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The Role of Lipid-Linked Activated Sugars in Glycosylation Reactions

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Introduction

In the prokaryotic eubacteria the single cytoplasmic membrane appears to serve many of the functions performed by the numerous subcellular organelles found in eukaryotic organisms. Thus, the cytoplasmic membrane of the bacteria is the site of oxidative phosphorylation; it is the site of active transport; and it is the site of biosynthesis of many of the macromolecular components of the cell surface. In this chapter attention will be focused on biosynthesis of the sugar-containing macromolecules that ultimately reside within or outside the membrane surface. More specifically it will deal with the mechanism of energy utilization in the biosynthetic processes involving glycosyl transfers from high energy, lipid-linked sugar intermediates first found in the eubacteria. It should be noted, however, that although it is in the prokaryotic bacteria where such systems have been most definitely established, preliminary reports suggest that analogous reactions may occur in eukaryotic cells.[1,2,3]

Relationship of High Energy Hydrophobic Sugar Intermediates to ATP Production

It is worthwhile to consider first the overall sequence of events involved in synthesis of the glycans of the cell envelope. Studies indicate that in most bacteria the origin of ATP is the cytoplasmic membrane, wherein the enzymes of oxidative phosphorylation reside. ATP thus formed presumably diffuses from the membrane to other locations for utilization in a great variety of energy-dependent biosynthetic processes. One class of compounds whose formation involves ATP either directly or indirectly is the other nucleoside triphosphates. Cytoplasmic enzymes catalyze the formation of these compounds. Also present in the cytoplasm are enzymes that catalyze the synthesis of the nucleoside diphospho sugars from the nucleoside triphosphates. These sugar nucleotides are, of course, the classical donors of glycosyl groups in formation of a great variety of polysaccharides, as first demonstrated

by Leloir and coworkers for the biosynthesis of glycogen.[4] However, in the bacteria some of the glycans that are found within or beyond the cytoplasmic membrane are formed in part not by direct glycosylation reactions via sugar nucleotides, but rather by glycosylations after the hexose is transferred to a carrier lipid. Since the membrane constitutes a hydrophobic permeability barrier, a lipid is particularly well suited to serve as carrier of activated glycosyl groups involved in reactions within the membrane.

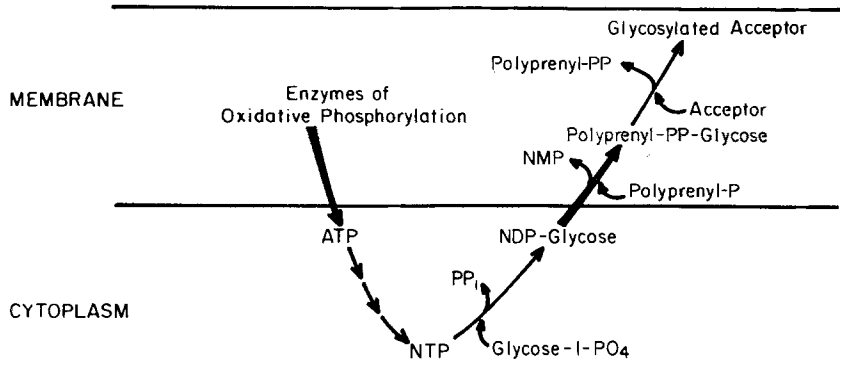

Figure 1. Production and utilization of high energy phosphate compounds for polysaccharide synthesis. NTP, NDP, and NMP represent nucleoside triphosphate, diphosphate and monophosphate, respectively.

Thus, as summarized in Fig. 1, following ATP synthesis at the membrane, a number of reactions in the cytoplasm lead to formation of sugar nucleotides. These sugar nucleotides can react with the carrier lipid in the membrane, thereby being converted into hydrophobic, high-energy glycosyl compounds that can participate in biosynthetic processes in the membrane. Thus, by this rather indirect route the energy of ATP synthesized in the membrane is utilized for biosynthesis of glycan components in the membrane.

