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MOLECULAR GENETICS OF MEMBRANE PHOSPHOLIPID SYNTHESIS

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INTRODUCTION

Biological membranes are comprised of hundreds of protein and phospholipid components (57, 110, 138, 237). Many of the proteins function in transport, energy transduction, and biosynthesis (6, 22, 83, 88, 98, 181, 235, 237). The significance of the various phospholipids (Figure 1), of which there are 100 to 1000 distinct chemical species in prokaryotes and eukaryotes, respectively (22, 88, 181, 235), is less obvious. Certain eukaryotic phospholipids esterified with arachidonic acid serve as precursors of prostaglandins and leukotrienes (205, 206), but the functions of most other phospholipid species remain obscure (22, 88, 181, 182, 235). Much has been written about the role of phospholipids in maintaining the physical properties of membranes (2, 31, 77, 219), yet the biological significance of physical studies is difficult to evaluate. The recent recognition of *sn*-1,2-diglyceride (derived from phosphatidylinositol-4,5-*bis*-phosphate turnover in animal cells) as a regulator of protein kinase C (14, 65, 94, 157), and the discovery of platelet-activating factor (an ether-containing phosphatidylcholine variant) (81, 221, 235), suggests that important biological functions of common lipid molecules may have been overlooked. Many other phospholipid functions likely remain to be discovered.

In this article I review the application of genetics to the problem of phospholipid heterogeneity. The isolation of mutants in phospholipid synthesis is a relatively recent endeavor (181, 182) and is important for the following reasons: (*a*) the biological relevance of pathways deduced from enzymological studies can be evaluated; (*b*) the number of structural and regulatory genes involved in the system can be determined; (*c*) the availability of mutants simplifies the molecular cloning of the genes; (*d*) a collection of mutants facilitates the testing of hypotheses concerning regulation and function; and (*e*) mutants in a system not previously characterized by genetics may reveal entirely new insights into biochemistry and physiology.

The properties of phospholipid mutants should be regarded in terms of the molecular structures affected. Since many different molecules are involved, I include a complete set of formulas. I focus primarily on the membrane phospholipids of *Escherichia coli* and discuss only briefly the extension of phospholipid genetics to animal cells (54). Recent progress with the yeast *Saccharomyces cerevisiae* is summarized elsewhere (90, 152, 153).

In the last five years mutant isolation and molecular cloning have progressed substantially. One of the most important discoveries to emerge from the genetics of the *E. coli* system was the identification of a new class of phospholipids, derived from glucosamine instead of glycerol, that act in the assembly of the outer monolayer of the outer membrane (4, 29, 154, 156, 183, 184, 194, 228). The development of sensitive techniques for the analysis

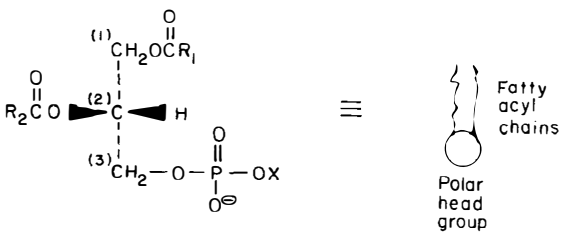


Figure 1 Generalized structure of a glycerophospholipid. In *E. coli* (181) the major polar moieties (*x*) are ethanolamine and glycerol, the whole molecule being referred to as phosphatidylethanolamine and phosphatidylglycerol, respectively. In phosphatidic acid, a key intermediate, X ≡ H. The predominant fatty acyl moieties (R_1 and R_2) are palmitate, palmitoleate and *cis*-vaccenate (181). A saturated fatty acid usually occupies the *sn*-1 position (number indicated in parentheses). Additional minor fatty acids include myristate, laurate, and the cyclopropane derivatives of palmitoleate and *cis*-vaccentate, both of which accumulate in stationary cells (181). The glycerophospholipids of *E. coli* contain no β-hydroxymyristate (181), which is only found on lipid A. In animal cells there are additional polar moieties, including *myo*-inositol and choline (22, 54). Further, there are many different polyunsaturated fatty acids, the incorporation of which depends on culture conditions, or nutritional factors (22, 54).

of the phospholipid compositions of mutants also led to the recognition of a vast number of minor, unidentified components (see below). In the future, phospholipid mutants must be exploited in new ways to provide insights into the more difficult problems of regulation and function.

LIPID MOLECULES IN *Escherichia coli*

Escherichia coli and other gram-negative bacteria are enclosed by an envelope (Figure 2) consisting of two membranes (166), separated by the periplasm (23) and the peptidoglycan (145). *E. coli* does not contain internal membranes or organelles (43). All lipids and related molecules are localized in the envelope. However, the early precursors of fatty acids and of polar headgroups are synthesized in the cytoplasm (181).

The biosynthesis and turnover of the predominant lipids from their precursors (Figures 3–5) are catalyzed by enzymes, most of which are minor membrane proteins. With a few exceptions (181), these enzymes function on the cytoplasmic surface of the inner membrane (12, 181, 243). The mechanisms by which lipids and proteins synthesized on the inner membrane are transported to the outer membrane are unknown. Zones of adhesion between the inner and outer membranes are visible by electron microscopy (9), and are postulated to play a role in intermembrane transport, but the composition and ultrastructure of these important domains are not well defined (106).

Phospholipids and related compounds in *E. coli* may be divided into four major classes. These divisions are: (*a*) the glycerophospholipids (Figures 1–4)

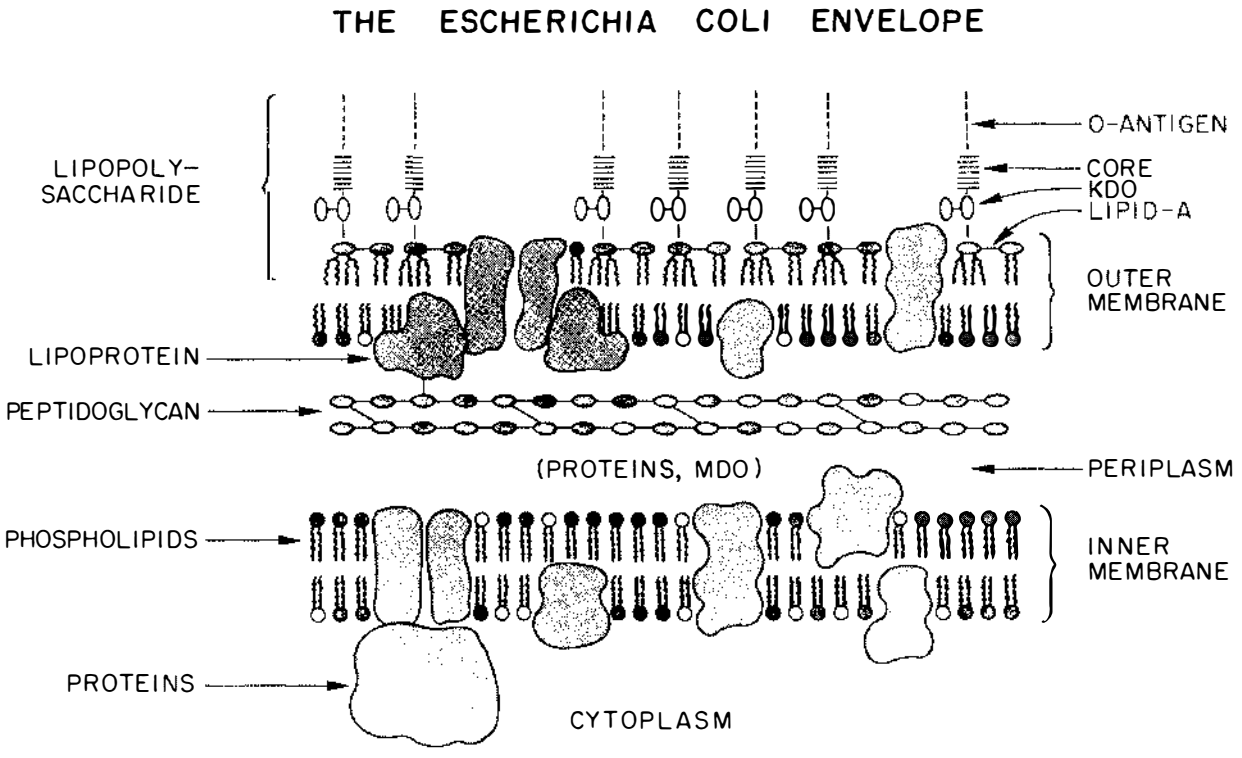


Figure 2 Schematic representation of the membrane lipids and proteins in the *Escherichia coli* envelope. Glycerophospholipids are depicted as in Figure 1; sugar residues are drawn as ovals. The proteins of the inner and outer membranes are different, and there are 50–100 distinct proteins in each membrane (3, 139, 181). The MDOs (membrane-derived oligosaccharides—Figure 6) are found in the periplasm (181, 209). Bacterial lipoproteins (24, 245) characteristically have the amino terminals shown in Figure 7. The biosynthesis and structures of the glycerophospholipids (181) are shown in Figures 3 and 4, and the biochemistry of lipid A (197) is shown in Figure 5. The minimal lipopolysaccharide required for growth of *E. coli* consists of the lipid A domain and at least two KDO (3-deoxy-D-*manno*-octulosonic acid) residues (20, 197).

