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BIOCHEMISTRY OF ENDOTOXINS

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CONTENTS

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OVERVIEW

In this review, I summarize the elucidation of the biosynthesis and molecular pharmacology of endotoxins. These substances are the lipopolysaccharides (LPS) that are found on the outer surface of gram-negative bacteria. I propose new experiments to explore the mysteries of LPS export and outer membrane growth. I also present new strategies for identifying LPS receptors on animal cells.

INTRODUCTION

The outer monolayer of the outer membrane of most gram-negative bacteria consists of a unique molecule, known as lipid A (Figures 1–3) (1–5). The *sn*-1,2-diacylglycerol moiety of classical membrane phospholipids (6, 7) is absent in lipid A (Figure 2), and is replaced by a 2,3-diacylglucosamine unit (2–6). The acyl chains that are linked to the glucosamine backbone of lipid A differ from those of glycerophospholipids in that they are 2–6 carbon atoms shorter and contain an *R*-3-hydroxyl substituent (Figures 2 and 3) (2–7). The unique structure of lipid A presumably reflects its specific roles in outer

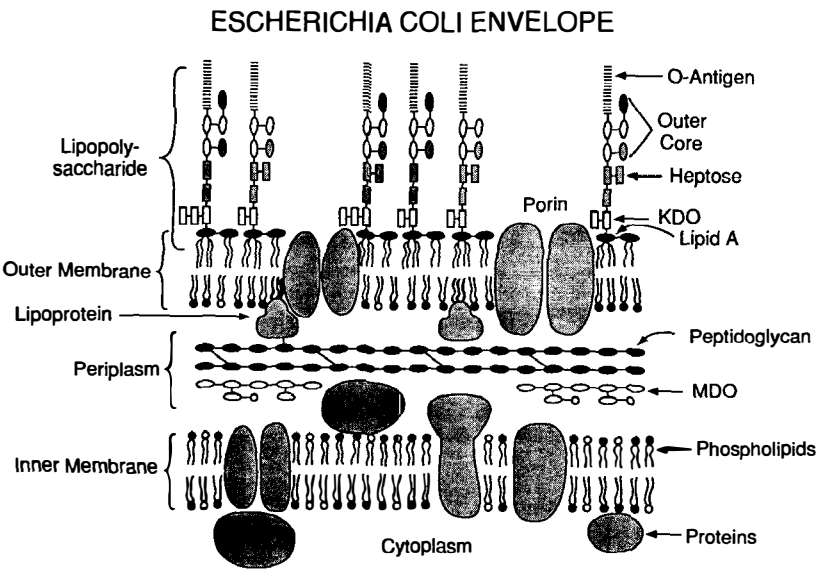


Figure 1 Schematic molecular representation of the *E. coli* envelope. Ovals and rectangles depict sugar residues. Circles represent the polar headgroups of phospholipids. MDO are membrane-derived oligosaccharides (6), and KDO is 3-deoxy-D-*manno*-octulosonic acid. KDO and heptose make up the inner core of LPS.

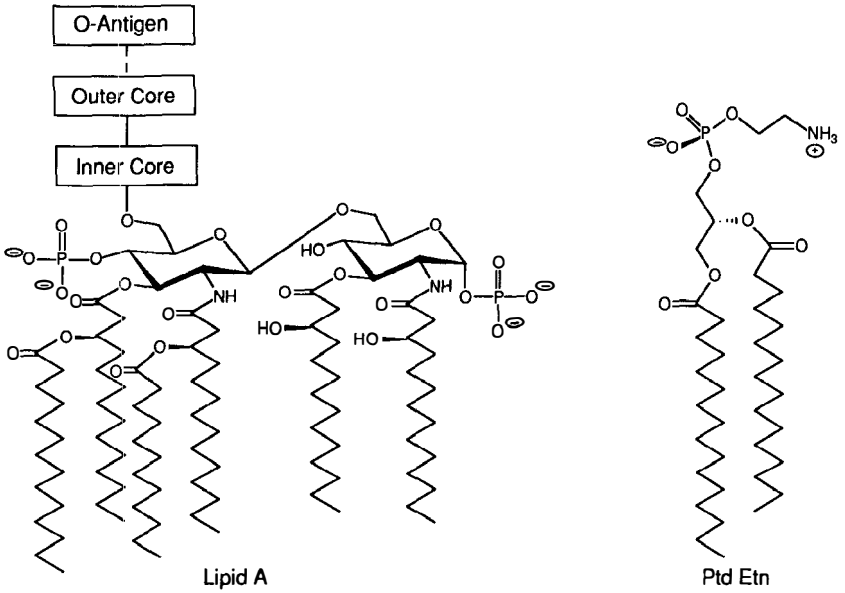


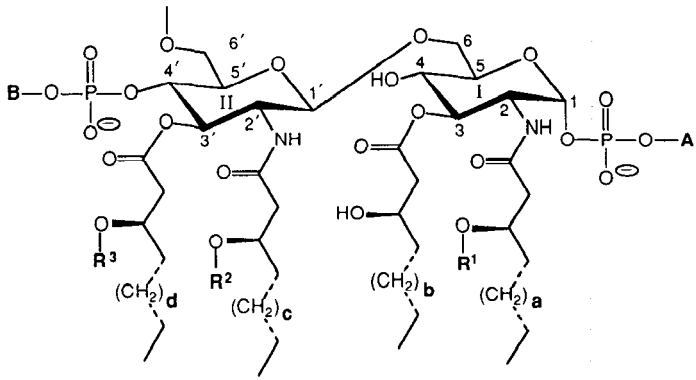
Figure 2 Covalent structure of *E. coli* and *S. typhimurium* lipid A. Phosphatidylethanolamine, the major glycerophospholipid (6) of these cells, is drawn to scale.

membrane assembly and function, and it ensures resistance to phospholipases.

Lipid A of *Escherichia coli* (8–12) and *Salmonella typhimurium* (13–15) is a β,1–6 linked disaccharide of glucosamine, acylated with *R*-3-hydroxymyristate at positions 2, 3, 2', and 3', and phosphorylated at positions 1 and 4' (Figures 2 and 3). The two *R*-3-hydroxy-acyl groups of the nonreducing glucosamine (8–15) are further esterified with laurate and myristate (Figure 2). The envelope of a single *E. coli* cell contains $\sim 2 \times 10^6$ lipid A residues and $\sim 2 \times 10^7$ glycerophospholipids (6), quantities consistent with the existence of one monolayer of lipid A and three monolayers of glycerophospholipids (Figure 1).

Lipid A is the membrane anchor of a larger structure, known as lipopolysaccharide (LPS) (Figure 1) (1, 4, 16–18). In LPS, the 6' position of lipid A (Figures 2–5) is glycosylated with a nonrepeating oligosaccharide, designated the core (18, 19). The eight-carbon sugar, 3-deoxy-D-*manno*-octulosonic acid (KDO), is linked directly to lipid A (Figure 5) (4, 16–18). Other core sugars include L-*glycero*-D-*manno*-heptose, glucose, galactose, and *N*-acetylglucosamine (4, 16–18, 20).

The LPS isolated from most clinical specimens additionally contains an O-antigen domain (Figure 1) (1, 4, 17, 18, 20). The latter is a distinct



	A	B	R^1	R^2	R^3	a	b	c	d
Escherichia coli K12	H	H	H	12:0	14:0	8	8	8	8
	(P)								
Salmonella typhimurium	H	H	H	12:0	14:0	8	8	8	8
	(AraN)	(PEtn)							
Salmonella minnesota	H	H	H	12:0	14:0	8	8	8	8
	(PEtn)	(AraN)	(16:0)						
Proteus mirabilis	H	AraN	H	14:0	14:0	8	8	8	8
			(16:0)						
Hemophilus influenzae	H	H	H	14:0	14:0	8	8	8	8
Neisseria gonorrhoeae	H	H	12:0	12:0	H	8	6	8	6
Pseudomonas aeruginosa	H	H	12:0	12:0	H	6	4	6	4
			(20H-12:0)	(20H-12:0)					

Figure 3 Predominant molecular species of lipid A found in pathogenic gram-negatives. The locations of the polar substituents, 4-amino-4-deoxy-L-arabinose (AraN) and phosphoethanolamine (PEtn), are the subject of a controversy (see text).

repeating oligosaccharide, attached to a distal glucose residue of the core (Figures 1 and 4) (1, 4, 16–18). The O-antigen repeat (often a tetrasaccharide) may be forty units long, but some LPS molecules within the same preparation may lack O-antigen (4, 18, 21). In *E. coli* K-12, the O-antigen is missing entirely (4, 17). O-antigen structures are highly variable, compared to those of the core and lipid A, possibly helping bacteria evade the immune system (4, 17).

LPS is often referred to as endotoxin (4, 22). This term was introduced in the 19th century to describe the component of gram-negative bacteria responsible for the pathophysiological phenomena associated with gram-negative

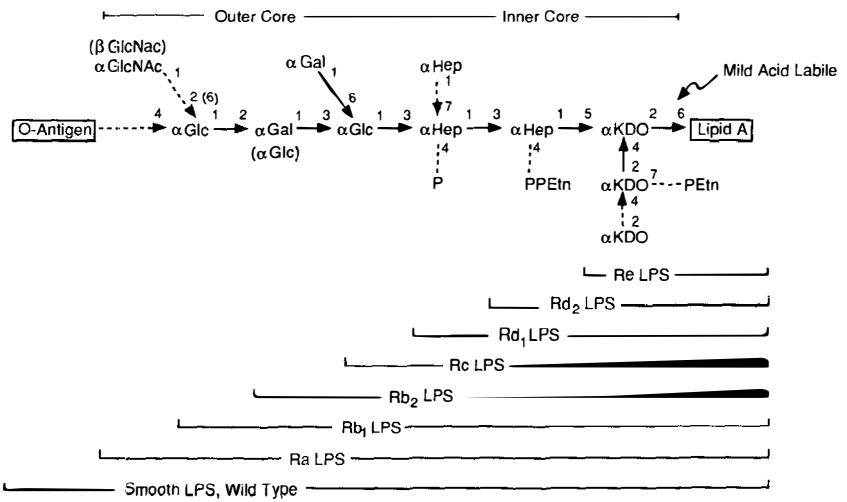


Figure 4 Definition of chemotype variants of the core domain. The core structures of *S. typhimurium* and *E. coli* K-12 (unique features of *E. coli* in parentheses) are very similar.

infections (4, 22, 23). It is now established that the lipid A moiety possesses most of the biological activities of LPS (3–5, 24). LPS and lipid A are potent activators of macrophages, resulting in the rapid induction of the syntheses of tumor necrosis factor (TNF) (25–27), interleukin 1 (IL 1) (28, 29), and other proteins (30). The generation of numerous lipid mediators, most notably platelet activating factor (PAF) (32), is also stimulated. When TNF is rapidly secreted into the circulation, a shock syndrome may occur, resembling that of gram-negative sepsis or LPS injection (31, 33–35). The high mortality associated with endotoxin-induced shock remains a major clinical problem, especially in debilitated and immunosuppressed patients (36, 37). The important studies of Beutler & Cerami (25) have clarified the role of TNF as a mediator in septic shock, and hold the promise of new therapeutic approaches (38, 39).

Given the prominent localization of lipid A in the gram-negative envelope (1) and the long-standing recognition of its importance in medicine (4, 22, 31), it is surprising that the true covalent structure of lipid A (8, 9) and the pathway for its enzymatic synthesis (2) were unknown before 1983. Progress with the chemistry of LPS was greatly accelerated by the development of fast atom bombardment mass spectrometry and high field NMR spectroscopy (2–6), combined with the use of molecular genetics to probe phospholipid metabolism (6). The latter approach led to the discovery of lipid X_n, a monosaccharide precursor of lipid A (9, 40) that provided the missing clue to lipid A biosynthesis (41, 42).

