3-P may be possible if enough phospholipid is taken up from the medium (T. McIntyre and R. M. Bell, personal communication). This approach could permit major substitutions of the normal phospholipids by non-physiological species. For instance, it is conceivable that phosphatidylethanolamine could be replaced with lecithin. Such modifications would not be possible by genetic means.

Mutants blocked in the second acylation reaction, i.e., in the conversion of 1-acyl-*sn*-glycero-3-P to phosphatidic acid, have not been identified. In *plsB* mutants, the specific activity of the second acyltransferase is normal (20). Although a preliminary report of a mutant in the second acyltransferase does exist (135), further studies by the same investigators revealed that this strain carried multiple mutations, probably not directly related to phospholipid biosynthesis (344).

Mutants defective in the synthesis of CDP-diglyceride have not been isolated. These would be of interest, since CDP-diglyceride formation may be rate making and the function of dCDP-diglyceride is unknown. The rapid autoradiographic screening techniques described below could be utilized for this purpose.

Mutants in Phosphatidylethanolamine Synthesis

Almost all of the mutants in the late stages of phospholipid biosynthesis have been isolated by the brute force assay of cell extracts derived from single colonies of mutagen-treated cells (133, 269a, 274, 275, 283). Because of the general success of the brute force strategy, Hirota and co-workers (143, 345, 346) have collected a bank of 5,000 temperature-sensitive, independently isolated mutants, which need only to be subscreened individually for the identification of specific biochemical lesions. A mutant lacking cardiolipin synthetase has recently been found in this collection (269a). Nevertheless, the assay and mapping of mutants by brute force is very cumbersome, especially if thousands of individual colonies must be examined for the presence or absence of a specific enzyme. For this reason, my laboratory (274) developed a rapid, direct technique for the autoradiographic assay of lipid enzymes in colonies that is applicable to most of the reactions of Fig. 4.

Mutant isolation by colony autoradiography. The rapid autoradiographic colony assay is carried out in the following way (274). A disk of filter paper is pressed down on an agar plate containing several hundred colonies of mutagen-treated cells, after which the paper is lifted off. In the process, the colonies are transferred to the paper, giving rise to a copy of the master plate. The few cells left on the master keep growing in the original pattern. The pattern of colonies is also retained on the filter paper, where they can be rendered permeable with lysozyme and ethylenediaminetetraacetate. Colonies treated in this manner remain adsorbed to the paper and can carry out in vitro phospholipid synthesis (such as the conversion of L-[*U*-^{14}C]serine to phosphatidyl-[*U*-^{14}C]serine dependent on added CDP-diglyceride). The radioactive phospholipid generated in situ does not diffuse very far and is precipitated around each colony with trichloroacetic acid after the reaction is over. The unreacted serine is washed away on a Büchner funnel. After autoradiography, the filter paper (to which the colonies remain tightly bound) is stained with Coomassie brilliant blue. This locates all colonies, including mutants. When the autoradiogram is superimposed on the stained filter paper, mutants stand out as blue colonies lacking a black halo (274).

The assay is applicable without significant modification to a variety of other bacterial colonies (220, 274) and to *Saccharomyces cerevisiae* (274). With appropriate modifications, it can even be adapted for use with colonies of

animal cells grown in tissue culture (108). With *E. coli*, the assay is very sensitive, and the frequencies of false-positives and false-negatives are relatively low (274). As a rule, one mutant (blue spot) is found in every 5,000 to 10,000 colonies examined (274). When cell extracts from such mutants are assayed by conventional methods, the specific activity of the enzyme under study is usually reduced by at least a factor of 10 (274, 275, 283). The screening technique makes no assumptions about possible associated phenotypes of such enzyme-defective strains. Consequently, both temperature sensitives and absolute defectives can be obtained (275, 283). A suitably motivated person can screen up to 40,000 colonies per day with this procedure.

Phosphatidylserine synthetase. Four strains defective in phosphatidylserine synthetase were isolated from a total of 20,000 colonies (274, 275). Only one of these mutations (*pss-8*) resulted in a sufficiently defective enzymatic activity to cause a temperature sensitivity for growth (Table 5) and a significant alteration of the phospholipid composition (275). The others were phenotypically silent and had a normal lipid content. All four mutations mapped at the same site (Fig. 6), being contransducible with *tyrA* and *nadB* (275). An additional mutation (*pss-21*, also temperature sensitive for growth) was recently isolated by mutagenesis of a strain already carrying a partially defective synthetase (Raetz, unpublished data). This organism was found to contain no phosphatidylserine synthetase when assayed in vitro. An especially striking reduction of the phosphatidylethanolamine level was observed with this strain.

Organisms bearing the *pss-8* and *pss-21* mutations grow at nearly normal rates at 25°C but contain less phosphatidylethanolamine (about 55 versus 82%) than does the wild type (275). Conversely, the mutants contain much more cardiolipin than normal, even during log phase (275). When the mutant is shifted to the nonpermissive temperature, the phosphatidylethanolamine content falls further (275). The best results are obtained with the *pss-21* mutation, in which the phosphatidylethanolamine content approaches 20% after 6 h at 42°C. As growth ceases, the mutants form long filaments (275). This defect in cell division is also observed with mutants in phosphatidylserine decarboxylase, which, like the *pss* mutants, accumulate anionic phospholipids (133). The content of phosphatidylglycerol does not vary by more than a factor of 2 under any condition.

Both phosphatidylserine synthetase mutants are hypersensitive to hydrophilic antibiotics, especially to the aminoglycosides (277). This suggests an increase in the permeability of the outer membrane related to the change in polar headgroup composition (277). Transduction and reversion analyses demonstrate that the same mutation is responsible for the temperature sensitivity, the antibiotic hypersensitivity, and the defective synthetase (277).

The characterization of the *pss* mutants demonstrates the usefulness of rapid autoradiographic screening for mutant isolation. It also reveals that: (i) phosphatidylserine synthetase is the major route to phosphatidylethanolamine in vivo; (ii) the various phospholipid genes are not close together on the chromosome (Fig. 6); and (iii) a twofold reduction in the amount of phosphatidylethanolamine in the membrane interferes (either directly or indirectly) with cell division. Partial purification of the synthetase from several mutants supports the view that the *pss* locus represents a structural gene (275).

Ohta and co-workers (245–248) have independently isolated an *E. coli* mutant defective in the *pss* gene (designated *pss-1*). This strain was found amongst the survivors of a serine suicide selection (245), similar to one described earlier by Cronan (68). It is not likely that this technique actually enriches for *pss* mutations, since Ohta et al. examined 300 survivors before finding *pss-1* (245). The serine suicide survivors originally described by Cronan (68) are not defective in synthetase activity (275), and their biochemical defect remains unknown.