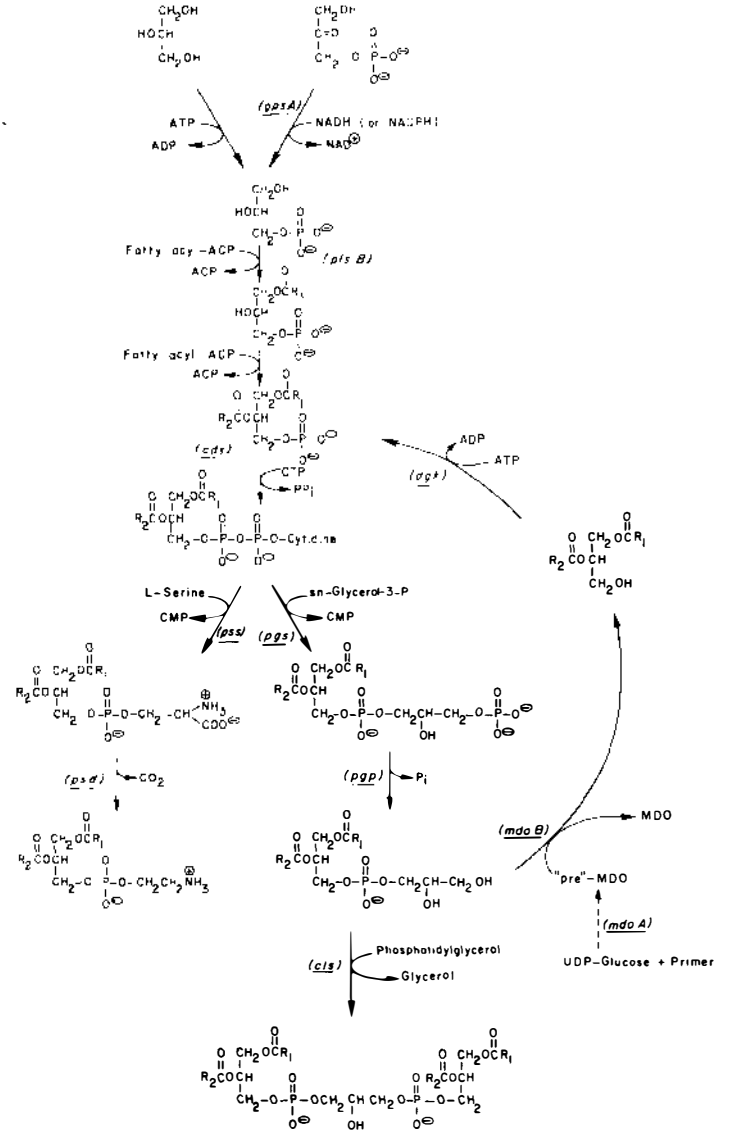


Figure 3 Enzymatic synthesis of glycerophospholipids in *E. coli*. Genetic symbols adjacent to specific reactions indicate the existence of mutants. R_1 and R_2 are defined in Figure 1.

($\sim 2 \times 10^7$ molecules per cell; 181); (*b*) the lipid A component of lipopolysaccharide (Figures 2 and 5), prominent in the outer monolayer of the outer membrane (197) ($\sim 2 \times 10^6$ molecules per cell); (*c*) the membrane-derived oligosaccharides (Figure 6), which are water-soluble substances (mol wt

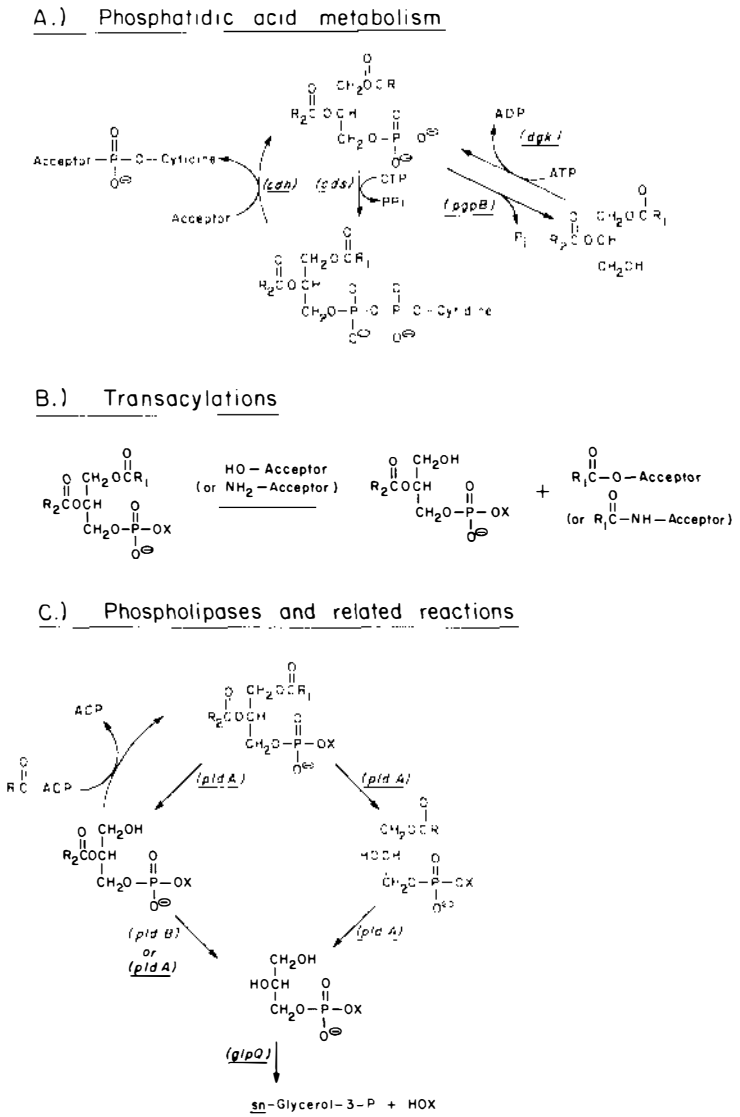


Figure 4 Ancillary glycerophospholipid interconversions and degradations in *E. coli*. The biological or regulatory significance of these reactions is not known (1, 27, 97, 101, 107, 113, 116, 117, 181, 201, 242). R_1 and R_2 are defined in Figure 1. Transacylations usually involve a diacylglycerophospholipid (phosphatidylethanolamine or phosphatidylglycerol) as the donor (97), and an acceptor, which may be a lysophospholipid (97), lipid X (C. R. H. Raetz & K. Brozek, unpublished results), or the unmodified amino terminal cysteine residue of a lipoprotein precursor (107, 245). A distinct acyl-ACP-dependent enzyme can re-acylate lysophosphatidylethanolamine (201).

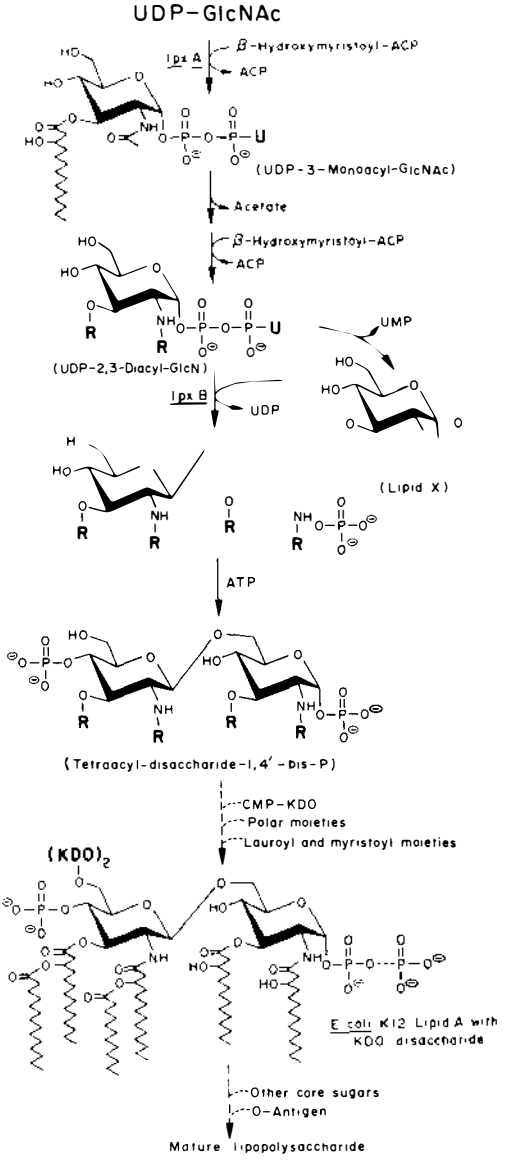


Figure 5 Enzymatic synthesis of lipid A in *E. coli*. *R* designates a β-hydroxymyristoyl moiety, having the 3*R* stereochemistry, and *U* designates uridine. The biochemistry of KDO is reviewed elsewhere (234). The structure shown at the bottom is the minimal one that appears to be required for cell growth (20, 197), but some of the molecules are substituted with a pyrophosphate residue, as indicated by the broken line. "Wild-type" lipopolysaccharide is much more complicated than this because many different core and O-antigen sugars, as well as other polar decorations, are added (197).

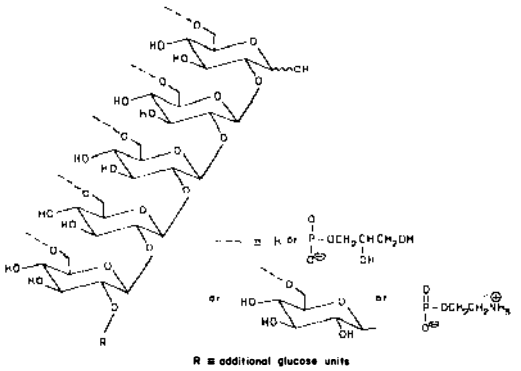


Figure 6 Partial covalent structures of the membrane-derived oligosaccharides. There are 6–10 glucose residues per MDO molecule (mol wt ~2,000), with 2–3 β,1→6 branches at unknown locations (115, 207, 236). MDO molecules contain one (or more) glycerol 1-phosphate residues (attached via phosphodiester linkage to the 6 position(s) of glucose), and a smaller subset of MDOs are similarly derivatized with phosphoethanolamine. A significant portion of the MDO molecules are also esterified with succinate and/or acetate (E. P. Kennedy, personal communication), but the locations of these residues are unknown.

~2,000) localized in the periplasm and formed in conjunction with glycerophospholipid turnover at low osmolarity (114, 181); and (*d*) the bacterial lipoproteins (Figure 7), which contain a 2,3-diglyceride residue attached by a thioether linkage to an amino terminal cysteine (24, 245). *E. coli* also contains small amounts of the polyisoprene, "bactoprenol," that functions as a carrier of sugar moieties during peptidoglycan and O-antigen syntheses (145, 151).

Each of the above molecular classes is a complex mixture of 10–100 distinct structures (115, 181, 197, 245). The diversity of *E. coli* phospholipids is dramatically illustrated by the following simple experiment. If $^{32}P_i$-labeled phospholipids are extracted and analyzed with an appropriate two-dimensional thin-layer chromatography system, followed by autoradiography, dozens of minor lipid components (not easily accounted for by the biochemistry shown in Figures 3–5) are detected upon prolonged exposure (Figure 8). The recognition of the extreme heterogeneity of these "minor" phospholipids, each representing 0.01–0.1% of the total, is very recent. A careful evaluation of the structures and functions of these unknowns is necessary.