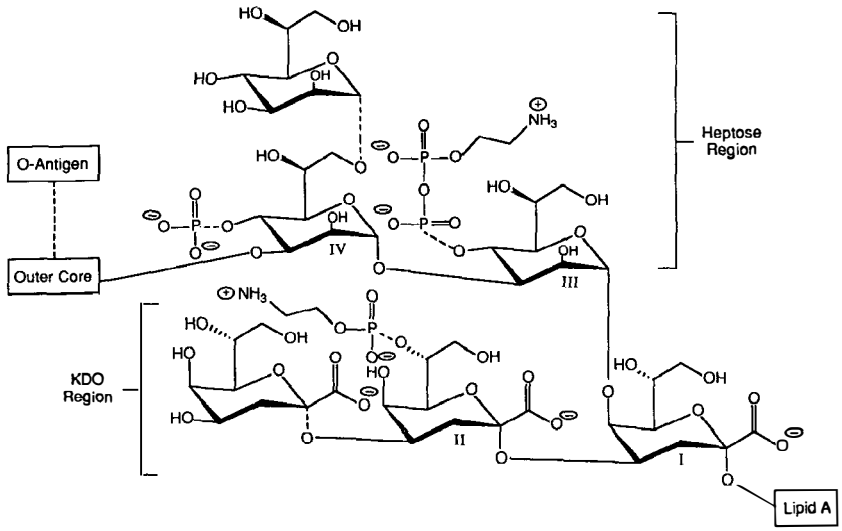


Figure 5 Proposed covalent structure of the inner core of *E. coli* K-12. Putative partial substitutions are indicated with dashed bonds.

Because lipid A is unique to gram-negative bacteria (1, 17) and is essential for growth (43), its biosynthesis is an attractive target for the design of novel antibacterial agents. In addition, pharmacological suppression of lipid A–induced TNF synthesis in macrophages could lead to new therapies for septic shock. The inhibition of interleukin 1 synthesis could be useful in the development of novel anti-inflammatory agents. The question of whether or not there are specific receptors for lipid A (and/or LPS) on animal cells (3, 44) remains a critical issue in this field.

BIOCHEMISTRY OF LIPID A AND CORE DOMAINS

Isolation, Structure, and Conformation of Lipid A

Lipid A and its precursors have solubility properties resembling those of glycerophospholipids (9, 45, 46). An LPS molecule bearing a complete core and O-antigen, however, is insoluble as a monomer, both in water and in most organic solvents (17). Commercial LPS preparations form opalescent dispersions in water, but their structures and critical micellar concentrations have not been thoroughly characterized (17). An important advance was made by Lüderitz & Westphal (47), who found that aqueous phenol could be used to extract LPS from bacteria. Galanos et al (48) found that mixtures of phenol, chloroform and petroleum ether were especially effective for extracting LPS lacking O-antigen. Acid and base hydrolyses of these preparations were used

to define the sugar and fatty acid compositions of LPS (4, 17, 20). Phenol-based solvent mixtures are not very useful for purifying subfractions of intact LPS to homogeneity.

The KDO–lipid A linkage (Figures 4 and 5) is labile to acid hydrolysis (4, 17, 20). When LPS is suspended in aqueous 0.1 M HCl and boiled for 15 min, the released lipid A forms a precipitate, while core and O-antigen remain in solution (4, 17, 20). Boiling in 0.1 M HCl also removes the anomeric phosphate of lipid A, any substituent (Figure 3) attached to the 4' monophosphate group, and some of the ester-linked fatty acids (13, 14). Selective removal of KDO without loss of the anomeric phosphate is possible, if the hydrolysis is carried out at pH 4.5 (14, 49). The discovery of an enzyme that cleaves the KDO–lipid A linkage under physiological conditions would greatly facilitate structural analysis.

In 1964, Burton & Carter (50) almost elucidated the covalent structure of lipid A as an incidental finding while searching for sphingolipids in *E. coli*. Using freezing point depression (50), they calculated a molecular weight of 1700 for lipid A released by acid hydrolysis, in excellent agreement with the FAB mass spectrometry of similar preparations, reported by Takayama and coworkers in 1982 (13, 14).

Five lines of evidence now provide unequivocal support for the lipid A structures shown in Figures 2 and 3. The most detailed work has been carried out with *E. coli* (2, 10–12), *S. typhimurium* (13–15), and *S. minnesota* (4, 51). (*a*) ^{31}P-NMR studies of crude LPS preparations ruled out earlier suggestions of pyrophosphate cross-bridges between lipid A units (52, 53). ^{13}C-NMR suggested that KDO is attached to lipid A by an α,2–6' linkage (19, 53). Prior to 1983, KDO was thought to be attached to position 3' (8, 54). (*b*) The application of FAB mass spectrometry to lipid A released from LPS by acid hydrolysis (13, 14) first defined the number and type of fatty acyl chains attached to the two GlcN residues (Figure 3). (*c*) Better methods for the purification (55), chemical degradation (49, 56, 57), and NMR analysis (10, 15, 19) of lipid A and its precursors (9, 45, 46) led to the conclusion that *R*-3-hydroxy fatty acids are attached exclusively to the glucosamine disaccharide backbone and that normal fatty acids are esterified exclusively to specific *R*-3-OH functions (*d*) The discovery of the monosaccharide precursor, 2,3-diacylglucosamine-1-P (lipid X), in which both acyl chains and *R*-3-hydroxymyristate, strongly supported the lipid A structures shown in Figures 2 and 3, and led to the elucidation of lipid A biosynthesis (2, 3, 8, 9, 40). (*e*) The chemical synthesis of the lipid A moiety shown in Figure 2 (12) verified that this material has the full biological activities of lipid A from enteric gram-negatives (24, 58). Chemically synthesized "lipid A," prepared according to earlier structural proposals (8, 17), lacked activity (58–60).

A high-resolution X-ray structure of lipid A has not yet been reported. Low

angle X-ray studies (61, 62) and model building (3, 61, 62) suggest that lipid A can fold into well-defined polar and nonpolar domains, compatible with insertion into phospholipid bilayers. Under some conditions lipid A may be able to assume an inverted hexagonal (H_{II}) conformation (63). Within biological membranes, unique lipid A–lipid A and lipid A–protein interactions must exist, and may play an important role in outer membrane assembly (see below). It has recently been possible to cocrystallize a defined lipid A disaccharide with the outer membrane porin encoded by *ompF*, but the structure is not yet solved (M. Garavito, personal communication).

Lipid A Structures in Non-enteric Bacteria

The structures of lipid A from the best characterized enteric and non-enteric gram-negative pathogens are compared in Figure 3. There is some heterogeneity within each organism, and there are several significant differences between species.

1. The lipid A of enteric gram-negatives [*E. coli* (10–13), *S. typhimurium* (14, 15), *S. minnesota* (51, 64–66), and *Proteus mirabilis* (56, 57, 66)] is "asymmetrically" acylated in the sense that four fatty acids are associated with the nonreducing end, while only two are linked to the reducing end glucosamine (Figure 3). With the exception of *Hemophilus influenzae* (67), the lipid A of the few non-enteric pathogens that have been characterized is "symmetrical" with respect to acyl chain placement (Figure 3) (67–70). The biological significance of this pattern is unclear.

2. Those non-enteric bacteria that tend to grow at lower temperatures [*Neisseria gonorrhoeae* (68) and *Pseudomonas aeruginosa* (69–70)] possess lipid A bearing somewhat shorter fatty acyl chains (Figure 3). This alteration might function to optimize the physical properties of lipid A for growth at lower temperatures. "Oxidizing" gram-negatives, such as *P. aeruginosa*, sometimes contain *S*-2-hydroxy fatty acids in acyloxyacyl linkage (Figure 3), in distinction to the *R*-3-hydroxy fatty acids that are attached to the glucosamine backbone (69, 70). The *S*-2, but not the *R*-3, oxygen atom (71) is derived from O_2.

3. Lipid A subspecies may exist within the same preparation that contain additional polar substituents at positions 1 and 4', including 4-amino-4-deoxy-L-arabinose and phosphoethanolamine (Figure 3) (4, 5). The functions of these moieties are unknown, but they generally reduce the net negative charge on lipid A. Polymyxin-resistant strains of *S. typhimurium* have been described with increased amounts of the 4-amino-4-deoxy-L-arabinose on lipid A (72).

The sites of attachment of phosphoethanolamine and 4-amino-4-deoxy-L-arabinose on lipid A require further investigation. Using the lipid A disaccharides precursors (45) that accumulate in KDO-deficient mutants of *S.*

typhimurium, Strain et al (73) demonstrated that phosphoethanolamine is attached via a pyrophosphate linkage at position 4' of lipid A, while 4-amino-4-deoxy-L-arabinose is attached via a phosphodiester linkage to the phosphate at position 1 of lipid A (Figure 3). These experiments were carried out with purified, undegraded lipids by several NMR methods. However, the results (73) conflict with all other published studies of polar headgroup localization on lipid A in which the positions of these substituents are reversed (4, 5, 17, 20).

With few well-documented exceptions (74), bacteria surrounded by two membranes (gram-negatives) contain a lipid A–like substance (4, 17). Even diverse phototrophic bacteria, which are assumed to have emerged relatively early in evolution, contain lipid A (75). In phototrophic bacteria, the *R*-3-hydroxy acids, attached to the glucosamine backbone, resemble those of *P. aeruginosa* (Figure 3) in length. In *Rhodobacter sphaeroides,* an N-linked, 3-keto-myristate variant may also be present (75–77) (see below), possibly functioning to limit lipid A acylation.

R. viridis and *P. diminuta* possess lipid A variants in which D-glucosamine is replaced with 2,3-diamino-2,3-dideoxy-D-glucose (75). It is also believed that some of these bacteria contain a monosaccharide form of lipid A in their LPS (75). Whatever the case may be, both *R*-3-hydroxy fatty acids are amide-linked in these organisms, conferring extra chemical stability (75). Although not found in living cells of *E. coli,* the 2,3-diamino-2,3-dideoxy-D-glucose analog of lipid X can substitute for lipid X as a substrate for the enzymatic synthesis of lipid A in cell extracts (78).

Most strains of *Rhizobium* contain LPS-like substances (17). It will be very interesting to elucidate the complete structure of *Rhizobium* lipid A (79) and to study the regulation of lipid A synthesis and LPS assembly during the penetration of root hair cells. A systematic search for lipid A–like molecules in *Archaebacteria* also might prove informative. The glycerophospholipids of *Archaebacteria* are extraordinary in that they contain a preponderance of ether linkages and because their hydrocarbon moieties are derived from isoprene, rather than acetate units (80). Identification of lipid A in *Archaebacteria* might provide new insights into the role of lipid A in membrane evolution.

There is no compelling evidence for lipid A–like molecules in eukaryotes. Given the diverse effects of lipid A on cells of the immune system (3–5, 31, 58), however, the possibility of lipid A homologs amongst the many, minor unidentified lipids of eukaryotes cannot be dismissed.

Definition of Chemotypes and the Inner Core

The core oligosaccharide of LPS (Figure 4) can be divided into an inner subdomain and an outer subdomain (4, 5, 16–18, 20). The inner core sugars of *E. coli* and *S. typhimurium* (18) (Figure 4) include certain unique species

that are characteristic of LPS, such as 3-deoxy-D-*manno*-octulosonic acid (KDO) (18, 81, 82) and L-*glycero*-D-*manno*-heptose (hep) (18, 83). The outer core consists of hexoses, primarily glucose, galactose, and GlcNac (18, 84). All of the core sugars are in the pyranose form (18, 84, 85), and in general, they have the α-anomeric configuration.

Certain bacteriophages attach to sugars of the O-antigen and core domains during infection (4, 18, 86, 87). It is very easy to select for bacterial mutants (referred to as "rough" or "R" because of their colony morphology) with truncated LPS structures (4, 17, 18, 86, 87). Only the lipid A and KDO moieties are essential for growth under laboratory conditions (3, 16, 43). Based on the sugar composition of the LPS isolated from various R mutants (4, 5, 16–18, 86, 87), it is possible to construct a classification of LPS substructures, termed chemotypes (Figure 4). The biochemical basis of these mutations is not adequately characterized. For example, mutations in at least three distinct genes confer heptose deficiency (88, 89), resulting in the Re chemotype.