Discovery of the Involvement of a Carrier Lipid in Glycan Synthesis

Cell wall peptidoglycan synthesis has been studied most extensively in *Staphylococcus aureus* and *Micrococcus lysodeikticus*. During investigations on peptidoglycan synthesis in these organisms, Strominger and his coworkers discovered that their particulate enzyme preparations catalyzed the incorporation of sugar residues supplied as UDP-*N*-acetyl-D-glucosamine and UDP-*N*-acetyl-D-muramyl pentapeptide into a lipophilic compound as well as into polysaccharide.[5] Synthesis of the lipid measured as a function of time indicated that it was formed prior to formation of the polysaccharide. It was also necessary to demonstrate that the lipophilic compound could function independently as a substrate for peptidoglycan synthesis. Therefore, the lipid intermediate

was generated in an initial incubation of particulate enzyme with the sugar nucleotides. The particular preparation was washed free of sugar nucleotide precursors and was supplied as the only substrate in a further incubation. Peptidoglycan was, in fact, produced in this second reaction. Thus, a disaccharide-containing lipophilic compound was proposed as an intermediate in synthesis of peptidoglycan from sugar nucleotides (Fig. 2, reactions 1 and 2). Subsequent investigations established the structure of this novel lipid to be undecaprenyl phosphate and elucidated the cyclical nature of its involvement in the

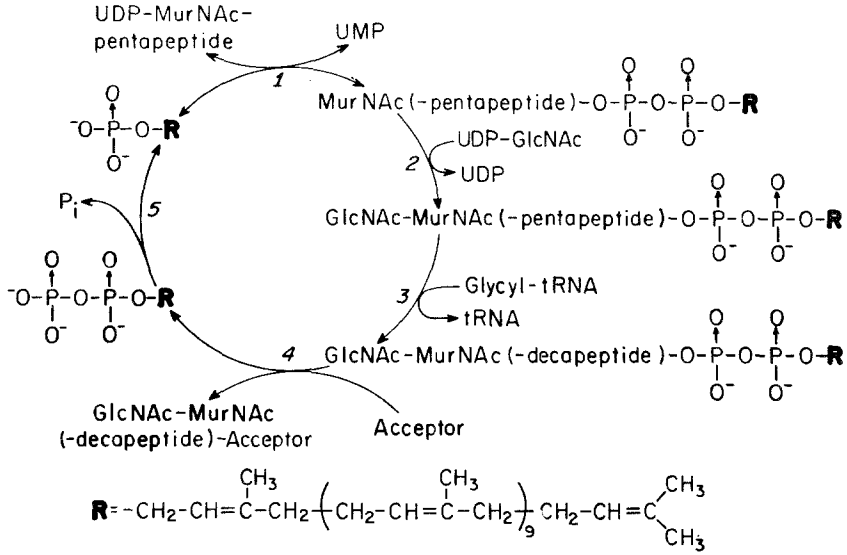

Figure 2. Sequence for the biosynthesis of the cell wall peptidoglycan. The abbreviations used here are: GlcNAc (*N*-acetyl-D-glucosamine); MurNAc (*N*-acetyl-D-muramic acid).

biosynthetic pathway. As indicated, the initial step in the biosynthetic pathway is reversible.[6] From the high energy bond of UDP-*N*-acetylmuramyl pentapeptide, initially a cytoplasmic component, a high energy glycosyl-1-phosphoryl bond is synthesized in the membrane.[7] This high energy bond remains intact until the final transfer of sugar residues from the lipid intermediate to form a glycosidic bond with acceptor polysaccharide. The second step in the reaction sequence produces the disaccharide containing lipid, and at this stage modifications of the peptide chain occur, such as the sequential transfer of glycine units from t-RNA to the lipophilic intermediate to form a pentaglycine side chain (reaction 3).[8,9] Reaction 4 is the transfer of the disaccharide to endogenous polysaccharide acceptor and concomitant release of pyrophosphoryl undecaprenol. It is not yet known if the disaccharide is further polymerized on the lipid before transfer to polysaccharide. Phosphoryl undecaprenol must be enzymatically

regenerated (reaction 5) before participating again as acceptor lipid. This reaction is the site of inhibition of peptidoglycan synthesis by bacitracin.[10] Stone and Strominger have only recently presented data which suggests that the mechanism of inhibition is the formation of a complex between bacitracin, C_{55}-polyisoprenyl pyrophosphate and divalent cations in which the cation serves as a bridge between the lipid and the antibiotic.[11] The synthesis of peptidoglycan is completed by transpeptidation resulting in cross-linking of the peptide chains via the pentaglycine bridge.[12] The biosynthesis of peptidoglycan, as well as other polysaccharides discussed in this review, has been described in depth in recent review articles.[13,14,15]