FUNCTIONAL IMPLICATIONS OF LIPID MOLECULES IN *E. coli*

All living organisms have the enzymes necessary to generate a diverse collection of lipid molecules, as illustrated for *E. coli* in Figures 3–7. Mutants

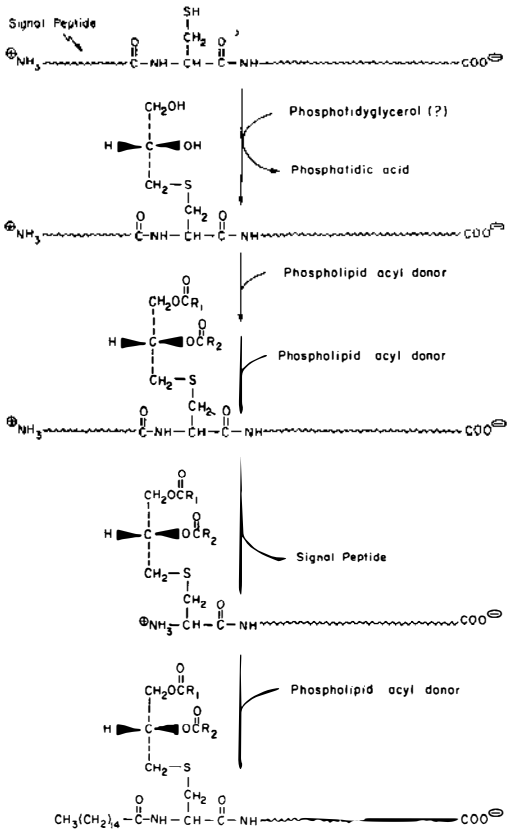
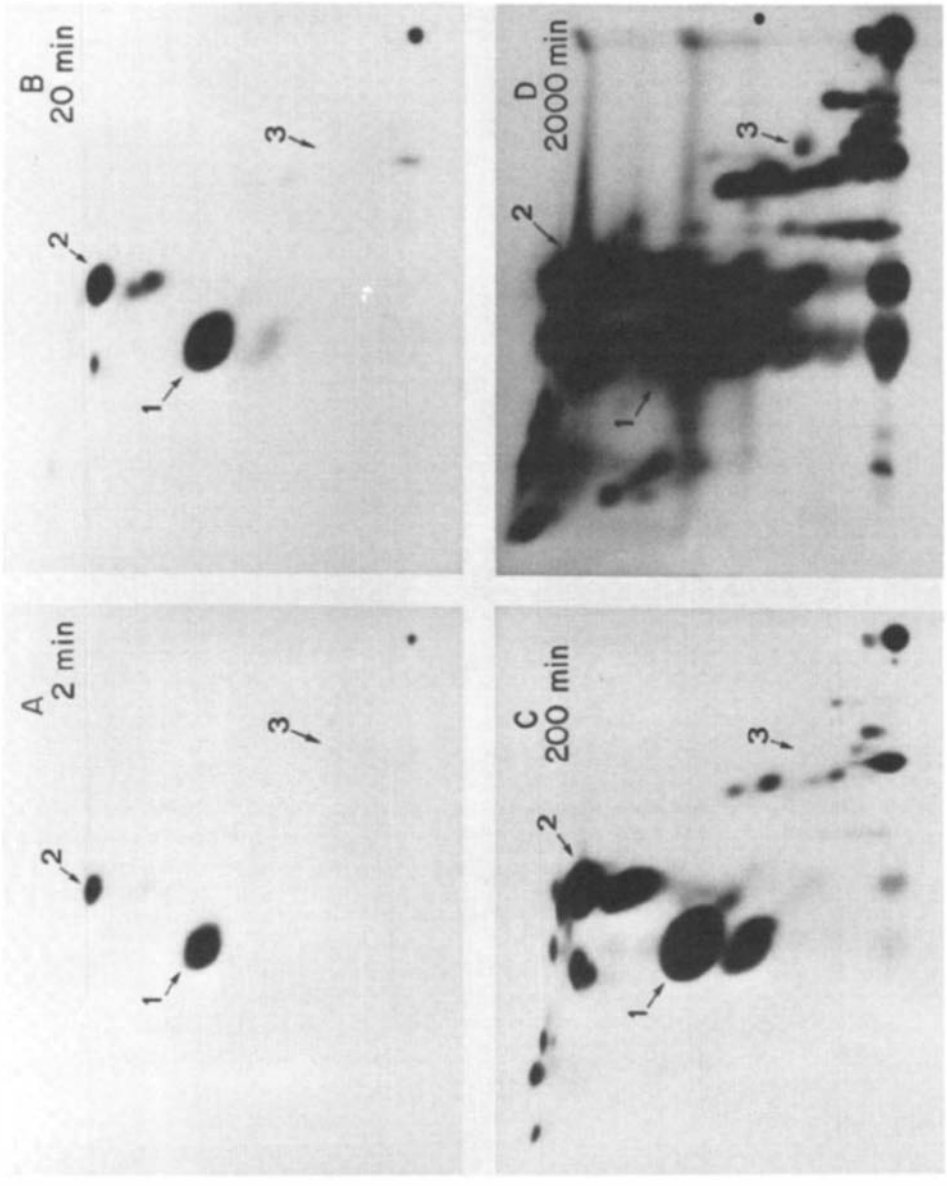


Figure 7 Hypothetical pathway for the biosynthesis of the amino terminus of a typical bacterial lipoprotein. A family of these proteins has been identified in *E. coli,* the most abundant of which is coded for by the *lpp* gene (Braun's lipoprotein) (24, 245). The above scheme is adapted from the work of Wu et al. (245). With the exception of the signal peptidase, none of the enzymes involved have been characterized in depth. The amino acid sequence at the cleavage site is always leu-ala-gly-cys (245).

defective in lipid synthesis are often conditionally lethal, as discussed in detail below (35, 90, 181, 182). This observation implies that lipids have important functions in biological membranes.

Lipid function is, however, difficult to evaluate because most lipid molecules were first discovered as chemical entities rather than as factors capable of triggering biological responses. The same problem is now frequently encountered with proteins isolated from high-resolution electrophoretic gels. Knowledge of the structure of such proteins rarely provides a clue to an enzymatic or a hormonal activity. For instance, many of the outer membrane proteins of *E. coli* may have functions not as yet appreciated (44). In the case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of lipids, the determination of function is made even more difficult by the fact that few X-ray structures are available (84) and almost nothing is known about lipid–protein interaction at the three-dimensional, molecular level.

The few lipids implicated in membrane functions (i.e. *sn*-1,2-diglyceride in protein kinase C activation and 1-alkyl-2-acetyl glycerophosphorylcholine in platelet aggregation) were not predicted a priori to have these roles, but rather were identified as biologically active fractions (14, 81, 157, 221). The "function" of *sn*-1,2-diglyceride, in particular, was studied only in higher eukaryotic cells (14, 65, 157) and was not genetically verified. As discussed below, *E. coli* also contains a small pool of diglyceride, the size of which increases dramatically in diglyceride-kinase mutants (181), but the role of this material in signal transduction has not been explored.

In the absence of more specific information, it is plausible that the major species of phosphatidylethanolamine and phosphatidylglycerol in *E. coli* function to maintain an optimal gel/liquid-crystalline transition temperature and a stable bilayer (2, 31, 77, 219). Some molecular species may be sequestered into nonbilayer domains (41, 199, 219), but unfortunately no reliable methods exist for demonstrating small, localized domains in biological systems. Members of the major phospholipid species of *E. coli* also serve as precursors of the phosphodiester moieties of the membrane-derived oligosaccharides (MDOs) (Figure 6) (56, 109), the latter possibly functioning in periplasmic osmoregulation (114). In addition, the major glycerophospholipids are the donors of the fatty acyl moieties present on the bacterial lipoproteins (Figure 7) (107, 233). The significance of fatty acylation as a posttranslation modification is unclear, however, and mutants defective in protein acylation are not yet available.

The function of lipid A and lipopolysaccharide may be related to their

Figure 8 Molecular complexity of the minor phospholipids of *E. coli*. Wild-type cells, growing exponentially on a low-phosphate medium (28), were labeled for several generations with $^{32}P_i$ (150 μCi/ml). The cells were rapidly harvested by filtration (28), and the phospholipids were extracted as described previously, except that 10 mM ammonium acetate pH 7 was used as the aqueous component, instead of phosphate-buffered saline (28). After adding 1 ml of $CHCl_3$ and 1 ml of 10 mM ammonium acetate, the phases were separated by centrifugation. The lower $CHCl_3$-rich phase ($\sim 10^7$ cpm) was dried under N_2, redissolved in a small volume of $CHCl_3$:MeOH:H_2O (66:33:4, v/v), mixed with carrier phosphatidylethanolamine, phosphatidylglycerol and lipid X (40 μg of each) (28), and spotted on a silica gel 60 plate (as indicated by the spot in the lower right corner of each panel). This was developed in two dimensions, as described previously (28), but with $CHCl_3$:MeOH:acetic acid:H_2O (35:15:3:2, v/v) as the first solvent (right to left), and $CHCl_3$:pyridine:88% formic acid:H_2O (35:30:8:0.8, v/v) as the second (bottom to top). The plate was dried and subjected to autoradiography for the times indicated. The method described above does not separate species differing only in the common fatty acyl moieties (R_1 and R_2 in Figure 1). Arrow #1: phosphatidylethanolamine; #2, phosphatidylglycerol; and #3, lipid X.

tendency to self-associate, which is especially observable in organic solvents when compared to glycerophospholipids (63, C. H. R. Raetz, unpublished). The relatively large polar headgroup of lipid A (Figure 5) may favor strong noncovalent interactions between adjacent molecules, particularly in the presence of divalent cations (183). A monolayer of lipid A might make *E. coli* more resistant to intestinal detergents and other environmental stresses. Physical studies of lipid A and its precursors are now possible and are important in understanding the role of lipid A in gram-negative envelopes.

The most specific and interesting lipid functions may eventually be assigned to the minor, unknown components (shown in Figure 8). As noted above, many types of transmembrane signalling in higher eukaryotes have been attributed to *sn*-1,2-diglyceride formation derived from the turnover of minor, phosphorylated metabolites of phosphatidylinositol (14, 65, 157). A prokaryotic counterpart to the "phosphatidylinositol cycle" has not been reported, but in this context, a role for some of the minor lipids of *E. coli* as modulators of chemotaxis (140, 218) deserves consideration. Certain minor lipids might also function as regulators of the enzymes involved in the synthesis of the major components (Figures 3–5) or in other membrane-associated processes.

The elucidation of lipid regulation and function requires the development of new hypotheses that must be tested with well-defined lipid mutants. In this context, I summarize the properties of the existing lipid mutants and indicate what additional mutants need to be isolated.