Because of the difficulty of analyzing intact lipopolysaccharides under nondestructive conditions, the proposed structure of the *E. coli* inner core (18) (Figure 5) cannot be considered definitive. Most gram-negative enteric bacteria certainly contain the two inner KDO residues (I and II) and the two inner heptose units (III and IV) (Figure 5) (4, 5, 16–18, 82–85, 90). A third KDO, a third heptose, and several additional phosphorylated substituents (Figure 5, dashed bonds) are thought to exist in some strains (5, 18), but the techniques used to show their presence are indirect and subject to analytical artefacts (5, 91). The strategy of utilizing FAB mass spectrometry to evaluate the structure(s) of the acid-released core oligosaccharide(s), recently described by Gibson et al (85) for *N. gonorrhoeae*, has not been applied to most other LPS cores.

The conformation and functions of the inner core are unknown. Like lipid A, the inner core is polyanionic and bears at least two net negative charges at pH 7 (Figure 5). The temperature sensitivity of mutants defective in KDO biosynthesis (16, 18, 45, 92, 93) suggests an essential role for this acidic sugar in outer membrane assembly. Heptose-deficient mutants are not temperature sensitive (4, 5, 86–89), but they are unable to grow in the presence of bile acids and are hypersensitive to hydrophobic drugs (1), consistent with a role for heptose in the maintenance of outer membrane structure. Heptose-deficient mutants also lack certain outer-membrane proteins (1, 94, 95). The space created by the absence of these proteins is filled in with glycerophospholipids, resulting in the appearance of glycerophospholipids on the outer surface of the outer membrane of Re mutants (1).

At least one KDO residue (or KDO-like sugar) is found in all gram-negative bacteria (4, 5, 17). In some organisms, subtle modifications of KDO

structure are observed. In *H. influenzae,* the innermost KDO may be phosphorylated (67, 96). In *Acinetobacter calcoaceticus,* an octulosonic acid isomer resembling KDO is attached to lipid A (97). The bond between the two is not cleaved by mild acid hydrolysis (97). In such bacteria the presence of lipid A may be overlooked.

The Outer Core

The presumed covalent structure of the outer core of *E. coli* K-12 LPS is shown in Figure 6. Some of the earliest progress in LPS biochemistry resulted from the finding that *S. typhimurium* mutants defective in the synthesis of UDP-glucose and UDP-galactose contained truncated LPS molecules of the Rd_1 and Rc chemotypes, respectively (Figure 4) (16, 18, 98). Shortly thereafter, enzymes were found that appear to transfer glucose or galactose residues from UDP-glucose or UDP-galactose to Rd_1, Rc, or Rb_2 LPS, in accordance with the sequence shown in Figure 4, but not to Re, Ra, or smooth LPS (98, 99). However, the structures of the substrates and products of these glycosyl transferases remain to be verified by spectroscopic methods, and synthetic substrates are not available.

The covalent structures of outer cores are more variable than those of inner cores (17, 18, 20, 84). As the methods for LPS isolation (100) and carbohydrate sequencing by NMR (101) improve, additional structural subtleties are likely to be discovered. Other than providing an attachment site for O-antigen, the function of the outer core is unknown. Mutants lacking outer-core sugars have no obvious phenotypes, other than bacteriophage resistance (1, 86, 87), but less obvious changes in outer membrane function cannot be excluded.

Interaction of LPS with Polyvalent Cations

Given the negative charge associated with the inner core and lipid A, it is not surprising that cationic drugs, such as polymyxin (72), bind tightly to LPS.

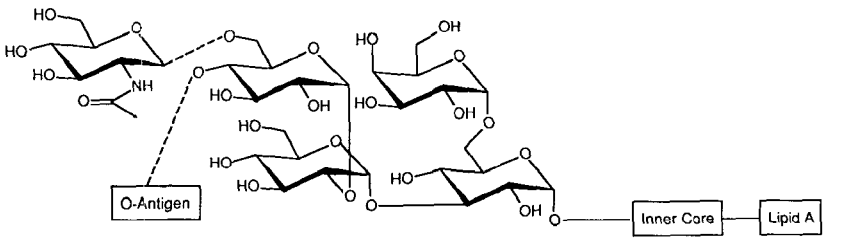


Figure 6 Proposed covalent structure of the outer core of *E. coli* K-12. Putative partial substitutions are indicated with dashed bonds.

When LPS is extracted from cells with phenol-containing solvents, it is frequently accompanied by organic counterions, such as polyamines (17, 102). These can be removed by electrodialysis (102), or preferably in the case of rough LPS, by partitioning in two-phase mixtures of chloroform, methanol, and water under acidic conditions (103). LPS also has high affinity for Mg^{2+} and Ca^{2+} (1, 17). Washing of intact *E. coli* with Tris-EDTA solutions causes the release of about 40% of the LPS from the bacterial surface and renders the cells permeable to hydrophobic organic compounds (1, 104). LPS is presumably released in the form of noncovalent aggregates.

Incubation of *E. coli* and related bacteria with high concentrations of Ca^{2+} at 0°C renders them competent to take up DNA, permitting genetic transformation without loss of viability (105). The phenomenon of transformation in *E. coli* is amazing, considering that the molecular weight exclusion limit of outer-membrane porins (Figure 1) to hydrophilic molecules is approximately 500 daltons (1). The role of Ca^{2+} in generating the competent state may involve unique interactions of the metal with LPS, which might be revealed by physical studies at the level of atomic resolution.

BIOCHEMISTRY OF O-ANTIGENS

Because O-antigen is usually attached to a terminal sugar of the outer core (Figures 1, 4, and 6), it is exposed and immunogenic (1, 4, 17, 18, 20). In *Salmonella,* there are more than a thousand distinct immunochemical variants of O-antigen (4, 17). Sometimes these differ by subtle modifications, such as acetylation of a single sugar residue (4, 18). In other instances, the O-antigen oligosaccharide may be totally distinct, requiring the presence and functioning of alternative biosynthetic genes (4, 18). The many modifications of O-antigen may help gram-negative bacteria evade the immune system, but some pathogenic gram-negatives, such as *Chlamidia trachomatis,* lack O-antigen (106, 107). The LPS of the latter organism consists only of lipid A and KDO.

The biosynthesis of O-antigen (see below) is independent of lipid A and core assembly (4, 17, 18, 20, 98). It occurs in association with bactoprenyl pyrophosphate (18, 108, 109), the same carrier lipid required for peptidoglycan biosynthesis (110, 111). When the synthesis and polymerization of O-antigen on bactoprenyl pyrophosphate is complete, it is transferred en bloc to the outer core of LPS (18, 20, 98).

Some O-antigens are repeating tri- or pentasaccharides. Others are branched tetra- or hexasaccharides (4, 17, 18, 20). Most O-antigen sugars are hexoses, but they also include unusual species, such as 6-deoxy or 3,6-dideoxy hexoses (4, 17, 18, 20). The well-studied O-antigen repeat of *S. typhimurium,* type B (4, 17, 18), is shown in Figure 7 (IV, III, II, and I are α-D-abequose, α-D-mannose, α-L-rhamnose, and α-D-galactose, respective-

ly). Since residue IV is an abequose branch attached to residue III, the actual repeat consists of residues I, II, and III. The lysogenization of *S. typhimurium* with P22 results in the introduction of a gene that modifies residue I with an additional glucosyl unit (not shown), linked α,1–6 (phage conversion) (18). This alteration is thought to increase the resistance of P22 lysogens to superinfection, since the binding of P22 to glucosylated O-antigen is reduced (18). Host-encoded modifications of O-antigen are also observed. For instance, residue IV may be acetylated at position 2, and residue I is sometimes glucosylated at position 4 (form variation), but the significance of form variation is not understood (4, 18).

Many *E. coli* strains, including K-12 and B, have lost their capacity to synthesize O-antigen (4, 17, 18). However, some gram-negatives harbor natural or artificial plasmids encoding the enzymes of the O-antigen pathway (112–114), which in *Salmonella* are clustered within the *rfb* locus on the chromosome (4, 88). Introduction into *E. coli* K-12 of hybrid plasmids coding for the genes of *Shigella* O-antigen assembly enables the cells to generate this material and to export it to the outer membrane (113).

The glycosidic linkage through which O-antigen is attached to the core has not been studied in very many organisms and requires reinvestigation with new analytical methods (4, 17, 18, 20, 98). Within one LPS preparation, there are some molecules that contain no O-antigen, and others bearing up to 40 repeats (18, 21, 115). The microheterogeneity of the O-antigen on LPS presumably reflects what is attached to the bactoprenyl pyrophosphate carrier. LPS molecules differing by one O-antigen repeat can be separated from each other by SDS gel electrophoresis (21, 115), permitting estimation of the extent of O-antigen polymerization. The conformation of O-antigen is unknown.

Mutants of some strains of *Rhizobium,* a genus of gram-negative soil bacteria, have been described that are unable to penetrate the root hair cells of

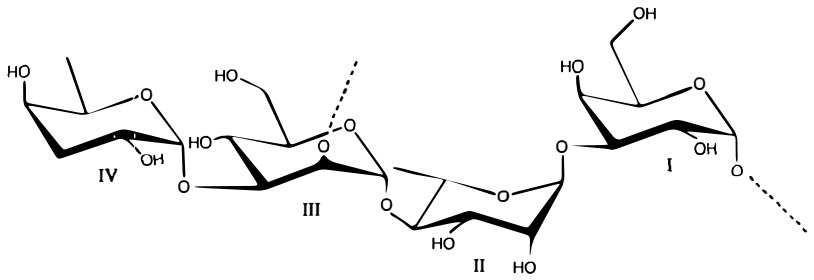


Figure 7 Structure of the O-antigen repeat of *S. typhimurium* type B. The dashed bond on the extreme right is the proximal end of the repeat.

legumes (116, 117). In some of these mutants there are alterations in the composition of O-antigen (116).

BIOSYNTHESIS OF LIPID A AND KDO

Lipid X and the β,1–6 Linkage of Lipid A

Early attempts to elucidate the biosynthesis of lipid A were hampered by the fact that the sites of acylation were unknown and that KDO was thought to be attached to position 3' (Figure 3) (16, 17, 20, 54). However, the biosynthesis of KDO and the existence of CMP-KDO as the activated form of KDO were defined 20 years before the enzymatic synthesis of lipid A was discovered (16, 20, 118, 119). Early recognition of the steps involved in KDO formation enabled Rick & Osborn (16, 18, 92, 120, 121) to isolate the first mutants defective in KDO synthesis. At nonpermissive temperatures, these strains accumulate a disaccharide precursor of lipid A that is not fully acylated (92, 121), demonstrating that the addition of KDO precedes the completion of lipid A. This precursor was later shown to have the structure designated as lipid IV_A in Figure 8 (45, 73).

Prior to 1983, it was thought that the glucosamine disaccharide of lipid A might be synthesized first, and then fatty acylated (16, 122). Attempts to demonstrate the existence of enzymes catalyzing the fatty acylation of lipid A, that had been O-deacylated with alkali, were unsuccessful (122). The discovery of previously unknown, monosaccharide substructures of lipid A (9, 123), which accumulate to high levels in some phosphatidylglycerol-deficient mutants of *E. coli* (40, 124, 125), provided the missing information needed to elucidate lipid A biosynthesis. The simplest of these molecules, designated lipid X (9), is shown in Figure 8. It is a derivative of α-D-glucosamine 1-phosphate, acylated with *R*-3-hydroxymyristate at positions 2 and 3. The relationship of lipid X accumulation to phosphatidylglycerol deficiency remains unexplained (6, 40). It may reflect the operation of regulatory mechanisms that balance the content of negatively charged glycerophospholipids in *E. coli,* since lipid X bears a formal structural resemblance to phosphatidic acid (41). The existence of lipid X in wild-type cells has been demonstrated (41), but its levels are very low (about 1000 molecules per cell).