Similar pathways involving undecaprenyl phosphate intermediates operate in the synthesis of the *O*-antigen portion of the lipopolysaccharide associated with the cell envelope of *Salmonella typhimurium*[16] and *S. newington*.[17] The *O*-antigen chain of *S. typhimurium* consists of branched tetrasaccharide repeating units. The repeating trisaccharide is mannosylrhamnosylgalactose, and abequose linked to mannose forms the branch. *S. newington* contains the same repeating trisaccharide but the configurations are different and there is no branch sugar. It was initially reported that *S. typhimurium* cell envelope preparations catalyzed incorporation of radioactive sugars from the appropriate sugar nucleotides into a lipophilic product which contained galactose-1-phosphate as the reducing terminal sugar residue.[16] The radioactivity of the intermediate in the lipid phase was transferred to the macromolecular product by raising the temperature of the reaction from 10° to 37°. At the same time Wright *et al.*[17] reported synthesis of a butanol-extractable radioactive compound with an *S. newington* preparation in the presence of the labeled sugar nucleotides UDP-galactose, TDP-rhamnose and GDP-mannose. The addition of GDP-mannose subsequent to incorporation of sugar residues from UDP-galactose and TDP-rhamnose into lipid caused a decrease in the level of radioactivity in the lipid, but an increase in radioactivity in the non-dialyzable polymer.

The initial step in the reaction sequence (Fig. 3) is the reversible transfer of galactose-1-phosphate from UDP-galactose to acceptor phosphate, with release of UMP.[16,17,18] Following formation of galactosyl-pyrophosphoryl-undecaprenol, rhamnose and mannose are transferred sequentially to the monosaccharide lipid intermediate. Galactosylpyrophosphoryl-antigen carrier lipid and rhamnosylgalactosylpyrophosphoryl-antigen carrier lipid have been isolated and used as acceptors for the addition of rhamnose and mannose, respectively.[19] At this stage polymerization occurs in the *S. newington* system. Addition of abequose catalyzed by *S. typhimurium* preparations requires the trisaccharide-lipid intermediate as a substrate, but the polymerized trisaccharide-lipid will not accept abequose (from CDP-abequose).

A dimer of the tetrasaccharide attached to lipid has been isolated and characterized, indicating that polymerization for the *O*-antigen system does occur at the level of lipid intermediates.[20,21] Polymerized oligosaccharide is then transferred to core lipopolysaccharide and undecaprenyl pyrophosphate is released. Undecaprenyl phosphate is regenerated by dephosphorylation of lipid pyrophosphate in a bacitracin sensitive reaction.[22]

Robbins and his coworkers also discovered that *O*-antigen synthesis proceeds by addition of trisaccharide repeating units at the reducing

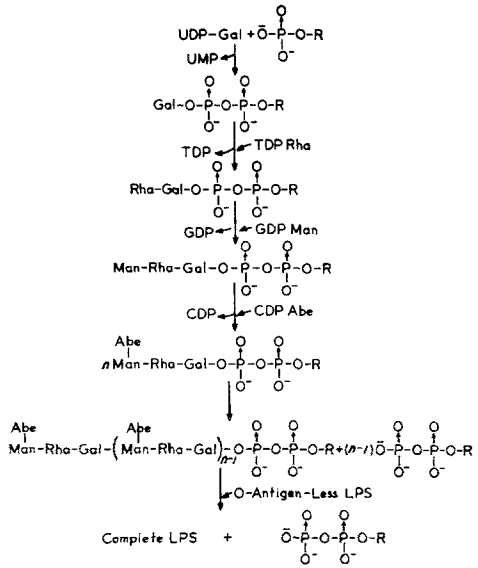

Figure 3. Sequence for the biosynthesis of the *O*-antigen chain of the lipopolysaccharide of *S. typhimurium*. The abbreviations used here are: Gal (D-galactose); Rha (L-rhamnose); Man (D-mannose); Abe [abequose (3,6-dideoxy-D-galactose)]; LPS (lipopolysaccharide); and R (undecaprenol).

end of growing chains.[23] Their study involved pulse-labeling of growing cultures with ^{14}C-glucose or pulse labeling a cell free system with UDP-galactose-^{14}C followed by borohydride treatment of *O*-antigen chains which converted the reducing terminal galactose unit to galactitol, thus permitting one to distinguish the reducing from the non-reducing end of the chain.