THE NUMBER OF "LIPID GENES" IN *E. coli*

The broad scope of lipid genetics can be appreciated by estimating the number of *E. coli* genes required for the assembly of the four molecular categories (Figures 3–7). The problem can be subdivided by considering the known (or surmised) components of metabolism: (*a*) biosynthesis and degradation of fatty acids (not shown; see 59, 158, 235); (*b*) synthesis and turnover of the glycerophospholipids (181) (Figures 3 and 4); (*c*) biosynthesis of the MDOs (109, 242) (Figures 3 and 6); (*d*) posttranslational modification of the bacterial lipoproteins (232, 233) (Figure 7); and (*e*) biosynthesis of the lipid A (184) (Figure 5). The minimum number of required structural genes is approximately eighty. Because regulatory genes (186, 223), proteins [to catalyze the flip-flop and translocation of lipids within the envelope (16, 25, 204)], and many minor unidentified components must also be considered (Figure 8), there might be several hundred lipid genes. This number represents 3–6% of the *E. coli* genome (8). Not more than one-quarter of these (Tables 1 and 2) have been identified. Important genes, such as those for acetyl CoA carboxylase or acyl carrier protein (both required for de novo fatty

Table 1 *E. coli* genes involved in the synthesis and degradation of phospholipid precursors

Gene symbol	Map position (min)	Enzyme affected[a] [mutant phenotype(s)]	References
acpS	43	Holo[ACP]synthase (pantothenate requiring)[b]	174
cfa	37	Cyclopropane fatty acid synthase (normal growth, absent CFAs)[b]	75, 76
fabA	22	β-Hydroxydecanoylthioester dehydrase (unsaturated FA auxotroph)[b]	38, 214
fabB	50	β-Ketoacyl-ACP synthase I (unsaturated FA auxotroph)[b]	47, 68, 214
fabD	24	Malonyl-CoA-ACP transacylase (TS growth)[b]	82
fabE	72	Putative acetyl CoA carboxylase (uncharacterized temperature sensitive)	216
fabF	24	β-Ketoacyl-ACP synthase II (normal growth without temperature regulation of FA composition)[b]	68
fadA	86	β-Subunit of FA oxidation complex (unable to grow on FA)[b]	8, 158, 249
fadB	86	α-Subunit of FA oxidation complex (unable to grow on FA)[b]	8, 158, 249
fadD	40	Acyl-CoA synthase (unable to grow on FA)[b]	8, 158
fadE	5	Electron transport flavoprotein of β-oxidation (unable to grow on FA)[b]	8, 158
fadL	51	Transport of long chain FA (unable to grow on long chain FA)[b]	17, 158
fadR	26	Negative regulatory gene for *fad* regulon and *aceBA* operon (constitutive for β-oxidation)	8, 158
glmS	84	Glucosamine phosphate synthase (glucosamine auxotroph)	8, 246
gpsA	81	*sn*-Glycerol 3-phosphate dehydrogenase (glycerol auxotroph)	8, 33, 36
pyrG	60	CTP Synthase (cytidine auxotroph)	8, 58
serA	63	Phosphoglycerate dehydrogenase (serine auxotroph)	8
serB	100	Phosphoserine phosphatase (serine auxotroph)	8
serC	20	Phosphoserine aminotransferase (serine auxotroph)	8
tesB	10	Acyl-coenzyme A thioesterase II (none known)	149

[a]Unless otherwise indicated, the available mutations reduce the level of, or delete, the affected enzyme. With the exception of *fadR*, all of the above appear to be structural genes coding for enzymes.

[b]Abbreviations: FA, fatty acid; TS, temperature sensitive; CFA, cyclopropane fatty acid; ACP, acyl carrier protein.

acid biosynthesis) (59, 158, 235) are still unknown. Only a few mutants exist that are specifically defective in lipid A, MDO, or bacterial lipoprotein assembly (Figure 9, Table 2).

FORMATION OF COMMON PRECURSORS

Fatty Acid Synthesis

A soluble system of enzymes catalyzes the synthesis of fatty acids in *E. coli* (59, 158, 235, 239). In yeast or higher eukaryotic systems, a large multifunctional enzyme complex catalyzes the same set of reactions (22, 240). The de novo fatty acid synthesis in *E. coli* occurs with the growing chain attached via a thioester linkage to the acyl carrier protein (59, 158, 235, 239) rather than to coenzyme A. At the ten-carbon stage *cis*-double bonds are introduced into the growing chain by means of a β,γ-dehydrase, coded for by the *fabA* gene (38, 214) (Table 1, Figure 9, part a). Oxygen is not required for this process, nor can *E. coli* synthesize polyunsaturated fatty acids (59, 235). The β,γ-hydroxydecanoyl acyl–carrier protein dehydrase is situated at a branchpoint, divering what would otherwise become saturated fatty acids (such as palmitate or β-hydroxymyristate) to palmitoleate or *cis*-vaccenate (59, 235).

The available collection of fatty acid–biosynthesis mutants *(fab)* is small, as most of them must be isolated as temperature-sensitive conditional lethals, rather than as auxotrophs (82, 214). The most likely reason for this requirement is that *E. coli* cannot utilize exogenous β-hydroxymyristate for the biosynthesis of lipid A (4) (Figure 5). (Exogenous β-hydroxymyristate that has been taken up from the growth medium is presumably converted to β-hydroxymyristoyl coenzyme A, and not to β-hydroxymyristoyl acyl carrier protein (ACP), the substrate required for lipid A biosynthesis; 4.) However, mutants defective in the *fabA* or *fabB* genes can be isolated as unsaturated fatty acid auxotrophs (37, 38, 214). Uptake of long-chain fatty acids from the medium requires an envelope-associated protein, the product of the *fadL* gene (17, 158). The known *fab* genes are scattered around the chromosome (Figure 9, section *a*). Much remains to be learned about the genetics of dc novo fatty acid synthesis (Table 1). Important control mechanisms are likely to exist in the early steps, since most of the energy required for biosynthesis of lipids is expended for the formation of the hydrocarbon chains (235).

Fatty Acid Degradation

The genes coding for proteins involved in fatty acid degradation are designated *fad* (8, 158). The important studies of Overath et al (169, 170) and Nunn (158) clarified the mechanisms by which fatty acid degradation is controlled. A repressor coded for by the *fadR* gene (158) (Figure 9, section *a*)

Table 2 *E. coli* genes involved in the biosynthesis and turnover of glycerophospholipids, membrane-derived oligosaccharides, and lipid A

Gene symbol	Map position (min)	Enzyme affected[a] [mutant phenotype(s)]	References
*cdh**	88	CDP-Diglyceride hydrolase (normal growth, CDP-diglyceride accumulation	29, 89, 100
*cds**	4	CDP-Diglyceride synthase (pH sensitive)	64, 66, 102
*cdsS**	69	(Suppressor of pH sensitive *cds*)	67
cls	27	Cardiolipin synthase (resistance to DHBP)[b]	99, 159, 172
*dgk**	92	Diglyceride kinase (sensitive to low osmolarity)	132, 137, 189, 190, 203
*dgkR**	94	Elevated diglyceride kinase (none known)	186
glpQ	49	Glycerophosphodiester phosphodiesterase (none known)	126
lpp	36	Major outer membrane lipoprotein (EDTA sensitivity)	8, 93
lpxA	4	UDP-GlcNAc acyltransferase (no mutants available)	4, 5, 40
lpxB	4	*(pgsB)* Lipid A disaccharide synthase (TS growth in certain backgrounds, lipid X accumulation)[b]	40, 154, 156, 194
mdoA	23	MDO β,1→2 Glucosyl transferase (chemotaxis defective)[b,c]	18
*mdoB**	99	MDO Phosphoglycerol transferase I (suppression of *dgk* phenotype)[b]	56, 108, 202
*pgpA**	10	Biosynthetic PGP phosphatase (none known, deletions not available)[b]	101
*pgpB**	28	Outer membrane PGP and phosphatidic acid phosphatase (none known)[b]	101
*pgsA**	42	PGP synthase (TS in certain backgrounds; no deletions available)[b]	71, 146, 156
pgsB	4	See *lpxB*.	
pldA	85	Detergent-resistant outer membrane phospholipase A (none known)	1, 96, 117, 181
*pldB**	85	Inner membrane lysophospholipase L_2 (none known)	116–118
plsB	92	Glycerol-3-P acyltransferase (glycerol auxotrophs)	10, 74, 132, 133
plsX	24	Unknown (accentuates phenotype of *plsB* mutations)	127
psd	95	Phosphatidylserine decarboxylase (TS growth)[b]	86, 181
*pss**	56	Phosphatidylserine synthase (TS growth, antibiotic hypersensitive)[b]	161, 162, 180, 181, 185, 187
*pssR**	84	Elevated phosphatidylserine synthase (none known)	223

[a]Unless otherwise indicated, the available mutations reduce the level of the affected enzyme by a factor of ten or more, when assayed in crude extracts. With the exception of *dgkR*, *pssR*, and *cdsS*, all of the above appear to be structural genes coding for enzymes.

[b]Abbreviations: DHBP, 3,4-dihydroxybutyl 1-phosphonic acid; TS, temperature sensitive; PGP, phosphatidylglycerophosphate; MDO, membrane-derived oligosaccharides.

[c]H. Rotering, W. Fiedler & V. Braun, personal communication.

*Mutants obtained by colony autoradiography.

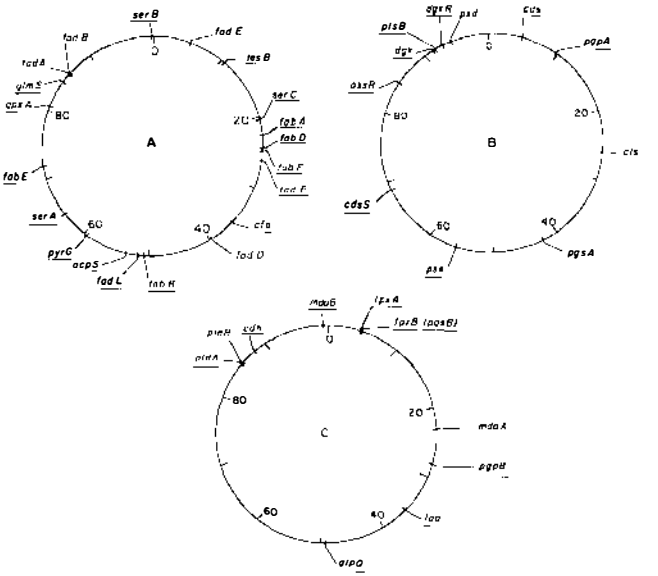

Figure 9 Locations of "lipid genes" on the *E. coli* chromosome. *A*) Genes for the biosynthesis and metabolism of precursors, including fatty acids, serine, glycerol 3-phosphate and CTP. *B*) Genes for the synthesis of the glycerophospholipids. *C*) Genes for the synthesis of lipid A, MDO, and lipoprotein, and for the degradation of glycerophospholipids. Genetic symbols and phenotypes are explained in Tables 1 and 2.

regulates the synthesis of enzymes involved in fatty acid degradation, which are required when *E. coli* is grown on a fatty acid as the sole carbon source. Fatty acid degradation occurs at the level of a fatty acyl thioester with coenzyme A (158, 235), a derivative formed shortly after, or possibly in conjunction with, transport. Transported fatty acids are not converted to the acyl-ACP form (158). Five of the enzymes required for fatty acid degradation are recovered in a complex (15, 248, 249).