Comparison of the structure of lipid X (Figure 8) to that of *E. coli* lipid A (Figure 2) suggested that lipid X might be a precursor (or breakdown product) of the reducing end unit of lipid A. However, both the reducing and the nonreducing end sugars of lipid A (Figure 2) may be viewed as substituted 2,3-diacylglucosamine moieties. This consideration led Ray et al (42) to prepare a nucleotide derivative of lipid X (designated UDP-2,3-diacyl-GlcN

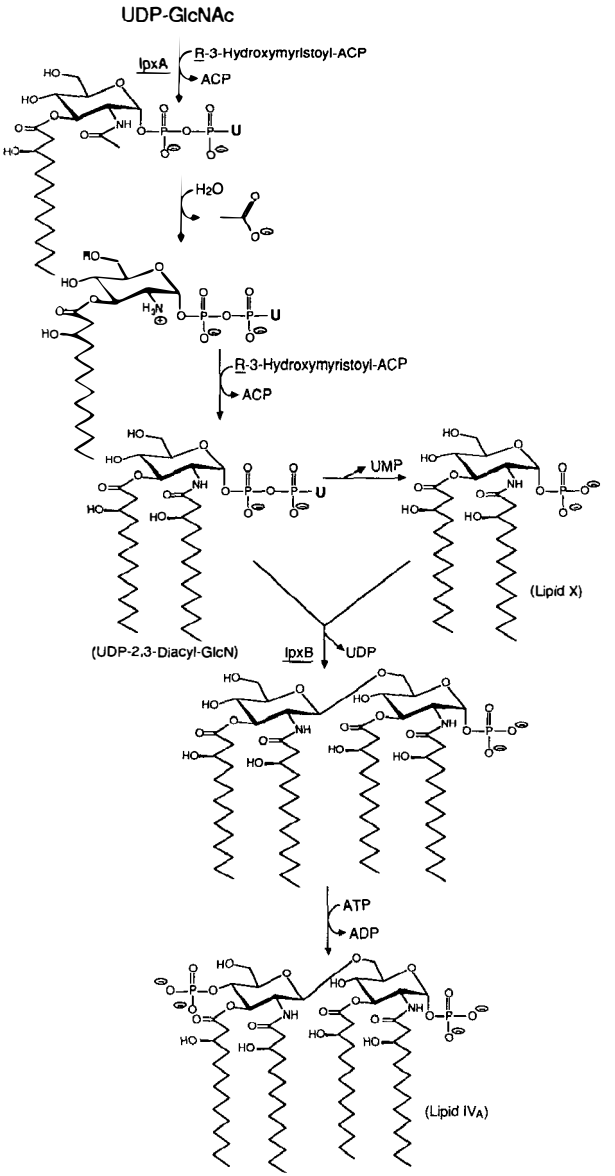


Figure 8 Early steps of lipid A biosynthesis. UDP-GlcNAc acyltransferase is the first committed enzyme (2, 3). The *lpxA* and *lpxB* genes code for the enzymes indicated. ACP, acyl carrier protein; U, uridine.

in Figure 8) to test the hypothesis that this substance might serve as a precursor of the nonreducing end sugar of lipid A. This experiment was done by incubating 1 mM lipid X and 1 mM UDP-2,3-diacyl-GlcN with a crude cytosolic fraction of *E. coli* (42, 126). Rapid formation of a new product, having the structure of the tetraacyl-disaccharide 1-phosphate shown near the bottom of Figure 8, was observed (42, 126). The demonstration that this product had the characteristic β,1–6 linkage of lipid A and that an enzyme catalyzing its formation existed in wild-type *E. coli* provided compelling initial evidence that lipid X was a precursor of lipid A. The identification of UDP-2,3-diacyl-GlcN as a natural product in living cells provided additional support for this scheme (41).

Fatty Acylation of UDP-GlcNAc

As noted above, lipid X bears a formal structural resemblance to phosphatidic acid (41). The 1, 2, and 3 positions of the GlcN-1-P backbone of lipid X are analogous to the 3, 2, and 1 positions of *sn*-glycerol-3-P of phosphatidic acid (41). The fatty acyl chains of lipid X are a little shorter than those of phosphatidic acid, and the polar headgroup of lipid X is somewhat larger. This may explain why under some conditions lipid X appears to form micellar rather than vesicular structures in water (126, 127).

Given the resemblance of lipid X to phosphatidic acid, it seemed reasonable to suppose that lipid X might be synthesized by the enzymatic acylation of GlcN-1-P, in analogy to the acylation of *sn*-glycerol-3-P in *E. coli* and other systems (6, 7). This did not prove to be the case. Instead, Anderson et al (128) found that UDP-2,3-diacyl-GlcN was a precursor of lipid X, necessitating the search for a system of enzymes capable of acylating UDP-GlcN or UDP-GlcNAc. As illustrated in Figure 8, the latter was discovered to be the key precursor of lipid A (129).

The three enzymes required for the synthesis of UDP-2,3-diacyl-GlcN (Figure 8) are localized in the cytosol (128–130). The first committed step is the acylation of the 3 position of the GlcNAc residue of UDP-GlcNAc with *R*-3-hydroxymyristate (129), followed by deacetylation, and N-acylation (130). The sole functional acyl donor in this system is *R*-3-hydroxymyristoyl-acyl carrier protein (128–130). The two acyltransferases do not function at appreciable rates with myristate, *S*-3-hydroxymyristate, or other *R*-3-hydroxy fatty acids differing in length (129). This finding is consistent with the fatty acid composition of lipid A isolated from *E. coli* and *S. typhimurium* (10–15, 17).

Strangely, glycerol-3-phosphate acyltransferase (6, 131) does utilize *R*-3-hydroxymyristoyl-acyl carrier protein efficiently, raising the question of how *R*-3-hydroxymyristate is excluded from glycerophospholipids. One obvious possibility is compartmentalization. All of the enzymes of lipid A and KDO

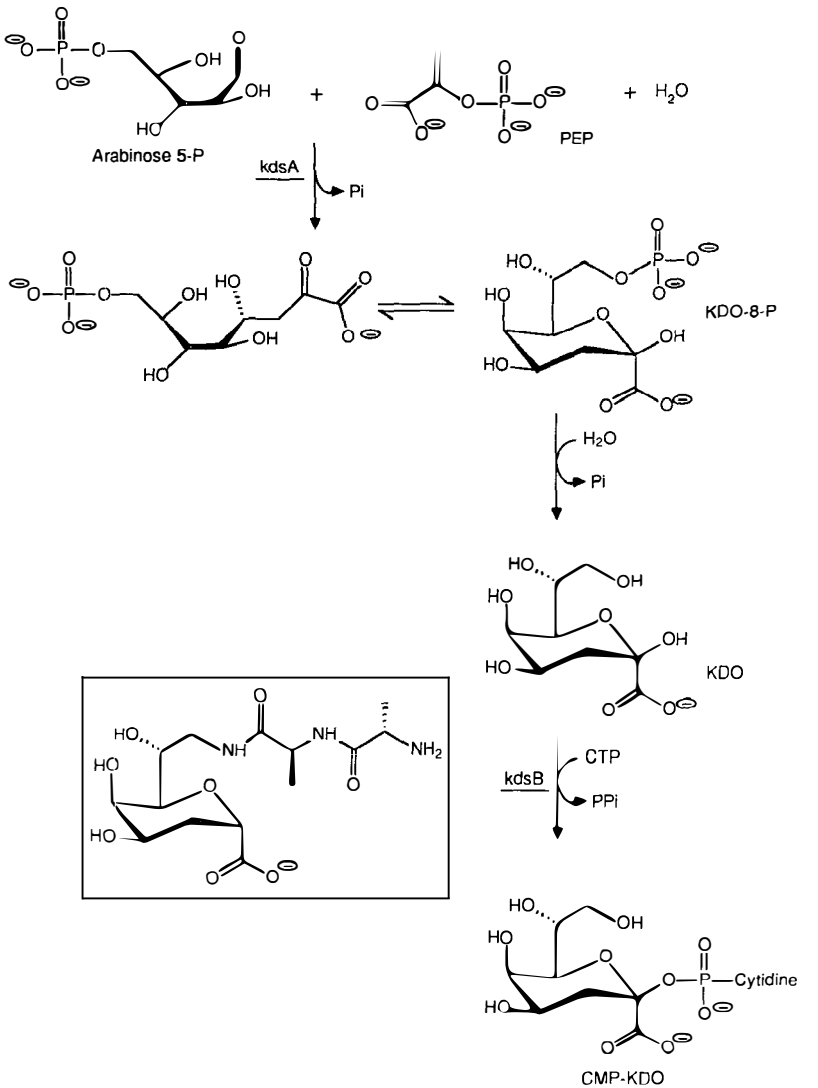


Figure 9 Enzymatic synthesis of CMP-KDO. The prodrug shown in the inset is converted to 2,8-dideoxy-8-amino-KDO in vivo, a competitive inhibitor of CMP-KDO synthase (147–151).

biosynthesis (Figures 8–10), with the possible exception of the 4' kinase (Figure 8; 132) and the last myristoyl transferase (Figure 10), are recovered in the cytosol, whereas most enzymes of glycerophospholipid synthesis are associated with the cytoplasmic membrane (6, 7). Perhaps, *R*-3-hydroxy-

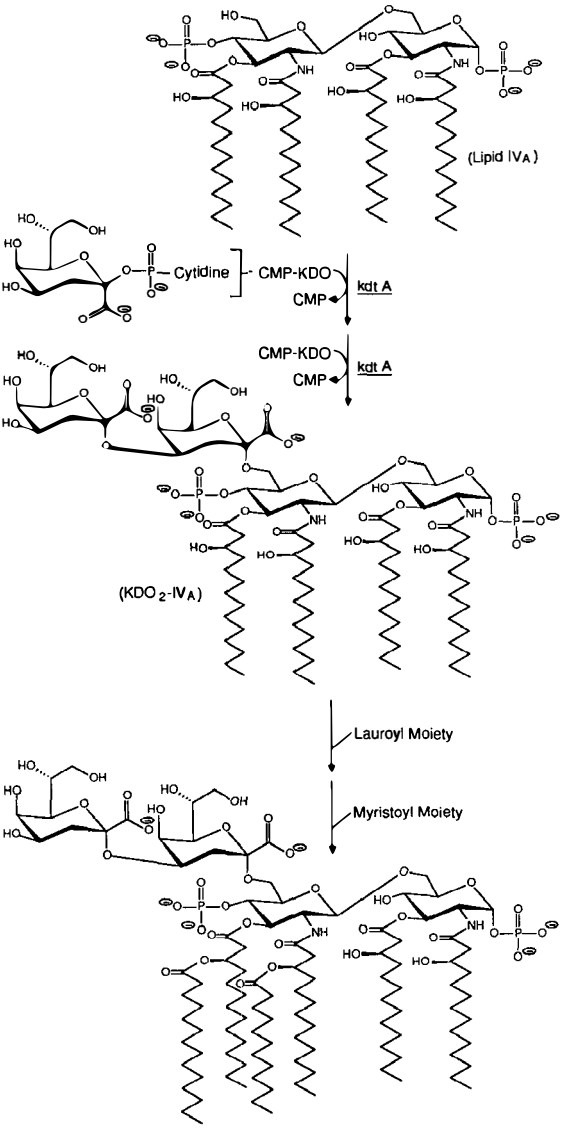


Figure 10 Late steps of lipid A biosynthesis. The structure shown at the bottom (Re endotoxin) is the minimal LPS required for growth.

myristoyl acyl carrier protein does not partition into the inner membrane, where glycerol-3-P acyltransferase could utilize it.

Formation of Lipid IV_A

Two enzymes cleave UDP-2,3-diacyl-GlcN to generate lipid X and UMP (Figure 8) (128, 129). One of these also attacks CDP-diacylglycerol (41, 133). The cleavage of UDP-2,3-diacyl-GlcN may be regulated, because an imbalance in the ratio of lipid X to UDP-2,3-diacyl-GlcN would interfere with lipid A biosynthesis. The disaccharide synthase cannot condense two molecules of UDP-2,3-diacylglucosamine directly (126).

The 4' monophosphate of lipid A is incorporated by a unique kinase (132) (Figure 8), distinct from diacylglycerol kinase (6). The 4'-kinase is very useful for the preparation of lipid A disaccharides of high specific radioactivity (46). The [4'-^{32}P]-lipid IV_A generated by the kinase is a sensitive probe for examining the later stages of Re LPS assembly (103), and for detecting the binding of lipid A–like molecules to the surfaces of animal cells (see below) (44).