The properties of *pss-1* closely resemble those of *pss-8* and *pss-21* (246, 275). However, complementation studies have not been performed. All strains carrying *pss* mutations are stabilized by 0.2 to 0.4 M NaCl, 0.5 M sucrose, and especially by 20 mM $MgCl_2$ (246; Raetz, unpublished data). The effect of the divalent cations is selective and cannot be attributed to the osmolarity or the ionic strength. Unlike *pss-8* and *pss-21* (275), strains bearing *pss-1* are able to grow at 42°C if sufficient levels of salt and divalent cations are added to the medium (246). However, the phosphatidylethanolamine level of *pss-1* only reaches a limiting value of 35 to 40% (246).

Ohta and Shibuya have found that macromolecular synthesis continues at 42°C, whereas phosphatidylethanolamine synthesis is selectively inhibited (246). Supplementation of their mutant with lysophosphatidylethanolamine results in a partial correction of the phospholipid composition, suggesting that a pathway exists in vivo for the reacylation of lysophosphatidylethanolamine (M. Nishijima, personal communication). However, the temperature sensitivity of *pss-1* is not suppressed by lysophosphatidylethanolamine. The effect of the *pss* mutation on

transport functions has not been examined, but there are no major changes in fatty acid and membrane protein composition (277) under permissive conditions (30°C).

The changes in the lipid composition of mutants in the *pss* gene resemble those of a sodium-sensitive mutant described earlier by Lusk and Kennedy (194). A careful study of this mutant failed to reveal any defect in phosphatidylserine synthetase activity in vitro (194). A similar alteration of lipid composition is observed during infection of *E. coli* with certain amber mutants of bacteriophage f1 (384). Perhaps the phosphatidylserine synthetase is rendered nonfunctional in vivo in these situations by not having access to its membrane-bound substrate, CDP-diglyceride.

Despite the considerable information that has accumulated concerning the properties of phosphatidylserine synthetase mutants, there are no methods for their direct selection. A preliminary survey of bacteriophage sensitivities (T4, T7, P1, and P2) revealed no significant differences from the pattern observed with isogenic wild-type strains (277). Antibiotic hypersensitivity (277) affords a potential method for selecting these mutants, but other mutations leading to antibiotic hypersensitivity are much more common than mutations in the *pss* gene (277). Colicin sensitivity studies have not been reported. The availability of additional *pss* mutants would be desirable, since existing mutants do not stop making phosphatidylethanolamine at 42°C as rapidly as is required for some physiological studies (246, 275).

Phosphatidylserine decarboxylase. Hawrot and Kennedy have isolated mutants in phosphatidylserine decarboxylase by using a combination of brute force screening and localized mutagenesis (133, 134). As in the case of phosphatidylserine synthetase, there appears to be a large excess of decarboxylase activity in vivo. Consequently, many partial mutants are found in these studies which are not sufficiently defective to cause a conditional lethality or an alteration of the lipid composition (133).

Provided that the decarboxylase is inactivated sufficiently, strains carrying *psd* mutations are temperature sensitive for growth (Table 5) and accumulate large amounts of phosphatidylserine at restrictive temperatures (133, 134). This material can replace over 80% of the phosphatidylethanolamine normally present in the membrane (E. Hawrot and E. P. Kennedy, personal communication). The ratio of serine-derived phosphatides to polyglycerophosphatides remains constant (133). The *psd* mutation is cotransducible with the *purA* and *ampA* genes (Fig. 6) (134). Some filamentation occurs as growth ceases at 42°C (133). As in the case of *pss* mutants, this may be related to the accumulation of anionic phospholipids.

The phosphatidylserine that accumulates in the decarboxylase mutants is found both in the inner and in the outer membrane (Hawrot and Kennedy, personal communication). When the mutants are shifted back to the permissive temperature (30°C), all of the phosphatidylserine is decarboxylated. Since the decarboxylase is localized in the inner membrane (22, 376), these results indicate that certain phospholipids (unlike lipopolysaccharide and proteins) can move freely between the two membranes. The mechanism by which this occurs is unknown. These results are consistent with the finding that phosphatidylserine can be fused with and decarboxylated by deep rough mutants of *S. typhimurium* (156, 157).

The three temperature-sensitive *psd* mutants examined so far fall into one complementation group (134). The envelope composition and the permeability to small molecules have not been examined in detail. Biochemical and genetic studies indicate that *psd* is a structural gene (133, 134). The kinetics of inactivation of the mutant decarboxylase suggest that the membrane exerts a stabilizing effect on the enzyme (133).

Mutants in Polyglycerophosphatide Synthesis

Phosphatidylglycerophosphate synthetase. The genetics of the reactions leading to the polyglycerophosphatides have not received as much attention as the pathway to phosphatidylethanolamine. Mutants unable to make anionic lipids in vivo have not been reported. It is possible (though unlikely) that anionic lipids are not essential for growth. Mutants defective in the synthesis of phosphatidylglycerol would be of great interest, since this compound has been implicated as an activator of enzyme II of the phosphotransferase system (179, 180).

Mutants lacking phosphatidylglycerophosphate synthetase in vitro have been isolated by colony autoradiography (274), and the location of the *pgs* gene is known (Fig. 6) (275). Some of these mutants contain no more than 5% of the wild-type activity, when assayed under optimal conditions (274). However, none of the mutants are temperature sensitive for growth, and all have 50 to 90% of the normal level of phosphatidylglycerol in vivo (274) (Table 5).

The significance of these findings is uncertain. It is conceivable that other biosynthetic pathways (or other phosphatidylglycerophosphate synthetases) predominate in vivo. However, there is no clear evidence for isoenzymes or

alternate routes. Studies with the *sn*-glycero-3-P analog 3,4-dihydroxybutyl-1-phosphonic acid strongly suggest that phosphatidylglycerophosphate is a major physiological intermediate in the formation of phosphatidylglycerol in living cells (360).

Since other enzymes, such as phosphatidylserine synthetase and phosphatidylserine decarboxylase, apparently are present in great excess (133, 275), it is possible that this is also the case with phosphatidylglycerophosphate synthetase. The small residual activity observed in all existing *pgs* mutants (274) may be sufficient to meet the bacterium's demand for phosphatidylglycerol. As in the case of phosphatidylserine decarboxylase, the instability of the enzyme observed in vitro may be partially compensated in vivo by its membrane association (133). It may be necessary to isolate second-step mutations, starting with the partial mutants already available, to observe major changes in the lipid composition of the cell. If the *pgs* gene is not essential, it should be possible to isolate insertion and deletion mutations with bacteriophage Mu (145).

Bell et al. (23) have characterized a mutant that has three times more phosphatidylglycerophosphate synthetase than normal and also contains a twofold excess of polyglycerophosphatides in vivo. The activity of phosphatidylserine synthetase was not examined, nor was the genetic location of this mutation determined. The altered lipid composition was shown to be unrelated to a mutation in DNA synthesis—also present in the original mutant isolate (23).