In the early 1970s certain strains harboring combinations of *fabA* and *fad* mutations were constructed that require unsaturated fatty acids for growth but are unable to degrade or alter them (39, 215, 217). In these strains a wide variety of unnatural fatty acids (including polyunsaturates) can be incorporated into the membrane phospholipids, since fatty acyl coenzyme A can substitute for fatty acyl-ACP as the substrate for glycerol-3-phosphate acyltransferase (73, 181) (Figure 3). Studies of the modification of fatty acid composition reveal that the gel/liquid-crystalline transition temperature of *E. coli* phospholipids can vary widely from its normal setpoint without significantly altering growth (37). However, these modifications change the kinetic properties of many membrane enzymes, as is evident by assaying kinetics as a

function of temperature (35, 37, 168). It appears that *E. coli* cannot grow efficiently without small amounts of both gel and liquid-crystalline fatty acyl moieties in its envelope (37). Under certain narrowly defined conditions, short, saturated fatty acids may substitute for unsaturated fatty acids (173). More studies defining the hydrocarbon requirements for growth would be desirable, especially in conjunction with a search for secondary mutations capable of suppressing the growth phenotypes associated with fatty acid perturbations.

sn-*Glycerol-3-Phosphate*

The concentration of *sn*-glycerol-3-phosphate (Figure 3) in *E. coli* is about 100–200 μM and is regulated (46, 134). Its concentration is orders of magnitude higher than that of the fatty acyl–acyl carrier protein (200). In cells grown on glucose, *sn*-glycerol-3-phosphate (Figure 3) is derived by reduction of dihydroxyacetone phosphate (181). Mutants with lesions in this enzyme (*gpsA;* Figure 3 and Table 1) require exogenous glycerol for growth and generate *sn*-glycerol-3-phosphate via glycerol kinase (Figure 3).

Mutants defective in either the *gpsA* or *plsB* genes (Figure 3) are useful for studying the effects of inhibition of total glycerophospholipid synthesis on other cellular functions (10, 141). In *E. coli,* inhibition of glycerophospholipid synthesis induced by glycerol starvation does not cause an immediate shutoff of macromolecular synthesis (141, 181). Growth, DNA, RNA, and protein syntheses continue for about one division, causing the membranes to acquire a greater buoyant density (141). Upon readdition of glycerol an immediate burst of glycerophospholipid synthesis occurs prior to resumption of macromolecular synthesis, indicating some kind of regulatory mechanism acts for balanced membrane growth (143). The effect of glycerol starvation on lipid A formation is unknown (Figure 5).

The coupling of glycerolipid synthesis with the formation of macromolecules deserves further study. Studies with *Caulobacter* (34) led to somewhat different conclusions than did studies with *E. coli* (141, 143). Inhibition of total glycerophospholipid synthesis in glycerol auxotrophs of *Caulobacter* (34, 210) causes an immediate cessation of DNA elongation, while RNA and most membrane proteins continue to be made. The reasons for the rapid cessation of DNA synthesis in glycerol-starved *Caulobacter* (in contrast to those in *E. coli*) are not obvious. Given the discrepancy, the coupling of phospholipid and macromolecular synthesis should be reexamined in a newly constructed set of *E. coli* mutants, with "tighter alleles" of the *gpsA* and/or *plsB* genes (Figure 3), but without the complication of secondary mutation(s) in *plsX* (127). Glycerol auxotrophs, concomitantly blocked in their ability to regenerate phosphatidic acid via diglyceride kinase (133, 189, 190, 203) (Figure 3), should also be examined.

Other Important Precursors

In addition to the above materials, the assembly of glycerolipids requires cytidine triphosphate and serine (Figure 3). Mutants blocked in the formation of CTP from UTP are cytidine auxotrophs (58, 66), and starvation of such strains for cytidine causes massive accumulation of phosphatidic acid (66). The effects of L-serine starvation on glycerophospholipid composition have not been examined in much detail (250), and the intracellular level of serine is not known.

As shown in Figure 5, the first committed step of lipid A synthesis is the fatty acylation of UDP-GlcNAc (4), catalyzed by a different enzyme than that involved in *sn*-glycerol-3-phosphate acylation (Figure 3). UDP-GlcNAc is also the starting material for the formation of peptidoglycan (Figure 2) (145), which together with lipid A accounts for most of the glucosamine found in the envelope. Partitioning of UDP-GlcNAc between lipid A and peptidoglycan is probably regulated. Glucosamine auxotrophs of *E. coli* (defective in conversion of fructose 6-phosphate to glucosamine 6-phosphate) have been available for many years (246), but have not been characterized in depth with regard to envelope biogenesis.

BIOSYNTHESIS AND ASSEMBLY OF GLYCEROPHOSPHOLIPIDS

Enzymatic Synthesis of Glycerophospholipids

The major glycerophospholipids of *E. coli* (Figure 3) are phosphatidylethanolamine (75%), phosphatidylglycerol (20%), and cardiolipin (1–5%). The gross phospholipid composition of exponentially growing wild-type cells is not strikingly altered by changes in culture conditions (181). Biosynthetic intermediates (Figure 3) and a large number of minor uncharacterized phospholipids (Figure 8) add up to about 1–2% of the total, $CHCl_3$-extractable material, but the levels of these compounds have not been examined as a function of growth conditions. Reactions of phospholipid degradation (181) and ancillary pathways (generally of unknown significance) are summarized in Figure 4. Genetic symbols indicate the steps at which mutants are available. The chromosomal locations of these *E. coli* genes are shown in Figure 9, and phenotypes of mutants are summarized in Table 2.

The validity of the scheme shown in Figure 3 is supported by the following: (*a*) identification and purification from *E. coli* membranes of most of the enzymes involved (73, 137, 181, 224); (*b*) detection of the intermediates among the minor phospholipids (Figure 8) (29, 64, 66, 181, 203); and (*c*) isolation and characterization of mutants (Table 2). In wild-type cells in-

termediates, such as phosphatidic acid, cytidine 5' diphosphate (CDP)-diglyceride, phosphatidylserine, phosphatidylglycerophosphate, and diglyceride, are present in the range of 0.01–0.1% of the total membrane lipid (29, 64, 66, 181, 203), equivalent to 2×10^3–2×10^4 molecules per cell. Consequently, the de novo intermediates of biosynthesis must turn over several thousand times during a single generation.

Starting with the acylation of glycerol 3-phosphate (or perhaps with the late stages of fatty acid synthesis on ACP), the reactions of phospholipid assembly (Figure 3) presumably occur on the cytoplasmic surface of the inner membrane (12, 181, 243). Most phospholipid enzymes (Figure 3) appear to be integral membrane proteins, and are part of the same structure that includes their precursors and products. However, the orientation of the active sites of the lipid synthetic enzymes of *E. coli* has not been probed as carefully as that of the corresponding enzymes in endoplasmic reticulum of animal cells (11).

The enzymes for glycerolipid synthesis (Figure 3) are present in about 10^3 copies per wild-type cell (181). This level is not much lower than that of metabolites like CDP-diglyceride (29, 181) and probably represents a large catalytic excess. Massive overproduction of each of the enzymes of Figure 3 by gene-cloning techniques has little effect on either composition or rate of synthesis (102, 182, 188, 222). [It may be necessary, however, to reexamine the synthetic "rates" with very short times of pulse labeling (W. Dowhan, personal communication)]. The failure of enzyme overproduction to perturb phospholipid composition is especially puzzling in the case of phosphatidylserine synthase and phosphatidylglycerophosphate synthase (222). These two enzymes compete for the common precursor, CDP-diglyceride (Figure 3). The fact that overproduction of one or the other of the enzymes of the CDP-diglyceride branchpoint has no effect on the ratio of the polar headgroups suggests that the excess enzyme has no access to the CDP-diglyceride pool, which could be explained by the existence of a noncovalent complex of some of the enzymes of Figure 3 that functions to partition the CDP-diglyceride. Excess phosphatidylserine (or phosphatidylglycerophosphate) synthase, not present in the complex, would have no access to the CDP-diglyceride. An alternative explanation is that the phosphatidyltransferases are closely regulated in vivo, perhaps by major changes in the levels of regulatory molecules, or by one of the minor lipids (Figure 8).

Phospholipid Turnover and Formation of MDO

The molecular species of phosphatidylethanolamine (altogether about 1.4×10^7 molecules per cell) are metabolically stable compared to those of phosphatidylglycerol (about 0.5×10^7 molecules per cell), which undergo rapid turnover (181, 182). If cells are labeled with $^{32}P_i$ for many generations, washed, and then incubated in the presence of nonradioactive phosphate, one

observes that 5–10% of the radioactivity is lost from phosphatidylethanolamine per generation (C. H. Raetz & K. F. Newman, unpublished results), while one-half to two-thirds of the radioactivity is lost from phosphatidylglycerol (172, 208). The slow turnover of phosphatidylethanolamine is not well characterized, and may reflect a combination of processes, including degradation by phospholipases (Figure 4) and transfer of phosphoethanolamine moieties to a subset of MDOs (Figure 6) or lipopolysaccharide (197). The more rapid turnover of phosphatidylglycerol is accounted for by cardiolipin formation (172) (especially in stationary phase), lipoprotein biosynthesis (Figure 7) (24, 245), and transfer of the glycerol 1-phosphate headgroup to MDO precursors (Figure 3) on the periplasmic surface of the inner membrane (19, 109). MDO formation is greatly enhanced at low osmolarities (114). Under these conditions MDOs can represent several percent of the dry weight of *E. coli*. If cardiolipin synthesis and MDO synthesis are both blocked by mutation, the rate of phosphatidylglycerol turnover is slow, comparable to that observed in wild-type cells for phosphatidylethanolamine ("Quadruple mutants" bearing additional lesions in the *lpp* and *pldA* genes have not been constructed.) (C. R. H. Raetz and K. F. Newman, unpublished results).