The lipid intermediates from the peptidoglycan and *O*-antigen synthesizing systems have been isolated and purified. In independent studies from the laboratories of Strominger and Robbins the presence of a pyrophosphate linkage between sugars and the lipid was established and the lipid moiety of each intermediate released upon acid hydrolysis was analyzed by mass spectroscopy.[24,25] The structure thereby established was a polyisoprenoid alcohol containing 11 isoprene units, each

unit having a double bond (cf. Fig. 2). Whether or not identical isomers of undecaprenol are involved in both polysaccharide synthesizing systems has not been determined, but the significance of this mechanism for synthesis of polysaccharides which are located external to the cytoplasmic membrane increased when it was discovered that undecaprenyl phosphates also mediate the synthesis of other steps in biosynthesis of *O*-antigen as well as the synthesis of a variety of other complex glycans.

The Role of Carrier Lipids in Other Bacterial Systems

Recently two enzymatic systems which utilize polyisoprenol intermediates to modify the *O*-antigen chain during lipopolysaccharide biosynthesis have been reported, and both of these systems involve transfer of a single hexose residue to a preformed oligosaccharide or polysaccharide. In addition the sugar residue in both intermediates is linked to the lipid via a phosphodiester bridge. Wright has studied the biosynthesis of the lipopolysaccharide in E group *Salmonella*[26,27] and has reported upon the mechanism of the modification reaction in which glycosyl groups are added to the *O*-antigen in lysogenic cells. The *O*-antigen of E group *Salmonella* is a heteropolysaccharide composed of mannosylrhamnosylgalactosyl units. In cells lysogenic for either bacteriophage ϵ^{15} or ϵ^{34} the galactosyl units are of the β-configuration. In cells doubly lysogenic for ϵ^{34} and ϵ^{15} the galactosyl units are also substituted with α-D-glucosyl groups. Incubation of particulate enzyme from doubly lysogenic cells with ^{32}P-UDP-glucose-^{14}C results in transfer of the ^{14}C but not ^{32}P to the particulate preparation. Lipid-linked ^{14}C-glucose comprises 54% of the radioactivity transferred and the remainder was characterized as ^{14}C-lipopolysaccharide. The lipid intermediate that upon further incubation can transfer ^{14}C-glucose to lipopolysaccharide has a ratio of glucose to phosphate of 0·9:1. The sugar moiety is β-glucose-1-phosphate. The free lipid produced by acid hydrolysis was analyzed by mass spectrometry and the results indicated that the lipid is a mixture of C_{55} and C_{50} polyisoprenoid alcohols, with each isoprene unit having one double bond. Thus, without knowledge of the geometric configuration, the lipid moiety is identical in structure to the lipid moieties involved in peptidoglycan, *O*-antigen, capsular polysaccharide, and mannan synthesis. The lipid is distinguished from peptidoglycan and *O*-antigen intermediates by the presence of a phosphodiester bridge rather than a pyrophosphoryl bridge but it is similar in this respect to mannosylphosphoryl-undecaprenol (see below).

The acceptor for C^{14}-glucose in the glucose transfer reaction is proposed to be the growing *O*-antigen chain attached to antigen carrier lipid. Experimentally, this hypothesis was tested in a system which contained incomplete core lipopolysaccharide that was incapable of

accepting *O*-antigen chains. In this system it was still possible to observe transfer of glucose from the carrier to *O*-antigen attached to lipid. Thus, it seems likely that glucosyl units are transferred from glucosyl lipid stepwise to the growing *O*-antigen chain still linked to lipid. A hexasaccharide ($x = 2$ in Fig. 4) would be the shortest possible sequence of repeating units that act as a glucose acceptor, since glucosylation requires β-galactosyl units and these are formed during polymerization of the repeating units catalyzed by the ϵ^{15}-specific *O*-antigen polymerase. Further experiments will be necessary to substantiate this proposal.