Regulation and Molecular Genetics

The discovery that UDP-GlcNAc plays a central role in the synthesis of lipid A (128–130) demonstrates that this substance is situated at an important branchpoint in *E. coli*, leading either to the synthesis of the lipid A monolayer or to peptidoglycan (Figure 1). In the latter case, UDP-GlcNAc is first derivatized with phosphoenolpyruvate at position 3 of the GlcNAc residue, followed by generation of the muramyl pentapeptide (110, 111, 134). The partitioning of UDP-GlcNAc between LPS and peptidoglycan synthesis may be regulated.

R-3-hydroxymyristoyl acyl carrier protein is also situated at a branchpoint. It can be incorporated into lipid A (128–130), or it can be extended to generate palmitate, a major component of glycerophospholipids (7). The finding that UDP-GlcNAc acyltransferase does not function with the coenzyme A derivative of *R*-3-hydroxymyristate (128) explains why exogenous *R*-3-hydroxymyristate cannot be incorporated into lipid A (16, 135), but can be utilized as a carbon source by *E. coli*. Fatty acids taken up from the medium are converted to coenzyme A derivatives, not to ACP thioesters (7, 16, 135). Although exogenous fatty acids may be incorporated into glycerophospholipids (which derive their fatty acids from either coenzyme A or ACP thioesters), exogenous fatty acids cannot be elongated prior to incorporation into glycerophospholipids in *E. coli*, since elongation would require conversion to an ACP thioester (7, 135).

Very few mutants defective in lipid A biosynthesis have been described (2,

3, 42, 43). Galloway & Raetz (43) have recently identified two defective alleles of the *lpxA* gene (136, 137) that codes for UDP-GlcNAc acyltransferase (Figure 8). These organisms are temperature sensitive for growth, are strikingly deficient in UDP-GlcNAc acyltransferase, and stop growing under nonpermissive conditions (42°C) when the cellular content of lipid A is reduced about twofold (43). The rate of lipid A synthesis is reduced tenfold under these circumstances, but glycerophospholipid and bulk protein syntheses are unaffected for at least an hour after the shift from 30 to 42°C (43). After 2 hours at 42°C, cell viability rapidly declines (43). The isolation of conditional mutants defective in UDP-GlcNAc acyltransferase provides compelling physiological evidence that lipid A is an essential molecule for the viability of *E. coli* and that the pathway (Figure 8) is the predominant one for the generation of lipid A (43).

DNA sequencing of the region around the *lpxA* gene near minute four on the *E. coli* chromosome has demonstrated that *lpxA* is part of a complex operon containing at least one additional gene of the lipid A pathway (designated *lpxB*) (137–139). The latter codes for lipid A disaccharide synthase (Figure 8) (42, 136, 138). The operon also contains the *dnaE* gene (the catalytic subunit of DNA polymerase III) and at least three additional open reading frames of unknown function (140, 141). Most of the RNA transcripts from this region carry both *lpx* genes and *dnaE*, but additional, minor internal promoters exist (including one inside *lpxB* that can transcribe *dnaE*) (140, 141). The function of the operon is unknown. Its existence hints at coordinated regulation of outer membrane biogenesis and DNA replication.

The *lpxA* gene is immediately upstream of the *lpxB* locus (136, 138). "Tight" conditional point mutations in *lpxB* are not available, but one allele does exist that causes massive accumulation of lipid X and UDP-2,3-diacyl-GlcN (41, 42, 124). The total content of lipid A in this strain is not actually reduced, and the organism is viable (124). However, lipid X accumulation accentuates the temperature sensitivity of mutants defective in phosphatidylglycerol synthesis, and reduces the residual level of phosphatidylglycerol in such mutants (40, 124). The availability of the cloned *lpx* genes has permitted their overexpression and the purification of both enzymes to homogeneity (126, 129). The genetic loci coding for the other enzymes of the early steps of lipid A biosynthesis (Figure 8) have not been identified.

Biosynthesis and Activation of KDO

Heath and coworkers (119, 122, 142) first demonstrated that KDO is activated to CMP-KDO, a sugar nucleotide that serves as the precursor of the KDO domain of LPS. The first committed step of the KDO pathway (Figure 9) is the condensation of arabinose-5-P with phosphoenolpyruvate (118, 143). The enzymology of this system is well characterized (143–145). Unlike

CMP–sialic acid, CMP-KDO is unstable under physiological conditions (half-life of about 10 minutes), requiring its enzymatic generation in situ for biosynthetic studies (103, 145).

Two classes of mutations (*kdsA* and *kdsB*) are available that block the synthesis of KDO in *E. coli* and *S. typhimurium* (Figure 9) (88, 89). Mutations in KDO biosynthesis confer temperature-sensitive growth and result in the accumulation of underacylated disaccharide *bis*-phosphate precursors of lipid A (45, 73, 92, 93, 121, 146), predominantly lipid IV_A (Figure 10) (45, 73) in the inner membrane. The temperature-sensitive phenotype of KDO-deficient mutants most likely reflects the essentiality of Re LPS for membrane assembly, but the alternative, that lipid IV_A is toxic, cannot be excluded. KDO may also have other essential functions in the cell, unrelated to LPS.

Several inhibitors of CMP-KDO synthase have been described (147–151). These have promise as antibacterial agents. One of these compounds (Figure 9, inset) is a 2,8-dideoxy-8-amino analog of KDO, in which the amino function is acylated with the dipeptide L-Ala-L-Ala (147, 148). The latter facilitates the uptake and concentration of the drug via the oligopeptide permease (147, 148). Inside the cell, the dipeptide is hydrolyzed away (147, 148). The 2,8-dideoxy-8-amino KDO analog is a competitive inhibitor of CMP-KDO synthase with a K_i of approximately 4 μM (151). These inhibitors are extremely useful for studying the biosynthesis of LPS in diverse gram-negative bacteria. In most organisms they cause the accumulation of an underacylated lipid A precursor (as observed in *kds* mutants) (70, 152), but in *P. aeruginosa,* mature hexaacyl lipid A (Figure 3) builds up (70).

In *S. typhimurium,* the inhibition of KDO biosynthesis causes the accumulation of several metabolites in addition to IV_A (45, 73, 146). These include minor species bearing phosphoethanolamine (45, 73), 4-amino-4-deoxy-L-arabinose (153), palmitate (45, 46, 73), or some combination (Figure 11) (45, 73). The palmitate-containing variants, such as lipid IV_B (45, 46, 73), are especially intriguing, since negligible quantities of palmitate are found in lipid A of *E. coli* or *S. typhimurium* (10–15), but in *S. minnesota* LPS (51, 64–66), an analogous palmitoyl residue is actually found in some mature lipid A moieties (Figure 3). A palmitate-containing derivative of lipid X has also been observed (40, 46, 123, 125). In this substance (termed lipid Y) (40, 123), the palmitoyl moiety is attached to the *R*-3-hydroxyl of the N-linked hydroxymyristoyl residue, as in IV_B (Figure 11). The function of palmitate-containing intermediates is unknown.

Transfer of KDO to Lipid IV_A

Lipid A, released from LPS by mild acid hydrolysis, can accept KDO residues from CMP-KDO in the presence of an *E. coli* extract (142). The underacylated lipid A precursors that accumulate in KDO-deficient mutants

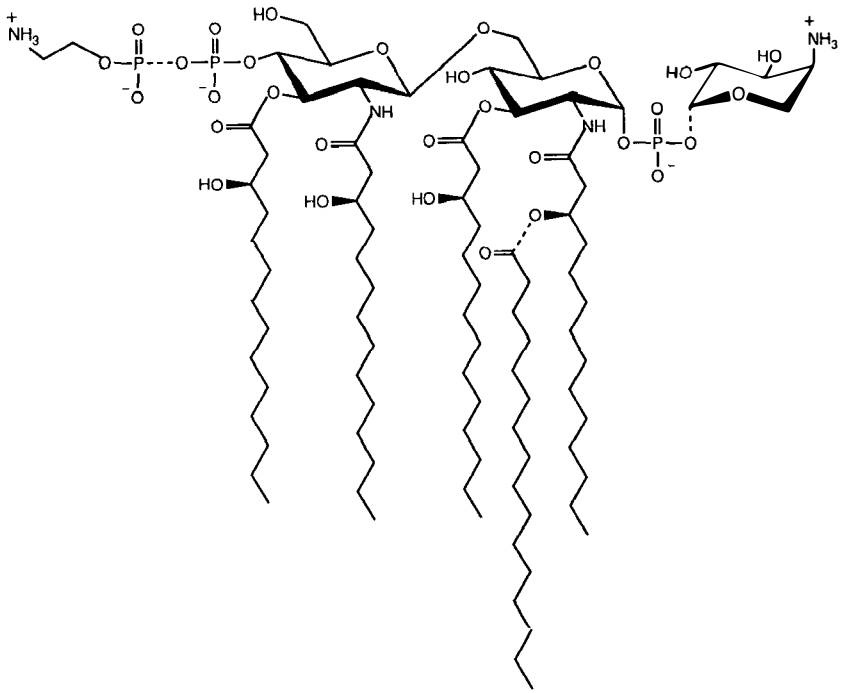


Figure 11 Minor modified forms of lipid IV_A that accumulate in KDO-deficient mutants. The substituents indicated by the dashed bonds may occur singly or in combination to give a total of eight derivatives (45, 73). Lipid IV_A is the predominant metabolite recovered from these mutants (45, 73). Lipid IV_B is the same as IV_A, but it is derivatized with palmitate.

(154) can also accept KDO. Based on radiochemical labeling studies, Munson et al (154) concluded that two KDO units were added in rapid succession. These investigators were unable to separate the two, putative KDO transferases from each other. The structures of the enzymatic products, generated in these early investigations, were not analyzed by spectroscopic methods (154).

Recently, Brozek et al (103) have re-examined the enzymology of KDO transfer in *E. coli* extracts, using lipid IV_A as the acceptor. A partially purified enzymatic system was developed that converts CMP-KDO and lipid IV_A to a single glycosylated product in high yield (103). FAB mass spectrometry and NMR spectroscopy showed the composition of the product (103) to be KDO_2-IV_A. Two-dimensional ^{1}H-NMR spectroscopy suggests that the two KDO moieties of the enzymatic product (103) have the same stereochemistry as the KDO residues of Re endotoxin (Figure 10) (82, 155, 156). The gene that codes for the bifunctional KDO transferase has recently been cloned

(157). It maps near min 81 between *rfa* and *pyrE* (157). Addition of a possible, third KDO residue (Figure 5) has not been observed in cell extracts (103, 154).

Formation of Acyloxyacyl Moieties

When KDO_2-IV_A is incubated with concentrated extracts of heptose-deficient mutants of *E. coli* or *S. typhimurium* (i.e. *rfaC, rfaD,* or *rfaE*) (88), it is converted, stepwise, to two less polar metabolites (K. Brozek and C. R. H. Raetz, unpublished). At least two, novel acyltransferases are involved (Figure 10). When a membrane-free cytoplasmic extract is used as the enzyme source, KDO_2-IV_A acylation is stimulated several hundredfold by lauroylacyl carrier protein (K. Brozek and C. R. H. Raetz, unpublished), but not by lauroyl coenzyme A or myristoyl acyl carrier protein. When membranes are present, myristoyl acyl carrier protein is also utilized, but not as rapidly as lauroyl acyl carrier protein. Analyses by FAB mass spectrometry and NMR spectroscopy confirm the presence of laurate and myristate residues in the products. Lipid IV_A cannot replace KDO_2-IV_A as an acceptor of laurate or myristate, demonstrating that the "late" acyltransferases of the lipid A pathway (Figure 10) recognize the KDO disaccharide (K. Brozek and C. R. H. Raetz, unpublished).

The palmitate moiety of lipid IV_B (Figure 11) (45, 73) or lipid Y (123) is incorporated by a distinct, membrane-bound acyltransferase that does not require a thioester substrate (46). Instead, the acyl donor may be any glycerophospholipid, bearing palmitate at the *sn*-1 position (46).

Acyloxyacyl residues may modulate the physical state of lipid A (61, 62). Mutants thought to be unable to add laurate and myristate are temperature sensitive and apparently accumulate something resembling KDO_2-IV_A (158). The enzymology and genetics of these mutants have not been studied. Mutants unable to incorporate palmitate are not yet available.