Although the MDOs (mol wt ~2,000) have not been characterized by structural techniques, such as NMR and mass spectrometry, many important features have been elucidated by biochemical studies (115, 207, 236). A "typical" MDO molecule consists of 6–10 glucose residues, generally linked β,1→2, but with two or three β,1→6 branches (Figure 6). MDO subfractions differ from each other in the extent of substitution with glycerol 1-phosphate and phosphoethanolamine (a minor component), as well as succinate and acetate (the locations of which are unknown). Transfer of glycerol-1-phosphate from phosphatidylglycerol to MDO precursors (designated pre-MDO in Figure 3) can be demonstrated in vitro (109), as can elongation of the β,1→2–linked oligosaccharide chain (242). In the coming years the complete covalent structures of the various MDO subfractions must be determined to elucidate fully MDO biosynthesis and function.

GENETICS OF GLYCEROPHOSPHOLIPID AND MDO SYNTHESIS IN *E. coli*

Isolation of Mutants by Colony Autoradiography

Precursors of membrane lipids, including long-chain fatty acids (17), glycerol-3-phosphate (7), L-serine (135), cytosine (58), and glucosamine (246), can be transported into *E. coli* from the growth medium. In contrast, the inherent insolubility of intact phospholipids and the barrier posed by lipopolysaccharide (Figure 2) prevent efficient phospholipid uptake (112, 142, 181). This inability makes it impossible to isolate phospholipid auxo-

trophs. (If conditions for efficient phospholipid uptake could be found phospholipid auxotrophs could possibly be isolated.) Specific inhibitors of the enzymes shown in Figures 3–5 are also not available. Therefore, direct mutant selections exist for only a few lipid enzymes (182).

For these reasons our laboratory developed general methods for the rapid, autoradiographic detection of specific enzymes of the lipid system (26, 179), applicable to bacterial (26, 179), yeast (95), or mammalian cell colonies (51, 193) immobilized on filters. This methodology was previously reviewed in detail (182) and yielded most of the mutants currently available. Our autoradiographic filter screening methods are compatible with preselections based on specific growth phenotypes (66) and may also be employed for genetic mapping (64, 156, 180, 189).

Mapping and Cloning of E. coli *Lipid Genes*

Figure 9 indicates the locations of the known lipid genes on the *E. coli* chromosome. They are primarily structural genes (Table 2) by the criteria that residual enzymatic activity in certain mutants is thermolabile relative to that of the wild type, and that cloning results in enzyme overproduction proportional to gene dosage. With the exception of *lpxA* and *lpxB* (40), these genes are not clustered into operons. Recently, the DNA sequences of *plsB* (74, 132), *dgk* (74, 137), *cds* (102), *pgsA* (71), *cdh* (89, 100), *pldB* (118), *pldA* (96), *lpxB*, *pgpA*, and *pgpB* (T. Icho, D. N. Crowell, J. Coleman & C. R. H. Raetz, unpublished results) have been completed. In most instances, some amino acid compositions and/or sequences were also available (96, 113, 118, 132, 137). Although the DNA sequences of the lipid genes have not yet been analyzed in detail, the enzymes fall into two classes; those that are extremely hydrophobic *(cds, dgk, pgsA, pgpB)* and those that do not differ greatly from soluble proteins in amino acid composition *(plsB, cdh, pgpA, lpxB, pldA, pldB)*. The former may have catalytic sites deeply imbedded in the bilayer and may have multiple bilayer-spanning segments, as determined by hydropathy analysis (71, 74, 89, 96, 100, 102, 118, 132, 137), while the latter may be anchored to the bilayer by small hydrophobic domains.

The enzymes of phospholipid metabolism are unique among the membrane proteins in that they interact with their surrounding bilayer both allosterically and catalytically. Future progress in elucidation of the molecular mechanisms and regulation of the lipid enzymes requires the use of structural techniques, especially X-ray crystallography and NMR. While these techniques are difficult to apply to membrane proteins, the availability of overproducing strains makes purifying many of the key enzymes in 100-mg quantities possible (73, 137, 163, 224).

Phenotypes of Phospholipid Mutants

The extent to which mutations in the phospholipid system can be used to modify membrane lipid composition has not been examined in great detail (182). In many cases mutations in the phospholipid genes give rise to conditional lethalities (Table 2), but lipid composition may be considerably perturbed before growth ceases.

The parameter most sensitive to mutations appears to be the total phospholipid content ($\sim 2 \times 10^7$ molecules per cell). Using mutations in either the *gpsA* or *plsB* genes (Figure 3), the total phospholipid content can be depleted (10, 141, 143). The cells stop growing with only 40% less glycerophospholipid than is ordinarily present. This finding indicates that there is not a large, nonmembranous reservoir of glycerophospholipids. Further, this finding is reasonable, given the surface area of *E. coli* ($\sim 5 \times 10^8$ Å^2, assuming a cylinder 2.4 μ in length and 0.6 μ in diameter for an "average" cell). It also correlates with the fact that there are three glycerolipid-containing layers (Figure 2) interspersed with protein and with the approximate surface area occupied by a typical glycerophospholipid molecule in model systems (~ 50 Å^2) (230).

The ratio of zwitterionic to anionic lipids (i.e. phosphatidylethanolamine to phosphatidylglycerol plus cardiolipin) appears to be regulated in wild-type cells (22, 164, 188), but in certain mutants wide deviations from the wild-type ratio ($\sim 3:1$) are possible (156, 161, 162, 180, 187), especially when cells are grown in the presence of $MgCl_2$ and sucrose (160, 212, 213). Deletions of the genes for either of the phosphatidyltransferases (i.e. *pss* or *pgs;* Figure 3) have not been reported. Why strains harboring mutations in *pss* are temperature-sensitive for growth is unknown (161, 162, 180, 187). Growth could be limited by bilayer stability or some specific protein–lipid interactions could be needed. Second-site suppressor mutations exist that permit more cell growth with abnormal lipid compositions than do single-step mutations (146). For instance, Miyazaki et al (146) recently showed that the temperature sensitivity of certain *pss* mutations could be partially suppressed by mutations in *pgsA*, resulting in strains with very low phosphatidylglycerol levels (0.1–1% of the total lipid).

Mutations in the genes for the enzymes involved in the processing of intermediates, such as CDP-diglyceride synthase *(cds)* (64, 66) or phosphatidylserine decarboxylase *(psd)* (85–87), must also be isolated as conditional lethals. The membranes of *E. coli* can accommodate levels of most lipid intermediates up to 10–50% of the total phospholipid content (66, 87), but there are limits beyond which certain modifications are not tolerated. For instance, if mutations in the *cds* gene (Figure 3) cause phosphatidic acid to accumulate to levels greater than 15% of the total lipid, growth is inhibited (66). Preliminary studies indicate that phenotypic suppressors can be found

for conditional CDP-diglyceride synthase mutants, as for phosphatidylserine synthase mutants, but the biochemical nature of these suppressors is not known (67).

Mutations in the gene for cardiolipin synthase *(cls)* differ from others in the system (Figure 3) in that they have no obvious growth phenotypes under ordinary conditions (172). However, the available alleles are not deletions, and result only in a 10–20-fold reduction of the cardiolipin content (172). The residual level of cardiolipin in such mutants is 0.1% of the total phospholipid ($\sim 2 \times 10^4$ molecules per cell), a number that is likely to be in excess of most membrane enzymes. Since deletions of *cls* have not been isolated, cardiolipin cannot be conclusively determined altogether nonessential. Some investigators have suggested that strains bearing *cls* mutations do not grow as well as wild-type cells in long-term chemostat experiments, and that lesions in *cls* enhance the temperature sensitivity of mutations in the *pss* gene (159). Mutants deficient in cardiolipin synthetase are also resistant to certain glycerophosphate analogs (99).

Mutants in Diglyceride Kinase, MDO, and Phospholipases

The transfer of the glycerol 1-phosphate headgroup of phosphatidylglycerol to the MDOs results in the formation of *sn*-1,2-diglyceride as a byproduct (109, 181) (Figure 3). Mutants defective in diglyceride kinase (189, 190, 203) accumulate diglyceride to about 50 times the normal level (5–10% of their membrane lipids). This amount of diglyceride is still compatible with a bilayer structure in model systems and does not cause a phase separation (80). A significant amount of diglyceride is probably also generated by reactions unrelated to MDO biosynthesis (203), including phosphatidic acid phosphatase (101) and phospholipase C (181). The latter is not well characterized in *E. coli* (181), but the possibility that diglyceride has a second messenger function in *E. coli,* as it may in higher eukaryotic cells (14, 65, 157), merits further investigation.

Strains harboring mutations in diglyceride kinase grow poorly at very low osmolarity (189, 190, 203) or in the presence of arbutin, an MDO-like glycerol 1-phosphate acceptor that can be provided from the medium (19, 108). This phenotype can be explained by the fact that these treatments greatly stimulate phosphatidylglycerol turnover and diglyceride accumulation (19, 114). E. P. Kennedy (personal communication) recently discovered that many phenotypic revertants of diglyceride-kinase mutants, able to grow at low osmolarity, posses second-site mutations in various stages of MDO assembly. The availability of many new MDO mutants should greatly facilitate biochemical dissection of the MDO system. The mechanisms of osmoregulation of MDO formation are unknown, but appear unrelated to the

better-characterized osmoregulation of outer-membrane proteins (79, 125, 131).

As shown in Figure 3, two classes of mutants defective in MDO synthesis were already isolated (18, 108). Strains with lesions in *mdoB* (Figures 3 and 9) are defective in the transfer of the phosphoglycerol moiety from phosphatidylglycerol to MDO precursors (56, 108, 109, 202), while strains defective in *mdoA* cannot synthesize the β,1→2–linked oligosaccharides (18, 242) (Figure 6). Although lesions in either *mdoA* or *mdoB* suppress the phenotype of diglyceride kinase mutants, they do not themselves cause obvious conditional lethalities (18, 108). Whatever functions the MDOs may have, they are not required for growth under laboratory conditions. H. Rotering, W. Fiedler & V. Braun (personal communication) recently made the provocative observation that *mdoA* lesions cause defects in chemotaxis.