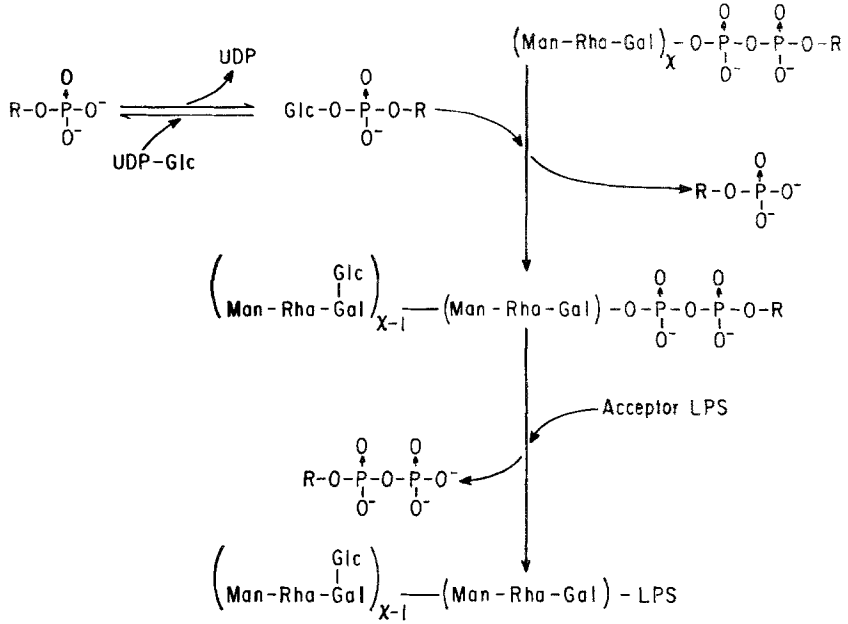

Figure 4. Schematic presentation of proposed sequence involved in glycosylation of *Salmonella* ϵ^{15}, ϵ^{34} *O*-antigen. The abbreviations used here are the same as those identified in Fig. 3 and Glc (D-glucose).

O-antigen factor 12_2 present in *Salmonella* groups A, B, and D, is determined by branches of the *O*-antigen which are nonreducing glucose residues linked to galactose units of the *O*-antigen side chain. Nikaido *et al.*[28] discovered that a cell envelope preparation from *Salmonella* strains possessing *O*-antigen 12_2 catalyzes the transfer of glucose from UDP-glucose to an endogenous acceptor. Results of chromatography of the radioactive product indicate that it is most probably lipopolysaccharide and not merely the *O*-antigen side chains. Moreover, the lipopolysaccharide is characteristic of strains which bear a modified *O*-antigen because radioactivity is present in the product as glucose and the oligosaccharide glucosylgalactosylmannosylrhamnose was obtained after partial acid hydrolysis. The time course of incorporation

of radioactivity from ^{32}P-UDP-glucose-^{3}H into lipid and lipopolysaccharide suggested that the glucosyl-lipid was an intermediate in the transfer of glucose, and glucosyl-lipid isolated from an incubation mixture served as a direct donor of glucose for lipopolysaccharide synthesis. The acid lability and chromatographic behavior after catalytic hydrogenation of this lipid intermediate and rhamnosyl-galactosyl antigen-carrier lipid were similar. However, phosphate is not transferred to the lipid along with glucose and the ratio of glucose to phosphate in the intermediate is approximately 1. Although the

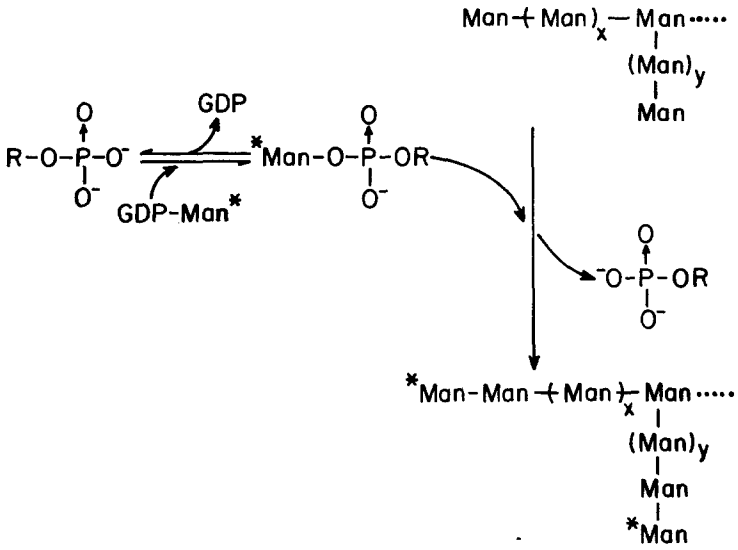

Figure 5. Role of mannosyl-1-phosphoryl undecaprenol in mannan synthesis in *M. lysodeikticus*. R is undecaprenol.