BIOSYNTHESIS OF THE CORE

The inner core of wild-type *E. coli* contains several substituents in addition to two KDOs. These include the two heptose units, designated III and IV (Figure 5), and possibly, a third KDO, a third heptose, and several phosphorylated moieties (Figure 5, dashed bonds). The evidence for these partial substituents is indirect (4, 5, 17, 18, 20, 91).

The heptose residues of LPS generally have the L-*glycero*-D-*manno* configuration (4, 5, 17, 18, 83). The biosynthesis of heptose has received little attention (18, 159). It is proposed (Figure 12) that this substance is derived from sedoheptulose 7-phosphate, and is activated as the D-*glycero*-D-*manno* isomer (Figure 12) (18, 159). The enzymes catalyzing these reactions have

not been adequately characterized. The identification of ADP as the activating nucleotide is plausible (160, 161), but tentative. A chemically synthesized standard has not been reported. Mutants defective in the *rfaD* gene are thought to lack the epimerase that converts ADP-D-*glycero*-D-*manno*-heptose to ADP-L-*glycero*-D-*manno*-heptose (162–164), but the supporting enzymology is inconclusive. Mutants in *rfaD* supposedly incorporate D-*glycero*-D-*manno*-heptose into LPS (162–164).

Brozek & Raetz have recently discovered that concentrated cytosolic extracts of wild-type *E. coli* convert KDO_2-IV_A to three, more polar derivatives (103). These reactions are stimulated by crude ADP-heptose preparations, isolated from *Shigella sonnei* (160, 161), but the structures of the enzymatic products have not been established (K. Brozek and C. R. H. Raetz, unpublished). Extracts from Re mutants of *S. typhimurium* (88) (defective in the *rfaC*, *rfaD*, or *rfaE* genes) are unable to generate these polar derivatives of KDO_2–IV_A (K. Brozek and C. R. H. Raetz, unpublished). In the case of *rfaD* and *rfaE*, the enzymatic activity can be restored by the addition of crude ADP-heptose from *S. sonnei*. These results suggest that *rfaC* encodes the enzyme that adds the innermost heptose (sugar III in Figure 5), while *rfaE* and *rfaD* are required for ADP-heptose biosynthesis. In extracts made from mutants harboring lesions in *rfaF*—having the partially heptose-deficient Rd_2 chemotype (88) (Figure 4)—the conversion of KDO_2-IV_A stops at the first polar intermediate migrating just below KDO_2-IV_A (103; K. Brozek and C. R. H. Raetz, unpublished), suggesting that *rfaF* codes for the enzyme that adds the second heptose (sugar IV, Figure 5). Since the heptosylation of KDO_2-IV_A proceeds in high yield (K. Brozek and C. R. H. Raetz, unpublished), the facile, semisynthetic preparation of LPS substructures or analogs of the Rd_1 and Rd_2 chemotypes should become feasible.

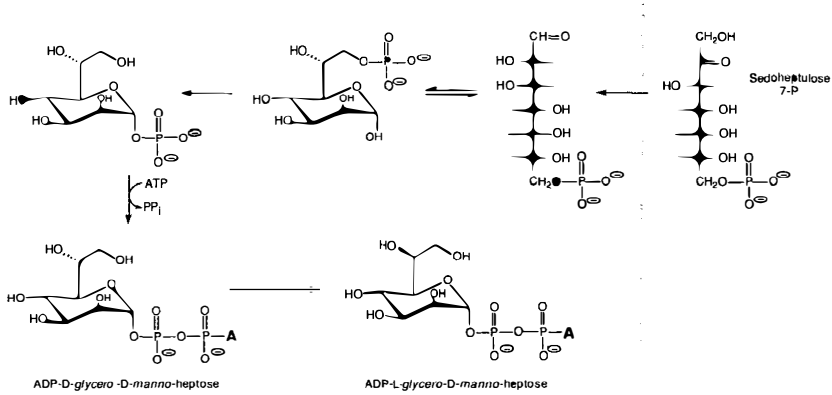


Figure 12 Proposed pathway for the biosynthesis of ADP-L-glycero-D-manno-heptose.

So far, well-defined in vitro systems have not been developed for the addition of the putative third KDO or the third heptose (Figure 5). *Salmonella* mutants defective in the *rfaP* gene (88, 91, 165, 166) appear to lack the phosphorylated substituents of the inner core. The LPS isolated from *rfaP* mutants can be phosphorylated in vitro (165) when incubated together with wild-type extract and ATP, but the structures of the resulting products have not been characterized.

Enzymatic systems for the addition of the polar decorations present on some forms of lipid A (Figures 3 and 11) have not been identified. Based on pulse-chase experiments, it has been suggested that the phosphoethanolamine of the ethanolamine pyrophosphate unit of the inner core is derived from phosphatidylethanolamine (167).

The hexoses of the outer core (Figure 6) are added one residue at a time (18, 98, 99). UDP derivatives function as the activated sugar donors, and the order of hexose addition is consistent with the chemotype scheme shown in Figure 4 (18, 98, 99). Most of the genes coding for these glycosyl transferases map at the *rfa* cluster near minute 81 (88, 89), but an answer to the question of whether the *rfa* genes are organized into operons awaits the complete sequencing of the region.

The glycosyl transferases that generate the outer core are peripheral membrane proteins. They require phosphatidylethanolamine for activity (18, 98, 99, 168). Several of them have been purified (18, 99). How they associate coordinately with their LPS substrates and phosphatidylethanolamine is unknown (18). The capacity of mutants defective in phosphatidylethanolamine synthesis (6, 7) to generate outer core has not been examined. Unequivocal proof for the structures generated by these enzymes and a chemical synthesis of standards are lacking (18, 98, 99).

BIOSYNTHESIS OF O-ANTIGEN

As noted above, O-antigen is synthesized independently of lipid A and core (4, 17, 18, 20). The cyclical formation of the O-antigen repeat and its polymerization require two molecules of bactoprenyl pyrophosphate (Figure 13), a 55-carbon polyisoprene that is also essential for peptidoglycan synthesis (18, 110). The number of bactoprenyl moieties per cell is small relative to the number of glycerophospholipid molecules (6), but this issue needs to be reinvestigated. The partitioning of bactoprenyl pyrophosphate during the biosynthesis of peptidoglycan (110, 111), O-antigen (18, 98), enterobacterial common antigen (169), and membrane-derived oligosaccharides (MDO) (170) may be regulated. The biochemistry of isoprene polymerization in bacteria is poorly characterized, and is assumed to be similar to that of

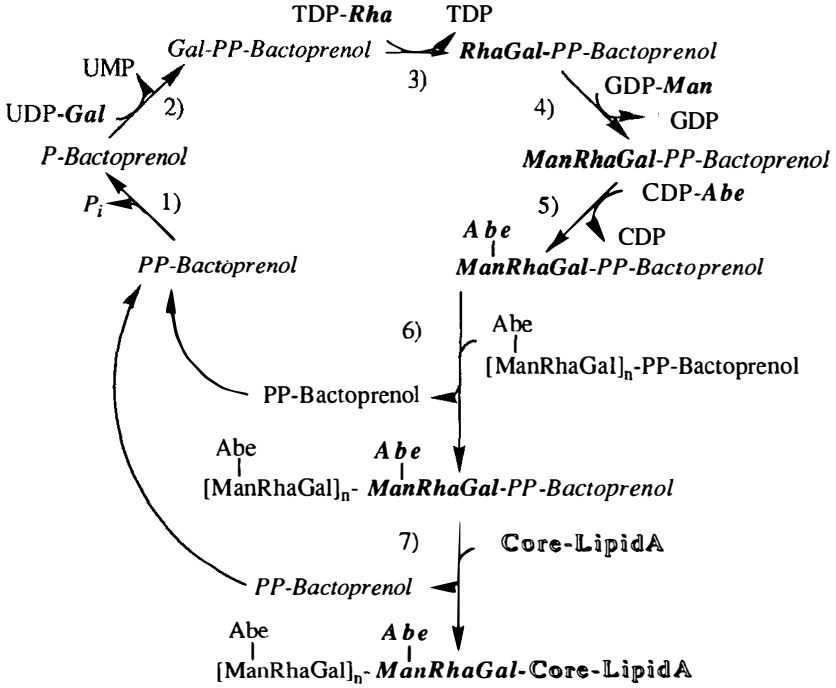


Figure 13 Biosynthesis of O-antigen and its transfer to core–lipid A.

eukaryotic systems (171). Despite the formal similarity of bactoprenol and dolichol as carriers of activated sugar residues (110, 172), protein glycosylation has not been observed in *E. coli*.

The reactions shown in Figure 13 illustrate the sequence of glycosylations that give rise to *Salmonella typhimurium* type B O-antigen, the covalent structure of which is shown in Figure 7 (18). The better-characterized *S. newington* O-antigen is identical, except that it lacks the abequose branch (18, 173, 174). The generation of the unusual dideoxy hexoses of LPS is of interest in its own right, since novel enzymatic reaction mechanisms may be involved (175).

An important feature of O-antigen polymerization is that the newly generated tetrasaccharide repeat is incorporated at the reducing end of the growing chain (18, 173, 174), as illustrated with bold italics in Figure 13. Peptidoglycan also grows in this manner (110). It has the advantage that the membrane-bound polymerase (enzyme 6) does not need to search for the nonreducing terminus of the growing O-antigen polymer. The latter is likely to be extended away from the membrane and the active site of the polymerase.

In other organisms, such as *Shigella* (112, 113) and certain strains of *E. coli* (114), genes of the O-antigen cycle may be encoded on plasmids. It is possible to express *Shigella* and other O-antigens in *E. coli* K 12 by introduction of appropriate plasmids (113, 114). Whatever the enzymology, the export of LPS is very permissive with respect to variations in O-antigen sugar composition, an observation that may have implications for LPS export (see below).

The ligase that transfers O-antigen to the outer core (reaction 7) has not received much attention (18, 176). Two genes (*rfaL* and *rfbT*) are required for ligase function (18, 176). When O-antigen polymerase (177) is rendered defective by an *rfc* mutation (176, 178), the ligase attaches a single O-antigen repeat to the outer core (18, 176, 178).

The generality of the cycle shown in Figure 13 has been questioned (18, 179). Apparently, growth of O-antigen in other systems may take place at the nonreducing end of the polymer (4, 18), but the enzymology has not been worked out.

LPS EXPORT AND OUTER-MEMBRANE BIOGENESIS

Topography of LPS Biosynthesis

During the last two decades, much has been learned about the structures of outer-membrane proteins (180, 181), phospholipids (6, 7), and LPS (2–5). In contrast, little progress has been made in unravelling the mechanisms by which outer membrane components are exported (16, 180, 182). The issue of how outer-membrane asymmetry (Figure 1) is achieved also remains unexplained. The many elegant studies that implicate signal peptides in the initial steps of outer-membrane protein biogenesis (180, 181, 183–185) fail to address the issue of coordinated export.

All of the enzymes of core–lipid A assembly, with the possible exceptions of the 4′ kinase (132) and the myristoyl transferase (Figures 8 and 10), appear to be peripheral membrane proteins (2, 3, 18). Presumably, these proteins are directly associated with the inner membrane during catalysis, since key intermediates (i.e. lipid X or lipid IV_A) are membrane bound (121, 125). Because cytoplasmic molecules, such as sugar nucleotides, ATP, and *R*-3-hydroxymyristoyl-acyl carrier protein are also required, the enzymes must function on the inner surface of the inner membrane. Modifications of LPS, involving the addition or removal of palmitate (46) or of polar decorations such as phosphoethanolamine (45, 167), could occur after export from the cytosol, since these moieties could be derived from glycerophospholipids (6, 7).

The enzymes of O-antigen tetrasaccharide biosynthesis (Figure 13) are

membrane bound (18, 98, 99). They must face the cytoplasm, since they utilize sugar nucleotides as substrates. Despite the availability of hybrid plasmids (4, 186, 187), these proteins have not been purified, severely limiting studies of mechanism and topography.