Strains lacking phosphatidylglycerophosphate phosphatase have not been reported.

Cardiolipin synthetase. Pluschke et al. (269a). have screened the random mutant collection of Hirota and co-workers (345, 346) for alterations in phospholipid composition. This led to the discovery of a strain lacking cardiolipin in vivo (269a). The defective gene (designated *cls*, Table 5) was found to cotransduce with the tryptophan locus (Fig. 6) (269a).

The *cls* mutants contain 10 to 50 times less cardiolipin than normal (269a). They are not temperature sensitive for growth (269a). The decrease in cardiolipin is compensated by an increase in phosphatidylglycerol, and the ratio of phosphatidylethanolamine to polyglycerophosphatides is unaltered (269a). Turnover of phosphatidylglycerol is reduced when compared with cls^+ (269a), but the gross level of MDO (as judged by glycerol labeling) is not strikingly reduced (Raetz, unpublished data). These findings suggest that a substantial portion of the *sn*-glycerol-1-P moieties present in MDO must arise from phosphatidylglycerol.

The bacteriophage f1 grows normally on mutants defective in the *cls* gene (269a). When amber mutants of f1 are used to infect *cls* mutants, phosphatidylglycerol accumulates (269a). This contrasts with wild-type cells, in which cardiolipin builds up (269a, 384). It suggests that amber mutants of f1 perturb the phospholipid composition of wild-type cells primarily by inhibiting phosphatidylserine synthesis, not cardiolipin turnover.

Mutants in Phospholipid Turnover and the Diglyceride Cycle

Genetic inhibition of polyglycerophosphatide turnover. The genetics of polyglycerophosphatide turnover have been analyzed by Schulman and Kennedy (320, 321). Situations in which the synthesis of MDO is blocked, for instance, in mutants unable to make UDP-glucose (320) or in phosphoglucose isomerase mutants grown on Casamino Acids (321), result in the cessation of polyglycerophosphatide turnover. In the latter case, MDO synthesis and polyglycerophosphatide turnover occur only when glucose is added to the medium (321). The biochemical mechanism by which the turnover is related to MDO synthesis has not been explored. A reduction in the rate of phosphatidylglycerol turnover is also observed in mutants lacking cardiolipin synthetase (269a), although considerable amounts of MDO are still made. Inhibition of phospholipid turnover obviously does not prevent cell growth (321).

Mutants in diglyceride kinase and role of the diglyceride cycle. Mutants lacking diglyceride kinase can be identified by a simple modification of the rapid autoradiographic screening assay originally developed for the phosphatidyltransferases (283). Four mutants lacking kinase activity have been isolated out of a total of 10,000 colonies examined (283). One of these organisms (RZ60) accumulates 20 times the normal amount of *sn*-1,2-diglyceride in its membrane fraction (Table 5), amounting to about 8% of the total lipid (283). Although RZ60 is not temperature sensitive for growth, it does not thrive in media of low osmolarity (283). In all four isolates, the gene responsible for the kinase defect (designated *dgk*) is cotransducible with the *malB* locus (Fig. 6) (283).

The accumulation of diglyceride in mutants lacking the kinase demonstrates that 1,2-diglyceride is the true substrate for the enzyme in vivo and that the kinase must represent a minor pathway for phosphatidic acid synthesis (283). This is not incompatible with results obtained with the *plsB* mutants, which indicate that the *sn*-glycero-3-P acyltransferase is the primary source of phosphatidic acid (19).

The origin of diglyceride in *E. coli* is not absolutely certain (283). The slow apparent turnover of diglyceride observed in wild-type organisms (54) argues that diglyceride is not an early intermediate in de novo phospholipid synthesis, since true early precursors, like phosphatidic acid and CDP-diglyceride, turn over very rapidly (280). The most likely sources of diglyceride would appear to be phosphatidylglycerol and/or cardiolipin (283). Indeed, the transfer of the unusual *sn*-glycero-1-P moiety from the polyglycerophosphatides to the MDO should generate *sn*-1,2-diglyceride as a by-product (see Fig. 4). This predicts that MDO synthesis, polyglycerophosphatide turnover, and diglyceride formation are all coupled. To test this hypothesis, a double mutant defective both in phosphoglucose isomerase (*pgi*) and diglyceride kinase (*dgk*) has been constructed (C. R. H. Raetz, manuscript in preparation). When MDO synthesis is inhibited in this strain by growing the cells on Casmino Acids, the diglyceride does not accumulate in large amounts unless glucose is added. This is the only direct evidence for the diglyceride cycle proposed in Fig. 4. Biochemical studies will be required to demonstrate the existence of this scheme. Whatever the origin of the diglyceride may be, the kinase presumably serves as a salvage enzyme for the reutilization of this material (283).

Since neutral lipids may be capable of increasing membrane fluidity (107), it may be of interest to examine the physical properties of membranes isolated from *dgk* mutants. Most of the diglyceride is localized in the cytoplasmic membrane (283; Raetz, manuscript in preparation). Additional diglyceride accumulates in RZ60 when the cells are shifted into a growth medium of low osmolarity (283).

Mutants in Catabolic Enzymes

Detergent-resistant phospholipase A. More genetic studies of phospholipid catabolism would be desirable to define the precise role of the individual phospholipases in lipid metabolism. Ohki et al. have developed an elegant general procedure for detecting mutants lacking the detergent-resistant lipase of the outer membrane (243). Their method is based on the release of free fatty acids from individual colonies (243). The fatty acids are detected by their ability to cross-feed an indicator strain that requires fatty acids for growth (243). Similar screening procedures could probably be developed for phospholipases C and D. The relatively slow, but reliable, microtiter dish procedure developed by Weiss and Milcarek (373) for obtaining mutants lacking various nucleases could also be adapted for use with the phospholipases. A lecithin agar for the detection of microbial phospholipases has been described (61) and might be useful in some cases.

Strains lacking the detergent-resistant phospholipase A (*pld*) appear to grow normally at all temperatures (1, 101, 243). The *pld* gene is cotransducible with *metE* (Fig. 6) (1). Both the A_1 and the A_2 activities are missing as a result of the same mutation, but the turnover of polyglycerophosphatides is not altered (101, 243). Bacteriophages, such as T4 and λ, grow normally on these mutants (243, 303). However, the free fatty acid release that usually accompanies lysis is not observed, which indicates that the outer membrane phospholipase is not essential for bacteriophage release (303). Colicin K and complement-mediated phospholipid hydrolyses are similar inhibited in *pld* mutants, indicating that these agents somehow lead to the activation of the detergent-resistant phospholipase, although this may not be the primary effect and is not required for cell killing (149, 195). Indeed, the factor(s) which inhibits the action of the detergent-resistant phospholipase in normal, growing cells has not been identified. An endogenous protein inhibitor of phospholipase A_1 has been described in *Bacillus subtilis* (166, 178).