The few mutants isolated in phospholipases (1, 45, 181) and ancillary lipid enzymes (29, 101) (Figure 4) also have no obvious phenotypes, but these mutants have not been examined under a variety of stressful conditions, nor have the levels of the various minor phospholipid species (Figure 8) been analyzed.

Mutants in Regulatory Processes

While a few strains are now available with mutations affecting most known steps of glycerophospholipid synthesis and degradation (Figures 3, 4; Table 2), there are not enough alleles of each gene to permit incisive studies of cell physiology. Alleles giving rise to altered enzymes that can be rapidly shut off (for instance, by a temperature shift) are not generally available. Furthermore, it would be of interest to search for mutants with altered phospholipid compositions, but with normal activities of the biosynthetic enzymes, possibly reflecting aberrations in important regulatory functions.

The regulation of the expression of the phospholipid genes also remains to be explored. Several mutants have been described in which the levels of specific enzymes are elevated (186, 223). The mutations causing the elevated enzyme level (for instance, of diglyceride kinase by *dgkR1* or of phosphatidylserine synthase by *pssR1;* Figure 9) map far away from the structural loci (186, 223). These regulatory mutations appear to act in *trans* on the wild-type structural genes, suggesting a diffusible product (186, 223). The interaction of *dgkR1* with the dgk^+ structural gene involves control at the level of transcription (136, 241). In addition to stimulating dgk^+ transcription, however, the *dgkR* mutation has subtle effects on diglyceride metabolism per se. In $dgkR^+$ cells harboring a cloned dgk^+ structural gene, one observes a threefold reduction in the diglyceride pool relative to vector controls, but paradoxically the *dgkR1* mutation abolishes this effect (241).

THE LIPID A COMPONENT OF LIPOPOLYSACCHARIDE

Biosynthesis of Lipid A

As shown in Figure 2, the outer surface of the outer membrane does not contain much glycerophospholipid. It is composed of a unique molecule, characteristic of gram-negative bacteria, termed "lipopolysaccharide" (43). Several recent books provide a detailed account of this complicated, but physiologically important, substance (92, 197). The literature prior to 1983 (198) is very confusing, because the correct covalent structure of lipid A and the pathway of its biosynthesis (Figure 5) were unknown (61, 165).

The lipopolysaccharide molecule may be subdivided into three domains (61, 165, 197) (Figure 2): (*a*) the O-antigen chains, which extend into the growth medium and are prominent antigenic determinants on the surface of clinical isolates of *E. coli*, but are absent in *E. coli* K12 (61); (*b*) the core region, which is relatively conserved among related gram-negative bacteria (61); and (*c*) the lipid A moiety (197), which constitutes the outer monolayer of the outer membrane and is also relatively conserved with regard to structure and composition. Because mature lipid A is covalently attached to the saccharide component of lipopolysaccharide (Figures 2 and 5), it is not soluble in $CHCl_3$/methanol mixtures, but must be extracted with phenol-containing solvents (63).

The polysaccharide portion of lipopolysaccharide functions as a receptor for many bacteriophages during infection of *E. coli* (20, 21, 144, 229). Therefore, a variety of O-antigen and core-deficient mutants, unable to support bacteriophage growth, can be isolated (20, 21, 144, 229). Genetic studies using bacteriophages have demonstrated that the O-antigen is nonessential for cell growth (197), and that all of the components of the core, with the exception of the two innermost 3-deoxy-D-*manno*-octulosonic acid (KDO) residues (Figure 2), are also nonessential (21, 129, 130, 195, 196, 229). Presumably, mature lipid A itself, as depicted at the bottom of Figure 5, is required for growth and outer membrane assembly, but there is little genetic evidence to prove unequivocally this idea (see below).

We have proposed the scheme for the biosynthesis of lipid A shown in Figure 5 (4, 28, 183, 184, 194). The first clue to this model was the discovery of the monosaccharide precursor (228), lipid X, which presented itself as an unknown, accumulating in certain phosphatidylglycerol-deficient mutants of *E. coli* (154, 156). The structure of lipid X (228), when compared to that of lipid A (Figure 5), suggested that it should be the precursor of the reducing-end unit of lipid A (194). Further careful inspection of the lipid A structure led us to hypothesize a nucleotide derivative of lipid X (UDP-2,3-diacylglucosamine; Figure 5) as the precursor for the nonreducing end (28).

Ray et al (194) provided compelling evidence for this notion by demonstrating an enzyme (the product of the *lpxB* gene; Figure 5) in wild-type extracts catalyzing the condensation of UDP-2,3-diacylglucosamine and lipid X to generate a tetraacyldisaccharide 1-phosphate intermediate with the characteristic β,1→6 linkage of lipid A (Figure 5).

The biosynthetic origin of lipid X from known metabolic intermediates was elucidated by Anderson et al (4). Pulse labeling with $^{32}P_i$ revealed that UDP-2,3-diacylglucosamine is a precursor of lipid X (4). A new system of fatty acyl transferases (dependent upon acyl-ACP; Figure 5) is capable of converting UDP-GlcNAc (also a key precursor of peptidoglycan) to UDP-2,3-diacylglucosamine (4, 40). Consequently, UDP-GlcNAc is situated at an important branchpoint in *E. coli,* leading either to the peptidoglycan (145) or to the lipid A layer (4) (Figure 2). The gene for the first UDP-GlcNAc acyltransferase *(lpxA)* appears to be in an operon with the gene for disaccharide synthase *(lpxB)* (40).

All of the reactions indicated in solid arrows in Figure 5 have been characterized in cell extracts, and their products have been verified by spectroscopic methods (4, 28, 184, 194, 228), as well as by chemical synthesis (104, 105, 124). However, the enzymes of KDO addition (148) and "late" acylation of the nonreducing end, leading to "mature" lipid A (Figure 5), are not fully understood (dashed arrows). Further, the possibility of alternative routes for lipid A biosynthesis cannot be excluded without additional mutants. So far, work on lipid A biosynthesis has been carried out only with *E. coli* (4, 28, 183, 184, 194), but the lipid A of *Salmonella typhimurium* (178, 227) and *Salmonella minnesota* (177) is virtually the same as that of *E. coli* (103).

As in the case of glycerophospholipid synthesis, very little is known about the mechanisms by which lipid A, synthesized in the cytoplasm and/or inner membrane, is transported to the outer membrane (167). Osborn and coworkers have shown that O-antigen chains are added on the periplasmic surface of the inner membrane in those organisms that bear these moieties (147), suggesting that a lipid A-specific "flippase" functions in the cytoplasmic membrane.

Mutants in the Lipid A Pathway

In addition to the enzymological evidence, two types of mutations provide biological evidence for our biosynthetic scheme. The first of these (designated *lpxB1*) causes a 100-fold reduction in the specific activity of the lipid A disaccharide synthase (194) (Figure 5). Cells harboring *lpxB1* accumulate high levels of 2,3-diacylglucosamine 1-phosphate (lipid X) and UDP-2,3-diacylglucosamine (28). These metabolites can also be detected in wild-type

cells, but only in the range of 0.01–0.1% of the total membrane lipid (28), requiring that they be carefully separated from the minor, unidentified lipids (Figure 8). Mutants defective in the *lpxB* gene are still capable of generating mature lipid A (K. Takayama & C. H. R. Raetz, unpublished observations), presumably because the accumulation of the precursor molecules compensates for the reduction in the disaccharide synthase. The *lpxB1* mutation can also accentuate the phosphatidylglycerol deficiency of strains harboring certain *pgsA* mutations (154, 156). Although the mechanism of interaction remains unknown, the discovery of monosaccharide lipid A precursors in such phosphatidylglycerol-deficient mutants (156) unraveled the pathway for lipid A biosynthesis (Figure 5) (183).

Insertion of *lpxB* into a multicopy plasmid causes overproduction of the disaccharide synthase proportional to gene dosage (40). Additional mutant alleles of *lpxB* (preferably ones conferring temperature-sensitive growth that is independent of lesions in *pgsA*) would be highly desirable to demonstrate that other pathways for the synthesis of the lipid A disaccharide do not exist in *E. coli*. At present only one allele of *lpxB* is available (154).

The *lpxB* gene was recently cloned and sequenced (40) (D. N. Crowell, J. Coleman & C. R. H. Raetz, unpublished results). It is very closely linked to, and situated between, *cds* (Figure 9) and *dnaE* (154). An open-reading frame (now designated *lpxA*) just upstream of *lpxB* codes for the first enzyme of the lipid A pathway (Figure 5) (40). The DNA sequence (D. N. Crowell, J. Coleman & C. R. H. Raetz, unpublished results) strongly suggests that *lpxA* and *lpxB* are in the same operon, but the ends of this operon have not been found. This operon could conceivably include the genes for CDP-diglyceride synthase *(cds)* (Figure 9) and the α subunit of DNA polymerase III *(dnaE)*, since both of these are transcribed in the clockwise direction, like *lpxA* and *lpxB* (102, 211).

Further genetic support for Figure 5 is provided by the characterization of mutants deficient in KDO biosynthesis (191, 225). These mutants were first described in *Salmonella typhimurium* (129, 130, 195, 196), but have recently also been obtained in *E. coli* (226). KDO-deficient mutants are temperature sensitive and are impaired in the formation of KDO from arabinose 5-phosphate and phosphoenolpyruvate (129, 130, 165, 195, 196). Under non-permissive conditions they accumulate a family of related disaccharide lipid A precursors, the most prominent of which is the tetraacyldisaccharide-1,4'-*bis*phosphate shown in Figure 5 (191, 225). The conditional lethality of KDO-deficient mutants may be due to the absence of mature lipid A or due to the accumulation of the disaccharide precursors in the inner membrane. Osborn and coworkers have postulated that KDO facilitates the translocation of lipid A molecules to the outer membrane (167).

Significance of Lipid A and Its Precursors

The molecules of the lipid A system (Figure 5) are important not only for the biogenesis of the *E. coli* envelope, but also for their complex pharmacological effects on animals (61). Mature lipid A is a potent toxin capable of inducing a severe shock syndrome in humans and other large mammals (61). Exposure to lipid A usually occurs during gram-negative infections (92). By unknown mechanisms lipid A and many of its precursors also stimulate proliferation of B lymphocytes and activation of macrophages, accompanied by prostaglandin release (60, 62, 119). The monosaccharide precursors of lipid A are nontoxic (30), but retain some of the "beneficial" immunostimulatory effects (192). [Early preparations of lipid X contained an additional lipid mitogen of unknown chemical structure not detectable by NMR or mass spectrometry. Synthetic and HPLC purified *E. coli* lipid X, however, do have modest mitogenic activity, and the ester-linked fatty acyl moieties are important for this biological effect (C. R. H. Raetz, unpublished observations).] Monosaccharide precursors of lipid A also have the capacity to desensitize animals, such as mice and sheep, against the lethal toxicity of mature lipid A and lipopolysaccharide (176). This finding may have important clinical applications. The enzymes shown in Figure 5 are very useful for the synthesis of lipid A substructures, as well as analogs and radiolabeled probes.