Classical studies with strains bearing *galE* mutations (16, 98, 99) have demonstrated that LPS is trapped in the outer membrane once export is complete. Even lipid IV_A cannot be reutilized as a substrate for KDO addition after export has occurred (16). In contrast, glycerophospholipids are able to move in both directions (6, 188, 189). The inability of lipid A and LPS molecules to return to the inner membrane may be explained by their high affinity for outer-membrane proteins (1, 190, 191) and for each other (1).

A remarkable feature of LPS export is its apparent lack of specificity for O-antigen and core structure (16). All chemotypes (Figure 4) are handled at about the same rate as smooth LPS (16). Even lipid IV_A, which accumulates in mutants unable to generate KDO, is exported at about one fifth the rate of LPS (16, 192). Inhibition of protein synthesis has no acute effect on LPS biosynthesis and export (16). These findings suggest that lipid IV_A itself contains the necessary information to permit vectorial translocation, and that the addition of KDO somehow accelerates the process.

Electron microscopy of *E. coli* suggests the presence of small zones of adhesion (Bayer's bridges) that can be visualized in thin sections of plasmolyzed wild-type cells (182). In addition, bands of adhesion, referred to as periseptal annuli, are observed (193). The appearance of the latter is synchronized with cell elongation and precedes the formation of the peptidoglycan septum between daughter cells, possibly restricting the movement of molecules around the septum (193).

Höltje et al (194) recently observed significant reductions in the number of Bayer's bridges in mutants lacking membrane-derived oligosaccharides (MDO, Figure 1). Since these mutants possess an outer membrane and are not conditionally lethal (6, 194), the role of Bayer's bridges is open to question. Some investigators have even disputed the existence of adhesion zones in wild-type cells, since they are not visualized by all types of microscopy (195). However, older studies demonstrating appearance of newly synthesized LPS and outer-membrane proteins over Bayer's bridges cannot be dismissed (1, 196, 197).

Periplasmic Intermediates and Flippases?

Four plausible alternatives for LPS translocation (Figure 14) have been considered by Osborn (16). Two of these (Figure 14 *A* and *B*) require flip-flop of O-antigen, core-lipid A, and/or mature LPS across one or two lipid

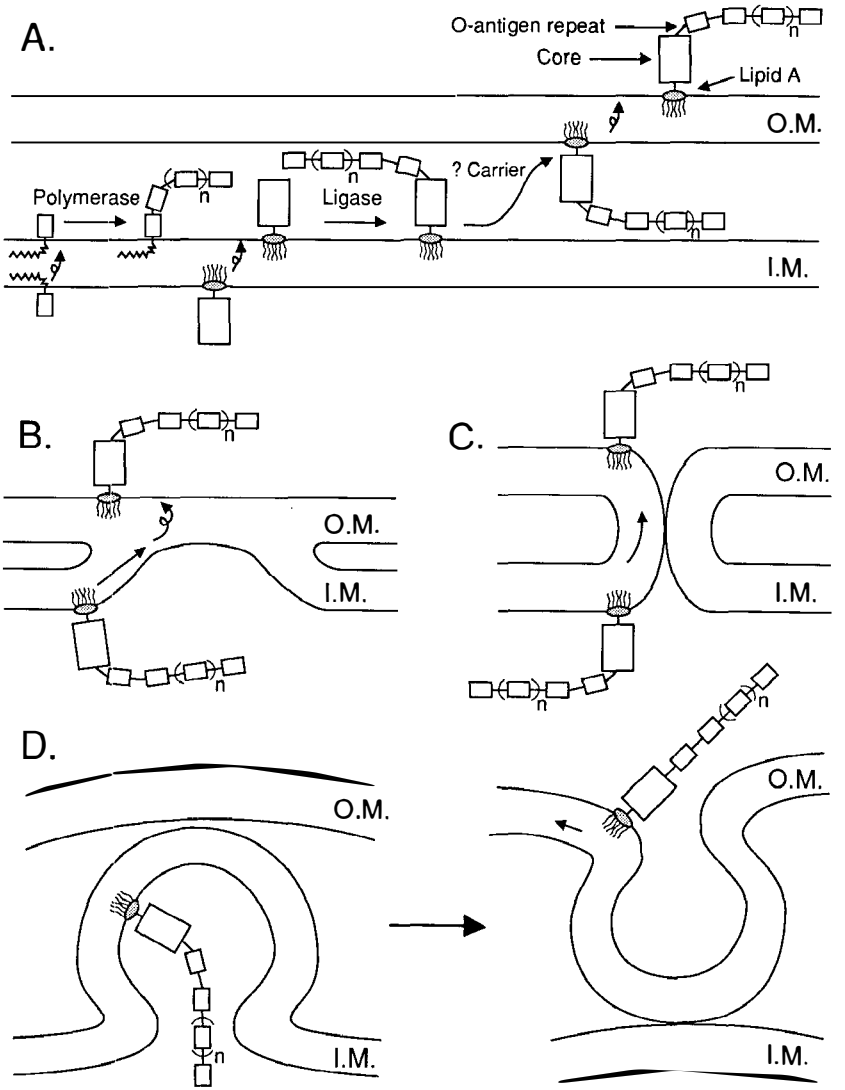


Figure 14 Possible schemes for the export of LPS in gram-negative bacteria. This figure is redrawn from Osborn (16) to provide a more accurate picture of the relative sizes of the polar and nonpolar moieties of LPS. The topographic implications of a periplasmic O-antigen ligase (202) are illustrated in (*A*). At the molecular level, a Bayer's bridge (182) might be a monolayer fusion (*B*) or a bilayer fusion (*C*). Given the presence of peptidoglycan (110) (not shown) and the dimensions of the periplasm (1), a vesicular model (*D*) is unlikely.

bilayers. A third alternative (Figure 14 *D*) involves the budding of a vesicle enriched in newly synthesized outer-membrane components from the inner membrane into the periplasmic space, followed by fusion with the outer membrane. A fourth model (Figure 14 *C*) postulates bilayer continuity between the inner and outer membranes, necessitating some kind of molecular filters (presumably proteins) to grant passage to LPS, outer membrane proteins, and some phospholipids.

None of the alternatives shown in Figure 14 is altogether appealing. Spontaneous flip-flop of LPS seems impossible, given its huge, negatively charged polar domain. Specific proteins that catalyze LPS flip-flop ("flippases") have not been identified, and their existence seems incompatible with the chemical diversity of LPS. Known sugar permeases do not transport large oligosaccharides, and resemble enzymes in their extreme specificity for stereochemistry and composition (198). Perhaps, short-chain lipid A analogs could be employed to search for putative lipid A flippases functioning as transporters of the polar headgroup of lipid A, in analogy to the approach suggested by Bell and coworkers for phosphatidylcholine (199–201).

Mulford & Osborn (202) have presented immunochemical evidence for the presence of O-antigen-containing intermediates on the periplasmic surface of the inner membrane. They suggest that O-antigen polymerization and ligation to core–lipid A occurs on the periplasmic surface of the inner membrane (Figure 14 *A*) (202). If so, it is necessary to postulate distinct flippases for an O-antigen unit attached to bactoprenyl pyrophosphate and for core–lipid A within the inner membrane, and another flippase for LPS within the outer membrane (Figure 14 *A*). An additional carrier protein to shuttle LPS across the periplasm might also be required. Outer-membrane proteins and phospholipids would be exported independently of LPS, and would have to find themselves again in the outer membrane.

The discovery that certain outer-membrane proteins transiently pass through the periplasm on their way to the outer membrane (203, 204) does fit with the notion that various outer-membrane components are translocated individually. In the case of porins, the periplasmic intermediate might be monomeric, whereas mature, functional porins in the outer membrane are trimeric (205).

Regrettably, the dispositions of the active sites of the O-antigen polymerase and ligase (18, 177) within the envelope have not been established. The studies of Mulford & Osborn (202) do not include statistical analyses of ferritin granule counts. The resolution is not as good as that of newer methods based on gold conjugates (206). The possibility of O-antigen reorientation during spheroplast preparation, a major problem at room temperature (202), cannot be ruled out unequivocally. Although the work of Mulford & Osborn is suggestive (202), it is open to other interpretations.

Vesicles or Zones of Adhesion?

Vesicular traffic (Figure 14 *D*) is an appealing mechanism to account for outer-membrane biogenesis and asymmetry. Vesicular traffic certainly plays a crucial role in eukaryotic systems (207, 208), but there is no evidence for it in the envelope of *E. coli.* About 7000 unilamellar vesicles (250 Å in diameter) would be required to generate the entire outer membrane during a cell generation. There is not enough space for a unilamellar vesicle in the periplasm (75 Å wide), and there are no conspicuous bulges when *E. coli* is viewed by electron microscopy (1, 180, 182). The discontinuities created in the peptidoglycan during the formation of a vesicle might also create osmotic fragility. Vesicular traffic in the *E. coli* envelope is therefore not likely.

Given present information, passage of at least some proteins and LPS through the zones of adhesion (Bayer's bridges) remains a possibility (1, 182, 196, 197). Unfortunately, Bayer's bridges are too fuzzy in thin sections to determine whether they are fused bilayers (Figure 14 *C*) or fused monolayers (Figure 14 *B*) (182). If small zones of bilayer continuity do exist, they must be tightly sealed to prevent loss of ions and small molecules. The adhesion zone models for LPS export (Figure 14 *B* and *C*) are compatible with the observed appearance of newly made LPS and outer-membrane proteins over the small zones of adhesion (182, 196, 197).

It is conceivable that the first event in outer membrane biogenesis involves the formation of patches of newly synthesized LPS and selected outer-membrane proteins within the inner membrane. Purified outer-membrane proteins have a very high affinity for the lipid A moiety of LPS, and LPS molecules associate preferentially with each other when dispersed together with glycerophospholipids in model bilayer systems (1). Outer membrane-targetting sequences, distinct from the standard signal sequence, have been described in some outer-membrane proteins (180). These targetting sequences could be lipid A/LPS recognition domains.

The coordinated assembly of the outer membrane in the bilayer fusion model (Figure 14 *C*) might involve the lateral diffusion of newly made patches of LPS and protein into the outer surface of the outer membrane. The asymmetry of LPS, phospholipids, and proteins is established during the formation of the original, inner-membrane patch. The opportunities for additional protein-protein, protein-LPS, and LPS-LPS interactions within the outer membrane could provide the driving force and prevent reverse flow. A requirement for patching in the inner membrane also might account for the deficiency of certain outer-membrane proteins in mutants lacking the heptose region of the inner core (1, 94, 95). The absence of heptose may weaken some LPS-protein interactions on the inner membrane, allowing glycerophospholipids to enter the patches destined for export to the outer surface of the outer membrane.

Experimental Approaches

If one of the adhesion zone models (Figure 14 *B* or *C*) is correct, the ligation of O-antigen to core-lipid A (18, 177) should occur on the inner surface of the inner membrane, or possibly in the outer membrane. Accordingly, the topographies of the active sites of the O-antigen polymerase and ligase (18, 177) must be established, requiring development of in situ assays and preparation of antibody probes. The immunochemical studies suggesting a periplasmic localization of an O-antigen intermediate (202) merit reinvestigation with newer techniques of immuno-electron microscopy based on colloidal gold conjugates (206). An accurate estimate of the number and type of bactoprenyl pyrophosphate conjugates per cell (18, 110) would also be of interest and might place some constraints on models for export.

The physical properties of lipid A, LPS, and LPS-protein complexes must be studied in order to establish what kinds of conformations they can assume. Model systems for measuring lipid A flip-flop and intermembrane transport must be developed. The search for proteins catalyzing these processes will be facilitated by the availability of the biosynthetic enzymes (Figures 8 and 10), which are ideally suited for the preparation of labeled substrates. It is worth noting that, despite decades of speculation (199–201), no glycerophospholipid flippase has ever been isolated. Perhaps, the unusual chemistry and large size of LPS will actually facilitate the identification of the first flippase!

Inhibition of the first committed step of lipid A biosynthesis in strains bearing *lpxA* mutations (Figure 8) (43) might interfere with the export of some outer-membrane components, depending on the mechanism of outer-membrane assembly. Accumulation of functional porins in the inner membrane might account for the observed bacteriocidal nature of the *lpxA* mutation (43). The structures of the zones of adhesion in *lpx* mutants (43) also merit investigation.