A mutation in the soluble, detergent-sensitive phospholipase A has recently been isolated, but genetic mapping has not been reported (101). The additional lesion in the sensitive phospholipase causes no obvious phenotypic alterations in strains which are already defective in the detergent-resistant enzyme (101), except for a slight increase in resistance to leukocyte-mediated killing (374). More studies will be required to analyze the significance of these findings.

The function of other catabolic enzymes, such as phosphatidic acid phosphatase, CDP-diglyceride hydrolase, and the lysophospholipases, might be elucidated if appropriate mutants were available.

Cloning of Genes Involved in Membrane Lipid Synthesis

The phospholipid enzymes are constitutive and present in very small amounts. Changes in growth conditions do not alter their specific activities by more than a factor of 2 (Raetz, unpublished data).

Recent advances in molecular cloning permit the construction of strains carrying multiple copies of specific genes or gene clusters (63, 139, 217). Clarke and Carbon have prepared a collection of 2,000 *E. coli* strains, each carrying a distinct hybrid ColE1 plasmid into which a frag-

ment of *E. coli* chromosomal DNA has been inserted (62). The hybrid plasmids (with their inserted DNA) are maintained at 10 to 20 copies per chromosome (62). The average molecular weight of the inserted fragment is 8×10^6, or about 0.25 min of the linkage map (62). The collection contains cloned fragments that represent almost all of the *E. coli* genes (62).

Distinct hybrid ColE1 plasmids carrying certain of the phospholipid genes (Fig. 6) have been identified (282). These are *plsB* (Bell, personal communication), *pss* (282), *psd* (Kennedy, personal communication), and *dgk* (Raetz, unpublished data). In all cases, specific overproduction (5- to 20-fold) of the cloned enzyme is observed when extracts from exponentially growing cells are assayed. This is very useful for the purification of these enzymes (282). The specific activity and subunit molecular weight of the overproduced phosphatidylserine synthetase are identical to those of the wild-type enzyme upon purification to homogeneity (282). This excludes the production of an activator by the plasmid-bearing strains and demonstrates that true overproduction is occurring (282).

In the case of *pss* and *psd*, gene amplification does not lead to a change in lipid composition (282). The overproduced phosphatidylserine synthetase is associated with ribosomes, indicating a large excess capacity to bind the polypeptide (282). Cloning of the *plsB* gene results in the overproduction of *sn*-glycero-3-P acyltransferase, but there is no major increase in the phospholipid content of the cell (Bell, personal communication).

The availability of the DNA and RNA corresponding to the genes of phospholipid metabolism provides a new approach to the study of the regulation of this pathway. The recent development of systems for the in vitro synthesis of membrane proteins will permit a direct examination of enzyme processing and of the chemical requirements for membrane insertion (52, 302, 380). As techniques for the overproduction of proteins by gene cloning are improved, it will be possible to obtain sufficient amounts of homogeneous lipid enzymes for chemical and physical studies.

REGULATION OF MEMBRANE LIPID SYNTHESIS AND ITS ROLE IN MEMBRANE ASSEMBLY

The regulation of membrane lipid synthesis in *E. coli* is not understood as well as that of other pathways. Unresolved problems include: regulation of total lipid content; regulation of polar headgroup ratios; quantitative determination of levels of biosynthetic intermediates; a physical substitute for Triton X-100 in vivo; coordination of membrane lipid, membrane protein, and macromolecular synthesis in growing cells; the mechanism of phospholipid flip-flop and generation of asymmetry; the mechanism of phospholipid translocation from the inner to the outer membrane; and the function of phospholipid molecular species and polar headgroups. The answers to the questions posed below are not known in many instances, although the increasing use of genetics in the study of this system should lead to the solution of these problems in the near future. More studies of lipid synthesis in synchronized cells would also be useful, since there appears to be a burst of lipid synthesis during the process of septation (89, 127), at least under some conditions (16, 89). Cell cycle-dependent variations in phospholipid content are well documented in the membranes of photosynthetic bacteria, but the mechanisms have not been elucidated (191).

What Regulates Lipid Content and Rate of Lipid Synthesis?

The lipid content of rapidly dividing *E. coli* represents about 5 to 7% of the dry weight, and modifications of growth conditions do not alter this value by more than 50% (80, 87, 111, 287). Most of the ATP required for the biogenesis of membrane lipids is expended during the synthesis of fatty acids (34, 368). Consequently, the control of the overall pathway at a rate-limiting reaction in fatty acid synthesis would seem to be most plausible. Acetyl coenzyme A carboxylase deserves consideration in this regard, but the mechanism for its regulation in *E. coli* is uncertain (34).

There appears to be a tight coupling of phospholipid and fatty acid syntheses in bacteria (215, 239). Starvation of glycerol auxotrophs, such as mutants defective in the *plsB* and *gpsA* genes, results in a rapid inhibition of both fatty acid and phospholipid syntheses (239). The inhibition of fatty acid synthesis occurs even though the level of ATP remains high and macromolecular synthesis continues for about one generation (202, 239). Obviously, some mechanism exists which rapidly shuts off fatty acid synthesis when the available pool of *sn*-glycero-3-P becomes limiting. Although the biochemical basis for the phenomenon is unknown, it could play an essential role in the regulation of lipid content.

Since free fatty acids do not accumulate in *E. coli* during glycerol starvation (239), there is obviously no phospholipid breakdown. All experiments of this kind have been carried out with mutants incapable of degrading fatty acids

(239) (i.e., *fadE*). Furthermore, Nunn et al. (239) have presented evidence that the pool of endogenous acetate becomes markedly depleted during glycerol starvation, since exogenous [1-^{14}C]acetate becomes much more effective as a label for the small amount of lipid that continues to be made (239). The apparent reduction of the acetate pool can be misleading in short-term radiochemical labeling experiments and explains why Cronan et al. (81) initially reported that fatty acid synthesis continues during glycerol starvation.

These observations (215, 239) suggest that the inhibition of fatty acid synthesis during glycerol starvation could involve an early reaction in fatty acid synthesis or even in the formation of acetyl coenzyme A. Since free fatty acids do not accumulate, it is possible that all molecules of acyl carrier protein become esterified with completed fatty acids. Perhaps the depletion of the free acyl carrier protein pool is sufficient to explain the inhibition of fatty acid synthesis, or the elevated level of fatty acyl acyl carrier protein might inhibit a critical enzyme. To resolve these alternatives, it will be necessary to determine the levels of intermediates, such as acetate, acetyl coenzyme A, and acyl carrier protein, during glycerol starvation and to examine the effects of certain of these intermediates on the enzymes of fatty acid synthesis. The isolation of mutants which contain more or less acyl carrier protein than normal would also be helpful in this regard.