Although the glucosamine-derived lipids have not yet been crystallized for X-ray diffraction, studies with space-filling models show clearly that they are quite similar to classical glycerophospholipids with regard to the conformations that they are likely to adopt (28, 194). In a formal sense, lipid X is an analog of phosphatidic acid, while UDP-2,3-diacylglucosamine is analogous to CDP-diglyceride (28). The fatty acyl chains of the lipid A precursors are a little shorter than those of the glycerophospholipids, while their polar domain (i.e. the glucosamine 1-phosphate residue) is larger than the corresponding *sn*-glycerol 3-phosphate backbone. It seems reasonable that the molecules shown in Figure 5 are capable of being inserted into membrane lipid bilayers.

BIOGENESIS OF BACTERIAL MEMBRANE LIPOPROTEINS

Figure 7 shows the putative pathway for the biosynthesis of the amino-terminal domain of a typical bacterial lipoprotein. The covalent chemistry of this posttranslational modification is unusual, and little is known about the enzymology (24, 233, 245). Radiochemical-labeling studies (233, 245) suggest that the glycerol moiety of the polar headgroup of phosphatidylglycerol is initially transferred to the cysteine residue of prolipoprotein that eventually becomes the amino terminus (Figure 7), but an S_N2-like displacement of

phosphatidic acid is an improbable mechanism. Perhaps other unknown intermediates are involved in thioether formation, since the relevant membrane enzyme(s) have not been purified.

Mutants defective in the enzymes involved in attaching the thioether-linked lipid residues also are not available, but the structural gene *(lpp)* for the most prominent of the membrane lipoproteins of *E. coli* is well characterized (Figure 9 section *C*) (24, 93, 245). Membrane proteins having a hydrophobic anchor of this type are trimmed by a unique signal peptidase (the product of the *lsp* gene), which apparently cannot function prior to the addition of the *sn*-2,3-diglyceride moiety (13, 231, 244, 247) (Figure 7). The N-terminal palmitoyl group is added by transacylation (107) (Figure 4), after removal of the signal peptide (Figure 7). Further study of the biochemistry and genetics of bacterial lipoprotein synthesis is clearly necessary. Although the structural gene for the major *E. coli* outer membrane lipoprotein *(lpp)* can be deleted (93), the lipid modification system per se may be essential, since penicillin-binding protein 3, which is required for cell division, is also a lipoprotein (Y. Hirota, personal communication).

GENETICS OF PHOSPHOLIPID SYNTHESIS IN ANIMAL CELLS

It is beyond the scope of this article to review the enormously complex system of phospholipids of higher eukaryotes, but three recent books discuss the biochemistry of these molecules in depth (22, 88, 220). Recent progress with the yeast system has been reviewed elsewhere (90, 152, 153). The few animal cell mutants defective in specific reactions of glycerophospholipid synthesis are described in Table 3. Obviously, much remains to be done in order to bring the animal cell system to the same level as *E. coli* (Tables 1 and 2).

We have developed general methods for isolating phospholipid mutants of animal cells (51, 193), primarily using the Chinese hamster ovary line (CHO-K1), a system in which many other mutations are already available (72). An important step was the development of a reliable technique for replica plating and autoradiographic screening of CHO cell colonies immobilized on filter paper or polyester cloth (51, 193). The details of this methodology are reviewed elsewhere (48, 182).

Chinese hamster ovary cells contain the typical mammalian phospholipids (53, 54), including phosphatidylcholine (about 50%), phosphatidylethanolamine (about 25%), phosphatidylinositol (5–10%), phosphatidylserine (5–10%), sphingomyelin (5–10%), and a very complicated mixture of minor species (1–5%). In CHO cells about half of the phosphatidylethanolamine is present as the plasmalogen form (53), in which the 1 position of glycerol carries a vinyl ether, rather than an ester substituent.

Table 3 Animal cell mutants defective in membrane phospholipid synthesis[a]

Parental cell line	Enzyme affected[b] (mutant phenotypes)	References
CHO-K1	CDP-Choline synthase (TS growth corrected by PC liposomes or by LPC)[c,d]	50, 52, 55
CHO-K1	Phosphatidylcholine/serine exchange (TS growth corrected by PS liposomes)[c,d]	121–123, 155, 238
CHO-K1	Putative inositol 1-phosphate synthase (inositol auxotroph)	51, 53
CHO-K1	Peroxisomal dihydroxyacetone-P acyltransferase (defective peroxisome assembly; plasmalogen-deficient; not TS)[c,d]	251
CHO-K1	CDP-Ethanolamine : diglyceride ethanolamine phosphotransferase (none known)[d]	175
$HSDM_1C_1$	Arachidonyl-CoA synthase (defective bradykinin-stimulated arachidonate release, not TS)[c,d]	150

[a]CHO mutants altered in sterol metabolism are reviewed elsewhere (32, 69, 70, 120).
[b]In every case the level of the enzyme affected is reduced by 5 fold or more.
[c]Abbreviations: TS, temperature sensitive growth; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PS, phosphatidylserine.
[d]Mutants obtained by colony autoradiography.

Although very few mutant alleles are actually available (Table 3), phosphatidylcholine and phosphatidylserine appear essential for growth of CHO cells, since mutants with depressed levels of these lipids appear to be temperature sensitive (50, 52, 55, 121–123, 155, 238). Interestingly, temperature-sensitive CHO mutants, deficient in phosphatidylserine (121–123, 155, 238) and phosphatidylcholine (50), can be "rescued" by supplementing the growth medium with liposomes of appropriate composition at 40°C. This contrasts with *E. coli* (see above), in which phospholipid supplementation is not efficient (181). In the two cases examined in the CHO system, cells appear to regulate the uptake of exogenous phosphatidylserine (121–123, 155, 238) or phosphatidylcholine (49, 50), depending on their need for it, and can maintain a nearly normal membrane lipid composition. The mechanisms of net phospholipid uptake by CHO mutants and the regulatory factors involved in maintaining composition require further study. The use of fluorescent probes for lipid uptake described by Pagano & Sleight (171), or the generation of CHO mutants defective in phospholipid uptake, might provide additional insights.

Several other lipid mutants of CHO cells have been developed that have no obvious growth phenotypes under usual culture conditions (Table 3). The most provocative of these mutants are defective in plasmalogen formation (251). These strains were identified by a colony assay for the *N*-ethyl

maleimide–resistant dihydroxyacetone phosphate acyltransferase (54, 251). This enzyme is localized in peroxisomes and is postulated to be the first committed step in plasmalogen biosynthesis (78, 111). Recent studies of these mutants in our laboratory show that their plasmalogen content is decreased by 10–20-fold (251). Interestingly, many of these strains not only are defective in the acyltransferase but also appear to lack peroxisomes altogether, possibly like the fibroblasts from patients with Zellweger's syndrome (42, 91, 128). These CHO mutants may facilitate dissection of peroxisome assembly (128) by somatic cell genetics, and in addition give some insights into the biological functions of plasmalogens (88).

None of the genes altered in the available phospholipid mutants of CHO cells (Table 3) have been cloned or characterized. Many additional glycerolipid mutants remain to be isolated in somatic-cell systems. Phosphatidylinositol synthesis, phosphorylation, and turnover in hormone-responsive somatic cells should be studied, given the considerable circumstantial evidence for involvement of phosphatidylinositol metabolites in transmembrane signalling (14, 65, 94, 157). At the very least, one would expect animal-cell mutants blocked in different stages of phosphatidylinositol metabolism to show alterations in hormone responsiveness. They might also be conditionally lethal, since inositol auxotrophs of CHO cells can be isolated (Table 3). In view of the ability of animal cells to take up exogenous glycerolipids, it might be possible to isolate auxotrophs requiring phosphatidylinositol and/or its phosphorylated metabolites for growth.

SUMMARY AND CONCLUSIONS

I have attempted to illustrate the genetic and biochemical complexity of membrane-lipid synthesis by focusing, primarily, on *E. coli*. The use of molecular genetics to probe membrane lipids is relatively new. Many important questions of phospholipid biochemistry (181, 182) remain unanswered. In the coming years our growing knowledge of the molecular genetics of phospholipids must be applied to the solution of the following problems: (*a*) How does a cell regulate its total phospholipid content in relationship to macromolecules, especially membrane proteins, cell wall components, and nucleic acids? Why do *E. coli* and *Caulobacter* behave differently in this respect (34, 141, 143, 210)? (*b*) How does a cell regulate its characteristic ratios of polar headgroups and fatty acyl chains? Why does overproduction of phosphatidylserine synthase have no effect on phospholipid composition (163, 182, 188, 222)? (*c*) How is lipid topography established, both in terms of intramembrane movement (flip-flop) and intermembrane movement? Are there transport systems (flippases) for short-chain diacylglycerophospholipids in *E. coli,* as in mammalian microsomes (16), and can flippase mutants be

isolated? (*d*) What are the functions of the many individual phospholipid species (Figure 8)? Does *E. coli* have a functional equivalent of the mammalian phosphatidylinositol cycle (14, 94, 157)?

A complete set of phospholipid mutants, together with phenotypic suppressors, should help to answer these questions by allowing selective perturbations in vivo and physiological studies of associated phenotypes. In addition, molecular cloning is already providing access to large quantities of the lipid gene products, opening the door to biophysical and chemical studies of lipid–protein interactions.

A unique feature of genetics, as applied to complex biochemical or physiological systems, is the high frequency of unanticipated discoveries that accompany the characterization of new mutants. In our work, this is best illustrated by the analysis of phosphatidylglycerol-deficient mutants of *E. coli* (Figure 3), which provided the clue (i.e. lipid X) that permitted the elucidation of lipid A biosynthesis (Figure 5) (156, 183). The interconnection of metabolic pathways and important control mechanisms are often revealed by the study of mutants. In the case of *E. coli* (Figure 2) it is best to consider the many lipids and proteins of the envelope as a whole. Considering how few mutant alleles are available for the lipid genes of *E. coli* (Tables 1 and 2), it will be important to create many more genetic lesions in order to gain a full understanding of regulation and function.

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