The hypotheses shown in Figure 14 are intended to highlight topographic issues. Many proteins must assist in outer-membrane assembly and/or LPS export. A search for novel, conditional mutants that are generally defective in outer-membrane biogenesis, but unaffected in LPS and protein biosynthesis, could provide important new insights. It will also be of interest to isolate temperature-resistant revertants of *lpxA* mutations (43), in order to search for bypass mutations that eliminate the need for lipid A. Perhaps, the entire outer membrane is unnecessary under some conditions.

Lastly, purified adhesion zones must be fractionated to identify unique protein and lipid components (209, 210). Sequencing of such proteins would permit cloning and regulated expression of the relevant structural genes, which might interfere with export in novel ways.

INTERACTIONS OF LPS WITH ANIMAL SYSTEMS

Evidence for Specificity

The complicated effects of LPS on animal systems include the induction of the characteristic endotoxin-shock syndrome (4, 22–26, 36, 37), the nonspecific activation of the immune system (31), and the activation of the complement cascade (211). The short-term administration of low doses of LPS results in an antibody-independent tolerance to otherwise lethal doses of LPS (212). As demonstrated by tests using chemically synthesized material, all of these effects of LPS are caused primarily by lipid A (5, 24, 58).

Evidence for the crucial role of lipid A in LPS-mediated effects is further supported by genetic studies of intact animals (213) and of animal cell lines (214). (*a*) C3H/HeJ mice are resistant to the toxic and immunostimulatory effects of either LPS or lipid A (213, 215, 216). The biochemical basis of this autosomal recessive mutation is unknown, and attempts to identify a receptor deficiency have been unsuccessful (217). Some investigators have found that certain LPS preparations are able to stimulate C3H/HeJ cells (213). This may indicate that there are multiple transduction pathways available to LPS or that the C3H/HeJ lesion is a point mutation, capable of responding under some conditions. (*b*) Mutants of the murine 70Z/3 pre-B lymphocyte tumor line can be isolated that are unresponsive to LPS or to lipid IV_A (214, 218). In this system, the response is monitored by measuring the synthesis of the kappa immunoglobulin chain, resulting in the presence or absence of surface IgM (214). Mutants unable to respond to LPS cannot respond to lipid A, and vice versa (214). However, some LPS-unresponsive strains are still able to synthesize kappa chains in response to γ-interferon (214). The latter mutants might include those defective in the lipid A/LPS receptor.

There is independent pharmacological evidence for specificity in the interaction of lipid A with animal cells. As first demonstrated by Mayer and coworkers (75, 76), lipid A from *R. sphaeroides* (Figure 15) is not toxic to animals and lacks the characteristic immunostimulatory effects of lipid A from *E. coli* or *S. typhimurium* (Figure 2). The three major differences between *R. sphaeroides* and *E. coli* lipid A are: (*a*) the presence of shorter O-linked hydroxy fatty acids in *R. sphaeroides;* (*b*) the presence of an unsaturated fatty acyl chain in *R. sphaeroides;* and (*c*) the presence of an N-linked β-ketomyristate residue in *R. sphaeroides* (75–77). The critical variable has not yet been identified, and synthetic standards are not available. Importantly, the lipid A of *R. sphaeroides* has the ability to block the effects of *E. coli* LPS in most cellular systems at a 10–100-fold excess (219). These findings, together with the genetic evidence (213, 214), provide persuasive evidence for the existence of some kind of lipid A receptor(s).

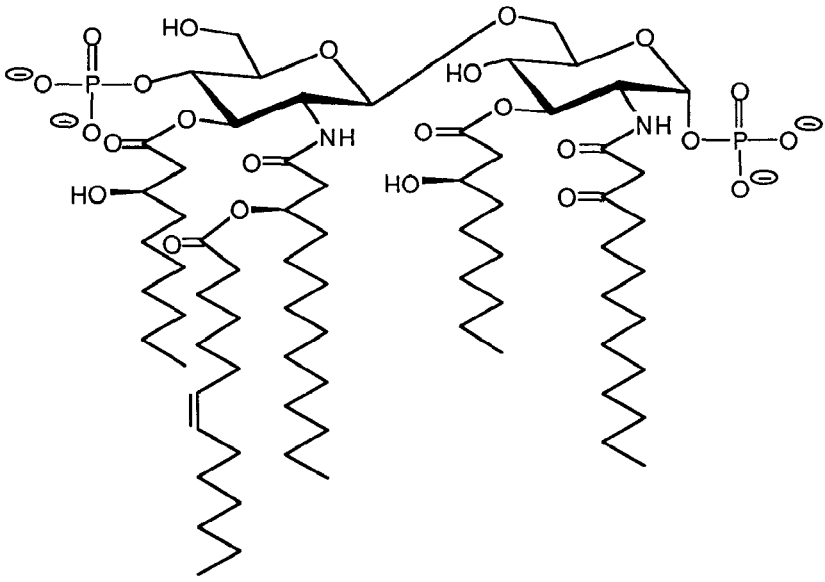


Figure 15 Structure of the nontoxic lipid A of *R. sphaeroides*. The structure shown is the major component of a mixture, consisting of at least three species (77).

Mediators and Transduction Pathways

Many different protein and lipid mediators are synthesized by immune cells in response to LPS (5, 31). Some of the key proteins include TNF (25), IL-1 (28), and colony-stimulating factors (31). Important lipids include PAF, leukotrienes, and prostaglandins (5, 31, 32). The functional interrelationships of these mediators are complex, because some of them modulate the syntheses or activities of the others (31), and different animals vary in their response to LPS (17). For instance, rodents are very resistant to LPS, unless pretreated with certain nonphysiological agents such as galactosamine (220), while many large mammals, including sheep (221) and humans (23), are extremely sensitive to LPS itself. Baboons, on the other hand, are seven orders of magnitude more resistant than humans (17, 222). The molecular basis for these enormous discrepancies is not immediately obvious. Paradoxically, mouse macrophages, human macrophages, and related cell lines are similar in their sensitivity to LPS in vitro (223–225).

A central question in this field is how the occupancy of a "receptor" by LPS, either on the surface or somewhere in the interior of the cell, triggers the synthesis of a few key proteins (3, 44, 217). In 70Z/3 cells, as in all other systems, the relevant LPS receptor has not been identified (214). An important downstream event, however, appears to be the activation of the transcrip-

tion factor, NFκB, involving its translocation from an inactivated, cytosolic pool to the nucleus, where it stimulates the synthesis of kappa mRNA (226). Phosphorylation of a cytosolic inactivating protein, termed IκB, by protein kinase C is thought to liberate NFκB (226). Phorbols also stimulate kappa chain transcription in 70Z/3 cells (226, 227), but the response is not as sustained as with LPS (227) and does not result in the generation of surface IgM (214). A thorough analysis of the LPS-unresponsive mutants of 70Z/3 cells, first described by Sibley and coworkers (214, 218), could help define the biochemistry of this transduction pathway.

Under some conditions, LPS itself does not stimulate macrophages to release arachidonic acid metabolites, but rather, "primes" the cells for the ability to respond to phorbols (225). Aderem and coworkers have shown that LPS pretreatment of mouse peritoneal macrophages results in the myristoylation of a 68-kd protein of unknown function, rendering this protein much more susceptible to phosphorylation by protein kinase C (228, 229). Myristoylation correlates well with priming. Perhaps, lipid A mimics an endogenous lipid mediator of animal cells or acts directly on an enzyme of a signal transduction cascade (230, 231).

In summary, a common theme in the action of endotoxin appears to be the activation of transcription of specific eukaryotic genes (25, 28, 214, 226). One of the earliest events may be the activation of the c-fos protooncogene, which regulates the transcription of additional selected genes (232).

The Search for Lipid A Receptors

Given the complexity of the response to LPS and the ability of the known mediators to modulate their own production (31), it would seem that an antagonist of the initial interaction of LPS with macrophages, like *R. sphaeroides* lipid A (77, 219), would have enormous therapeutic potential. Specific agents, such as antibodies to TNF (38, 39) or antagonists of PAF (233), also are likely to have considerable utility.

The identification of the relevant lipid A/LPS receptors on cells of the immune system remains the highest priority in the effort to elucidate endotoxin pathophysiology (3). The difficulties encountered in searching for LPS receptors on animal cells include: (*a*) the microheterogeneity and uncertain purity of LPS isolated from biological sources (4, 5, 17, 20); (*b*) the unknown effects and uncertain locations of reporter groups introduced into LPS, including fluorescent (234) and photoactivatible moieties (235), or ^{125}I; (*c*) the possible existence of multiple receptors (44, 236); and (*d*) the unknown physical state of lipid A dispersions in water.

We have approached this problem by studying the interaction of chemically defined precursors (Figures 8 and 10) of Re endotoxin (itself fully active in all responsive systems) with cell lines in which it is possible to generate somatic

mutations (3, 44, 214, 223). The monosaccharide precursors (Figure 8) have little or no biological activity (58, 214, 223). In some systems they may be weak antagonists (221, 237), but they do not possess the striking antagonistic properties of *R. sphaeroides* lipid A (219). The initial suggestion that lipid X is a potent agonist (238, 239) is now attributed to minor impurities present in early preparations (214), the identities of which remain elusive. In contrast, the disaccharide *bis*-phosphate substructures of lipid A, like lipid IV_A, possess significant biological activity (45, 58, 214), which may be enhanced several orders of magnitude in some systems by the additional presence of acyloxyacyl residues and KDO moieties (5, 29). Species-specific effects have also been uncovered. In mouse cells, lipid IV_A is a potent agonist (214, 223, 240), but in human cells it is an antagonist (29, 241).

We have used [4'-^{32}P]-lipid IV_A as a nonperturbing, direct probe for lipid A receptors (44, 242). This substance is generated at very high specific radioactivity with *E. coli* 4'-kinase (Figure 8), and it can be dispersed by sonic irradiation in phosphate-buffered saline (44, 46). Under conditions similar to those used in studies of lipoprotein receptors (243, 244), Hampton et al (44) have observed saturable, "specific" binding of lipid IV_A dispersions on intact macrophages and on macrophage tumor cells, but not on fibroblasts, such as CHO-K1 cells. Although the protein responsible for this binding has not been isolated, the ligand specificity of the binding resembles that of the acetylated-LDL (scavenger) receptor (3, 244). Indeed, acetylated LDL, but not native LDL, competes effectively with lipid IV_A (3). The recent purification of the acetylated LDL receptor (245) will help to clarify the significance of these observations.

Even if the lipid IV_A binding function that we have observed is identical to that of the acetylated LDL receptor (245), or one of its putative subtypes (246), it is not certain whether this receptor is actually involved in endotoxin action. Acetylated LDL does not appear to be an inducer of tumor necrosis factor, and it does not block the action of LPS (R. Hampton and C. R. H. Raetz, unpublished), as does *R. sphaeroides* lipid A (219). It possible that the receptor we have identified functions to remove LPS from the circulation and to clear it from the body. We are currently attempting to isolate macrophage tumor cell mutants lacking the lipid IV_A binding function and to assess their responsiveness to LPS.

Significant insights into lipid A–protein recognition will come not only from the identification of the true endotoxin "receptor" in animal cells, but also from the analysis of the structures of other proteins that interact specifically with lipid A. These include: (*a*) antibodies to lipid A (5, 37, 247); (*b*) acute phase serum proteins (248); (*c*) bacteriocidal proteins specific for gram-negatives that are localized in neutrophil granules (249); (*d*) enzymes involved in the late stages of Re endotoxin biosynthesis (103, 157), such as

the *kdtA* gene product (Figure 10); (*e*) proteins in crude cellular mixtures that interact with radioactive (44) or photoactivatible LPS probes (235, 250); (*f*) adhesion-promoting receptors or something associated with them (251); (*g*) enzymes of macrophages that partially deacylate lipid A (252); and (*h*) the outer-membrane proteins of gram-negatives (180). Although some sequence information is already available (180, 248, 249), the tertiary structures of these proteins have not been solved. Within the next few years, common functional themes should emerge as the structures and conformations of lipid A–recognizing proteins are elucidated.

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