Additional regulation of the overall pathway could occur at the level of formation of common intermediates, such as phosphatidic acid and CDP-diglyceride. However, cloning and 10-fold overproduction of the acyltransferases does not lead to an increase in the total amount of membrane lipid (Bell, personal communication). The control of CDP-diglyceride synthesis has not been investigated. Since there is 20 times less CDP-diglyceride than phosphatidic acid and both intermediates turn over rapidly (280), it is conceivable that CDP-diglyceride synthesis is at least partially limiting for the pathway (280). Mutants and overproducers of this enzyme have not been isolated. An accurate determination of the levels of the intermediates of Fig. 4 in living cells would also be desirable.

As techniques for the fusion of exogenous lipid vesicles with intact cells are improved (156, 157), it should become possible to increase the phospholipid content dramatically by direct supplementation. The effect of such modifications on the rate of endogenous lipid synthesis could provide further insight into regulatory mechanisms.

Finally, it should be noted that most of the enzymes that make phospholipids are themselves embedded in the bilayer membrane (22, 376). Purification studies have revealed that these enzymes can be separated from each other without loss of activity (103, 142, 281, 318, 339). Although it is impossible to exclude protein-protein interactions in vivo, there is no evidence that these enzymes form a complex. If this is true, then the surface area of the bilayer might exert a regulatory influence on these enzymes, which could serve to balance the ratio of lipid to protein present in the membrane. For instance, when too much lipid is present, the surface density of the biosynthetic enzymes is reduced, which should reduce the rate at which new phospholipids are formed. Conversely, when too little phospholipid is present, the capacity to synthesize new lipids should increase, since the enzymes (which are membrane proteins) are closer together. This model of surface-dependent regulation is compatible with the results of Bell and co-workers, who examined the rate of lipid synthesis upon readdition of glycerol to glycerol auxotrophs which had been starved for this substance and had lipid-depleted membranes (202). They found it to be greater than for comparable supplemented cells (202).

Does Guanosine Tetraphosphate Play a Direct Role in Regulation of Lipid Synthesis?

The rate of total lipid synthesis can be influenced by the *rel* gene of *E. coli*. When a stringent strain (*rel*$^+$) is starved for a required amino acid, there is usually a two- to fourfold inhibition of the rate of phospholipid synthesis (123, 340, 341). The aeration of the culture must be carefully controlled in such experiments (240, 356). The inhibition of RNA synthesis that occurs during the stringent response is generally of a much greater magnitude than the effect on phospholipid biosynthesis (340).

Several laboratories have examined the interaction of guanosine tetraphosphate (ppGpp), which accumulates during the stringent response, with the enzymes of fatty acid and phospholipid metabolism (209, 270). The inhibition of acetyl coenzyme A carboxylase, characterized by Lane and co-workers, suggests that ppGpp might act at the level of fatty acid synthesis (270). Recent genetic studies support this view but point to an additional inhibition of phospholipid synthesis (234, 236, 238). Several investigators have demonstrated that ppGpp inhibits *sn*-glycero-3-P acyltransferase (192, 209). However, this depends on whether fatty acyl coen-

zyme A or fatty acyl acyl carrier protein is used as the substrate (192). The report (209) of an inhibition of phosphatidylglycerophosphate synthetase by ppGpp in vitro is especially puzzling, since the inhibition of lipid synthesis in vivo is not restricted to phosphatidylglycerol (234, 236, 238).

The precise role of ppGpp in the control of lipid synthesis may be difficult to define. Furano and Wittel (115) have demonstrated that the synthesis of dozens of different proteins is greatly altered during the stringent response. With the use of two-dimensional gel electrophoresis, they found that the levels of some proteins rise, whereas those of others fall (115). In view of this unexpected complexity, the partial inhibition of lipid synthesis that occurs during the stringent response may have multiple causes.

Recent work by Gallant et al. (117) indicates that the role of ppGpp may be quite different than that which had been envisaged on the basis of early studies of the stringent response. When wild-type (*rel*$^+$) cells of *E. coli* are shifted from 23 to 42°C, there is a dramatic rise in the level of ppGpp, which exceeds that observed during the stringent response both in magnitude and duration (117). Such a temperature shift does not lead to an inhibition of RNA synthesis, and actually it is accompanied by an acceleration of the growth rate and of RNA synthesis (117). These findings led Gallant et al. (117) to conclude that ppGpp has no direct inhibitory effect on RNA synthesis, as had been supposed from results obtained by starvation of amino acid auxotrophs. Though not measured directly, it is unlikely that there is any inhibition of lipid synthesis under the conditions described by Gallant et al. (117), despite the high intracellular levels of ppGpp. Therefore, the inhibition of lipid synthesis observed during the stringent response (123, 234, 236, 238, 240, 341) (i.e., starvation of an amino acid auxotroph) cannot be attributed to the rise in intracellular levels of ppGpp, and the partial inhibitions of specific lipid enzymes by ppGpp in vitro are unlikely to have any physiological significance (209, 270).

What Regulates the Length and Unsaturation of Fatty Acids?

In procaryotic and eucaryotic systems, the lengths of the hydrocarbon chains of the fatty acids are determined by the enzymes involved in their biosynthesis (34, 126, 368). Cronan et al. (81) have suggested that an additional control may exist at the level of phosphatidic acid formation, since the small amounts of free fatty acids that accumulate during the inhibition of lipid synthesis by *sn*-glycero-3-P starvation are slightly longer than normal (81, 239). Presumably, when palmitoyl or *cis*-vaccenoyl acyl carrier proteins cannot be utilized for phosphatidic acid synthesis, they undergo one or two additional rounds of elongation.

As in the case of chain length, the temperature-dependent control of fatty acid unsaturation (34, 96, 204, 250, 254, 368) can occur both at the level of fatty acid synthesis and at the acylation of *sn*-glycero-3-P (34, 69, 70, 334, 368). The former is probably mediated by the β,γ-hydroxydecanoyl coenzyme A dehydrase, and the latter is probably mediated by the two acyltransferases. The temperature dependence and specificity of the acyltransferases observed in vitro qualitatively account for the fatty acid distribution that exists in vivo (334). The inverse relationship between the amount of unsaturated fatty acid present in the membrane lipids and the growth temperature presumably reflects the need to regulate membrane fluidity (75, 329, 335).

What Regulates the Ratio of Polar Headgroups in Membranes?

Although many modifications of polar headgroup composition are now possible, the control of the normal headgroup ratios remains a mystery. Presumably, the enzymes and substrates of the CDP-diglyceride branch point play a critical role in this process (Fig. 4). The invariability of the ratio of the serine-derived phosphatides to the polyglycerophosphatides is particularly striking. Even when the decarboxylation of phosphatidylserine is blocked, the sum of phosphatidylserine plus phosphatidylethanolamine divided by the sum of phosphatidylglycerol plus cardiolipin is the same as in normal, growing cells (133).

Several models for the regulation of the headgroup ratio have been proposed, but there is little evidence to support these hypotheses. Separate pools of phosphatidic acid have been observed in some membrane preparations (151), although most studies indicate that there is a common pool of phosphatidic acid and liponucleotide available to both branches of the pathway (264, 353, 375). The preferential use of CDP-diglyceride for one branch of the pathway and of dCDP-diglyceride for the other (280) is improbable, since both phosphatidyltransferases (upon purification) utilize both liponucleotides effectively in vitro (142, 181). The phosphatidylethanolamine level is not limited by the amount of phosphatidylserine synthetase present in the cell, since a 10-fold overproduction of the enzyme does not significantly alter the polar head-

group composition (282).

Since various phospholipids influence the rate of phosphatidylserine synthesis in vitro (150), it is possible that the ratio of neutral to negatively charged phosphatides present in the membrane regulates the activity of the phosphatidyltransferases. This should be examined with the homogeneous transferases, using model bilayer membranes of defined composition. The surface charge may be an essential determinant of enzyme activity, as suggested by the work of Dennis and co-workers (92, 95). Another possibility that remains to be explored is the contribution of the pools of L-serine and *sn*-glycero-3-P to the relative rates of polar headgroup synthesis. This could be examined easily in chemostat cultures by growing auxotrophs on limiting concentrations of L-serine or *sn*-glycero-3-P.

In the case of phosphatidylserine synthetase, which is not membrane bound, one must consider the possibility that certain changes in membrane structure may limit the access of the enzyme to its membrane-bound substrate, CDP-diglyceride. Such an uncoupling of the synthetase would result in an inhibition of phosphatidylserine synthesis in vivo without any apparent effect on the enzymatic activity in vitro. Several mutants exist which appear to have this property, but their biochemical alterations and membrane compositions have not been determined (12, 68, 188, 194).

What Factors Activate Phospholipid Enzymes In Vivo?

As indicated in Table 3, almost all phospholipid enzymes are activated in vitro by nonionic detergents, such as Triton X-100 (103, 142, 181, 281, 318). In some cases, this requirement is absolute. What substitutes for the detergent in the cell is uncertain, but the existence of an endogenous detergent seems improbable. Perhaps the proper insertion and orientation of these enzymes in the membrane are critical for their biological function. This could be examined with several of the enzymes available in homogeneous form, using liposomes of defined composition (see Addendum in Proof). In any case, the in vitro turnover numbers and intracellular concentrations of phosphatidylserine synthetase (181), phosphatidylserine decarboxylase (103), and phosphatidylglycerophosphate synthetase (142) are sufficient to account for the rate at which membrane lipids are synthesized in vivo.

What Coordinates Membrane Lipid, Membrane Protein, and Macromolecular Syntheses?

Using glycerol and fatty acid auxotrophs to inhibit the de novo biosynthesis of membrane phospholipids, Fox and co-workers (112, 146, 357, 358, 382) concluded that ongoing lipid synthesis was required for the insertion of a functioning lactose permease into the cytoplasmic membrane. On the basis of facilitated diffusion assays at two temperatures (382), these investigators further concluded that the temperature dependence of the newly inserted permease was determined by the fatty acid composition of the newly made lipids and not by the fatty acid composition of the whole membrane. The relevance of these findings for the synthesis and insertion of other membrane proteins was questionable, since Mindich and co-worker could not demonstrate a lipid requirement for the biogenesis of several transport systems in gram-positive bacteria (214, 381).

Recently, several laboratories have reexamined the possibility of a lipid requirement for the induction of the *E. coli* lactose permease (235, 261, 350, 372). When the conditions of lipid deprivation are carefully controlled to minimize cell damage, there is no obvious lipid requirement for the induction of lactose transport in the cases of both fatty acid and glycerol auxotrophs (235, 372). Furthermore, a detailed examination of the temperature dependence of facilitated diffusion by Overath et al. (261) revealed that the newly made permease does indeed sense the average fatty acid composition of the membrane and that the earlier assays used by Wilson and Fox (382) were misleading.

Bell and co-workers (19, 200, 202) have used mutants defective in the *plsB* gene to study the synthesis of cytoplasmic and outer-membrane proteins under conditions resulting in the inhibition of total phospholipid synthesis. Both membranes have the capacity to accept about 50% more protein than is ordinarily present in exponentially growing cells (200). Continued membrane protein synthesis in the absence of lipid synthesis causes substantial increases in the buoyant densities of both membranes (200). As in the case of membrane proteins (200, 286), the synthesis and secretion of periplasmic proteins can also occur independently of lipid synthesis (17, 168). The recent development of in vitro systems for the synthesis of integral membrane proteins provides additional evidence against a lipid requirement for membrane protein insertion, both in procaryotic and in eucaryotic organisms (52, 302, 380). Instead, leader sequences at the amino termini of the nascent polypeptides appear to be critical for the insertion process (33).

On the basis of studies with mutants defective in the *plsA* gene, Glaser et al. concluded that all macromolecular synthesis in *E. coli* is rapidly

inhibited upon cessation of phospholipid synthesis (119). Subsequent work by the same investigators revealed that this could be attributed to the defective adenylate kinase present in these organisms (120). When phospholipid synthesis is inhibited selectively in mutants defective in the *plsB* gene, then macromolecular synthesis does not stop immediately but continues for about one generation (19, 200, 202). Furthermore, the nucleotide levels remain high throughout the period during which phospholipid synthesis is inhibited (202). Thus, macromolecular synthesis, like membrane protein synthesis, is not directly dependent on continual de novo phospholipid synthesis, nor does the initiation of DNA replication have a lipid requirement (351), as had been postulated earlier (113). Nevertheless, there must be some coordinate control of these processes in living cells, but the mechanisms of this regulation remain unknown.

When strains carrying the *plsB* or *gpsA* mutations are deprived of glycerol for one or two generations, they stop growing and their membranes contain more protein than normal (200, 202). When glycerol is again made available to such organisms, growth and macromolecular synthesis do not resume immediately (202). Initially, there is a burst of lipid synthesis, whereas there is a lag in the resumption of DNA, RNA, and protein synthesis (202). Presumably, the ratio of protein to lipid in the membrane must be normalized before these processes can resume.

TOPOLOGY OF MEMBRANE LIPID SYNTHESIS

With the exception of phosphatidylserine synthetase (279), all of the biosynthetic lipid enzymes are associated with the cytoplasmic membrane (22, 365, 376). Since the common precursors (i.e., *sn*-glycero-3-P, L-serine, CTP, and fatty acids) are formed in the cytoplasm, it is likely that the active sites of these enzymes are oriented towards the interior of the cell. This could be examined directly by preparing antibodies to those enzymes available in homogeneous form (103, 142) and studying the binding of these antibodies (or antibody subfractions) to isolated membranes. The cytoplasmic membrane can be prepared as right-side-out or inside-out vesicles (116, 159, 295) and can be separated from the outer membrane by ultracentrifugation (259, 260).

If the de novo synthesis of membrane lipids occurs on the inner leaflet of the cytoplasmic membrane, then mechanism(s) must exist for the translocation of the newly made lipids to the opposite surface of the bilayer. In model systems, such phospholipid flip-flop is barely detectable (301). However, in growing gram-positive bacteria this process is very rapid, since a lag of no more than several minutes is observed between the time that new phosphatidylethanolamine is made and the time at which it appears in the outer surface of the membrane (299, 300). It is possible that catalytic factors (flippases) are needed for the translocation of phospholipids across the bilayer, although no specific factors have been identified. It is conceivable that some of the biosynthetic enzymes themselves are responsible for phospholipid flip-flop, and this could be examined with homogeneous enzymes in model systems. Alternatively, the preferential growth of the inner leaflet of the cytoplasmic membrane may generate a driving force for flip-flop not requiring specific catalytic proteins.

Although well documented in gram-positive bacteria (14, 26, 30, 299, 300), very little information is available on the possible asymmetry of polar phospholipid headgroups in the cytoplasmic membrane of *E. coli*. More could be learned about this problem by examining isolated inner membranes of known orientation (116, 159, 295). In the outer membrane of *S. typhimurium*, less phospholipid is exposed on the external surface than on the internal side (338). Presumably, the lipopolysaccharide (which is largely oriented towards the outside) can substitute for phospholipid molecules in the external leaflet of the bilayer (Fig. 1). The periplasmic localization of the MDO may also be related in some way to polar headgroup asymmetry in the cytoplasmic membrane.

The mechanism and topology of outer membrane assembly are especially perplexing. All outer membrane components, including the lipids, are first synthesized on the inner membrane and subsequently assembled to form the outer membrane (258, 260). Preliminary studies indicate that lipids, unlike proteins or lipopolysaccharide, can move freely from the inner membrane to the outer membrane and back (156, 157). Even non-physiological lipids, such as phosphatidylcholine, which can be introduced into the outer membrane by vesicle fusion with deep rough mutants, rapidly distribute themselves between both membranes (156, 157). The distribution of the abnormal lipids that accumulate in various polar headgroup mutants (Table 5) has not been examined.

Two general mechanisms have been proposed for the translocation of phospholipids from the inner membrane to the outer membrane (156, 157, 260). (i) Specific phospholipid translocators may be present in the periplasmic space, resembling the soluble phospholipid exchange proteins of eucaryotic cells which transfer phosphatidyl-

choline and other lipids from microsomes to mitochondria and vice versa in vitro (35, 383). As yet, proteins of this kind have not been found in *E. coli*. (ii) Zones of adhesion between the two membranes are observed by electron microscopy, and it is conceivable that phospholipids can flow freely through these regions (157, 186, 218). Unfortunately, no direct biochemical evidence exists to support this possibility. Characterization of mutants defective in outer membrane synthesis and assembly might shed some light on these problems.

ROLE OF POLAR PHOSPHOLIPID HEADGROUPS IN MEMBRANE FUNCTION

The elucidation of polar headgroup function in cell membranes is a major goal of phospholipid genetics. The importance of fatty acids in membrane processes is well documented (see references 75, 226, and 329 for reviews), but comparable studies of polar headgroup function do not exist. The situation should change in the next few years with the availability of specific headgroup mutants (133, 246, 269a, 274, 275, 283).

Involvement of Specific Lipids in Transport and Antibiotic Permeation

The most convincing evidence for the involvement of a specific phospholipid headgroup in a transport system has been presented by Kundig and Roseman (179, 180). They found that phosphatidylglycerol activates enzyme II of the phosphotransferase system (179, 180). Attempts to inhibit the transport system by incubation of membrane vesicles in vitro with specific lipases did not lead to clear-cut results (190, 213). An examination of the function of the phosphotransferase system in membranes containing more or less phosphatidylglycerol than normal would be of considerable interest.

As yet, no transport functions have been studied in the existing mutants altered in polar headgroup composition. However, Ohta et al. (249) have used hydroxylamine to cause the accumulation of phosphatidylserine in vivo and have examined the activity of various amino acid transport systems in membrane vesicles prepared from such cells. Significant stimulations of serine and glutamate transports were noted. Unfortunately, hydroxylamine interferes with the activities of many different enzymes, and therefore secondary effects cannot be excluded.

From studies with mutants in the *pss* gene, it is clear that polar headgroup substitutions can lead to striking changes in the permeability of the outer membrane (277). Mutants defective in phosphatidylserine synthetase are hypersensitive to several hydrophilic antibiotics, especially to the aminoglycosides (277). Since the overall membrane protein, fatty acid, and lipopolysaccharide compositions are relatively normal under conditions leading to pronounced antibiotic hypersensitivity (277), the polar headgroups may play an important role in maintaining the barrier function of the outer membrane. Specific inhibitors of phosphatidylserine synthetase might be useful for potentiating the action of numerous antibiotics currently used for the treatment of gram-negative infections (277). Some antibiotic hypersensitivity is also observed in mutants defective in phosphatidylserine decarboxylase (277), whereas little or no change in the antibiotic sensitivity is observed with mutants defective in cardiolipin synthetase and diglyceride kinase (Raetz, unpublished data).

Despite an earlier report to the contrary (189), chemotaxis does not appear to depend on the fatty acid composition or fluidity of the membrane lipids (211). This process has not been studied in mutants altered in polar headgroup synthesis.

Phospholipids as Activators of Membrane-Bound Enzymes

A large number of membrane-bound enzymes are not catalytically active in the absence of phospholipids (136). Usually, this is discovered when the purification of such enzymes is attempted. Of the enzymes involved in phospholipid biosynthesis that have been studied, only the *sn*-glycero-3-P acyltransferase exhibits a strong dependence upon added phospholipids after extraction from the membrane (339). Various different polar headgroups suffice for this purpose (339). Diglyceride kinase is stimulated preferentially by cardiolipin (318), but the physiological significance of this phenomenon is unclear, since mutants defective in the synthesis of cardiolipin (269a) do not accumulate diglyceride in their membranes (Raetz, unpublished data).

Additional enzymes which are dependent on or influenced by, the presence of phospholipids include the C_{55} isoprenoid alcohol kinase (140, 305), enzyme II of the phosphotransferase system (179, 180), the membrane-bound adenosine triphosphatase (109, 265), reduced nicotinamide adenine dinucleotide oxidase (88, 106), and several others (109, 136, 167, 319, 337). The homogeneous UDP-galactosyltransferase involved in lipopolysaccharide formation has been studied extensively and is active as a ternary complex with phosphatidylethanolamine and lipopolysaccharide (141, 297). The mutants with altered polar headgroup compositions (Fig. 6) should be

examined for changes in the activities of these membrane-bound enzymes.

The recent report of phosphatidylserine as the covalent amino terminus of certain membrane proteins in gram-positive organisms (4, 387–389) makes it necessary to search for similar structures in *E. coli*. One must also consider the possibility that certain membrane enzymes contain tightly bound (but noncovalent) phospholipid molecules at their active sites. These lipids might not readily exchange with the bulk of the phospholipids in the membrane bilayer or be removed during purification. None of the homogeneous enzymes of phospholipid biosynthesis now available (103, 142, 181) (Table 3) have been examined for the presence of stoichiometrically bound lipid. Recently, Khorana and co-workers have synthesized a series of fatty acids substituted with various photoactivable side chains (50, 125). These can also be incorporated into the membrane phospholipids by the use of appropriate fatty acid auxotrophs (125), opening additional chemical approaches to the study of lipid-lipid and lipid-protein interactions.

Phospholipid Biosynthesis During Bacteriophage Infection

Infection of *E. coli* with bacteriophage T4 has two major effects on the biogenesis of membrane lipids. It causes the release of free fatty acids by activation of the detergent-resistant phospholipase A (40, 43, 158, 227, 303, 366), and it partially inhibits the production of phosphatidylethanolamine (208, 266). It seems unlikely that either of these effects is essential in the life cycle of the bacteriophage (243), and de novo phospholipid synthesis is not essential for the growth of T4 (237). The release of free fatty acids does not occur in appropriate lipaseless mutants (243, 303). The suggestion (333) that an increased ratio of cardiolipin to phosphatidylglycerol interferes with bacteriophage assembly is equally improbable, since the mutants in phosphatidylserine synthetase (which accumulate substantial amounts of cardiolipin even under permissive conditions) support the growth of T4 (277).

A more striking inhibition of phosphatidylethanolamine synthesis occurs during infection of *E. coli* with amber mutants of bacteriophage f1 (56, 244, 384). As in the phosphatidylserine synthetase mutants, this results in the accumulation of cardiolipin with a concomitant reduction in the amount of phosphatidylethanolamine (275). When a cardiolipin synthetase mutant of *E. coli* is similarly infected, phosphatidylglycerol accumulates, arguing against a stimulation of cardiolipin synthesis in wild-type cells (269a). In view of the results obtained with the cardiolipin synthetase mutant (269a), it seems unlikely that cardiolipin and coat protein normally enter the membrane together in vivo (51). In the absence of total lipid synthesis, insertion of M13 coat protein into the membrane continues, though at a reduced rate (49). It is conceivable that the presence of a large amount of coat protein in the inner membrane somehow inhibits the enzymes involved in phosphatidylethanolamine synthesis.

Sands and others have characterized a novel bacteriophage of *E. coli* (designated PR4) which contains tightly bound phospholipids enriched in phosphatidylglycerol (39, 44, 307–309) and, possibly, phosphatidylserine (309). This does not infect *E. coli* unless the host cell also carries a specific drug resistance plasmid (39), which presumably directs the synthesis of an R-pilus required for virus attachment. This system affords several new approaches to the study of membrane assembly in *E. coli*, since PR4 probably utilizes host functions to acquire its phospholipid coat.

ESSENTIAL AND NONESSENTIAL MEMBRANE PHOSPHOLIPIDS

The studies of the past 5 years have made it possible to determine which aspects of lipid structure and metabolism are essential for cell growth and which are not.

(i) The total phospholipid content is essential. The cell does not possess a large unused reserve of phospholipids. Genetic inhibition of total lipid synthesis causes a cessation of growth after one cell division (19, 137, 202).

(ii) The membrane must contain some fluid and some nonfluid fatty acids at all temperatures. At any given temperature, the membrane fluidity can be varied widely in fatty acid auxotrophs before growth is inhibited (75, 329). However, some saturated and some unsaturated fatty acids are needed under all conditions that have ever been examined (74, 75, 329). Cyclopropane or branched fatty acids may serve as substitutes for unsaturated fatty acids (329, 331). Presumably, some fluid and some solid domains must always be available (9, 74). At lower temperatures, the minimum amount of required saturated fatty acid is less than at higher temperatures (75, 329).

(iii) The ratio of dipolar ionic to negative polar headgroups is essential. Negatively charged polar headgroups constitute about 15 to 20% of the total in wild-type strains (133, 275). When this amount is doubled (by genetic means), there is

already a marked impairment of the growth rate (133, 275). When the phosphatidylethanolamine level falls below 50%, growth gradually ceases (246, 275). As a rule, this is accompanied by filamentation, suggesting an inhibition of cell division (133, 275). In contrast to phosphatidylethanolamine synthesis, the genetic inhibition of cardiolipin synthesis has no effect on growth and is not accompanied by a change in headgroup charge (269a). The importance of phospholipid charge has also become apparent from studies of choline and inositol auxotrophs of yeasts and fungi (18, 147). If charge is essential, then mutants unable to make phosphatidylglycerol should also prove to be conditional lethals. An examination of physical properties (such as microviscosity) in membranes with altered polar headgroup composition will be of great interest.

(iv) Phospholipid turnover and catabolism are not essential for growth. This conclusion is based on studies with mutants unable to synthesize MDO or lacking certain of the phospholipases (101, 243, 320, 321). Obviously, specific membrane functions not needed for cell division may be altered. These have not been identified. Turnover and catabolism may also be essential in membrane adaptation to rapid environmental changes, but not for growth under laboratory conditions.

The above generalizations about essential and nonessential membrane lipids are based primarily on genetic studies. In the next 5 years, these principles will be refined as additional techniques for the modification of the lipid composition become available. Many of the questions concerning the regulation and assembly of membrane lipids (see Regulation of Membrane Lipid Synthesis and Its Role in Membrane Assembly) should be answered, and generalizations about the mechanism, structure, and insertion of phospholipid enzymes in membranes should emerge.

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ADDENDUM IN PROOF

Instead of 5 M NaCl, much lower concentrations of spermidine (but not putrescine) facilitate the removal of the phosphatidylserine synthetase from ribosomes (W. Dowhan, unpublished data). Furthermore, the synthetase physically interacts with mixed micelles of Triton X-100 and CDP-diglyceride, as judged by glycerol gradient centrifugation, and a high ionic strength, which is required for catalytic activity (Table 3), is also required for this interaction of the enzyme with mixed micelles (W. Dowhan, personal communication). G. M. Carman and W. Dowhan (unpublished data) have prepared mixed liposomes consisting of *E. coli* phospholipids and CDP-diglyceride (10%, wt/wt) and have found these to function as an effective substrate for the homogeneous synthetase in the absence of Triton X-100.

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