degree of unsaturation was not determined, the glucosyl-lipid carrier appeared to be the same as antigen carrier lipid and the structure of the intermediate was therefore proposed to be glucosyl-1-phosphoryl-polyisoprenol.[29] Using a *Salmonella* mutant which could not transfer completed *O*-antigen chains from antigen carrier lipid to the defective core of the lipopolysaccharide produced by this organism it was demonstrated that ^{14}C-glucose was transferred from UDP-glucose-^{14}C to the *O*-antigen side chains attached to antigen carrier lipid[30] in agreement with the hypothesis of Wright discussed above.

A phosphodiester linked lipophilic sugar intermediate is also involved in mannan synthesis catalyzed by a *Micrococcus lysodeikticus* particulate preparation.[31] The system utilizes GDP-mannose as the hexose source and the acceptor lipid required for the initial transfer of mannose has been isolated, purified and characterized as undecaprenyl phosphate[32] (Fig. 5). The lipid intermediate produced in this reaction

has also been purified, and analysis established the structure to be mannosyl-1-phosphoryl-undecaprenol.[33] The isoprenoid moiety of the intermediate appears to be identical to the intermediates involved in peptidoglycan and *O*-antigen synthesis, although the sugar is linked to the lipid via a phosphodiester bridge rather than a pyrophosphoryl bond. Clearly, more than one sugar residue is attached to the lipid intermediates of peptidoglycan and *O*-antigen synthesis, but it has not been possible to detect a lipid intermediate containing mannose oligosaccharides in the mannan biosynthetic system. Moreover, ^{14}C-mannosyl units are transferred only to the non-reducing termini of endogenous mannan. Mannosyl-1-phosphoryl-undecaprenol thus seems to function only to complete the synthesis of endogenous mannan, in a manner similar to the glucosyl-1-phosphoryl-undecaprenol intermediates which serve in the formation of branches in lysogenic *Salmonella* *O*-antigen. Addition of mannosyl units to the non-reducing termini is analogous to the classical method of synthesizing glycogen and starch by addition of glucosyl units to the non-reducing ends of growing chains[34] and is in contrast to the mechanism of *O*-antigen synthesis.

Biosynthesis of capsular polysaccharide with the participation of lipid intermediates in *Klebsiella* (*Aerobacter*) *aerogenes* has been reported by Troy *et al.*[35] The polysaccharide is composed of the repeating trisaccharide galactosylmannosylgalactose with glucuronate branches on each mannose residue (Fig. 6). Initially it was observed that ^{14}C-galactose accumulates in the lipid phase as a result of incubation of a cell envelope fraction with UDP-galactose-^{14}C at 12°. The first step of the reaction cycle is reversible and results in production of galactosyl pyrophosphoryl-undecaprenol. The acceptor lipid has been definitively characterized primarily by means of mass spectrometry as undecaprenyl phosphate. This lipid restores enzymatic activity to a lipid depleted particulate preparation and it is active as acceptor lipid for mannan synthesis.[36] The functional equivalence of the acceptor lipids from *M. lysodeikticus* and *A. aerogenes* does not necessarily indicate identical structures since the enzymes may not have absolute specificity for the acceptor. Mannosyl and glucuronyl units are sequentially added to the galactosyl lipid intermediate. Glucuronic acid must be incorporated into the lipid intermediate before the fourth sugar, another galactose, can be added to the repeating units. An octasaccharide which consists of 2 tetrasaccharide repeating units was obtained after release from the carrier phospholipid indicating that polymerization occurs at the level of lipid intermediate. Ultimately the radioactive oligosaccharide is transferred to an acceptor to form polysaccharide product. The identification of endogenous acceptor for the polymerized oligosaccharide attached to lipid remains unknown. The polysaccharide formed by the system was characterized as capsular

material with the aid of specific phage induced capsular polysaccharide depolymerase which has endogalactosidase activity. Participation of a lipid intermediate, the structure of which is not clearly defined, has been described in synthesis of capsular polysaccharide from another strain of *Klebsiella*.[37]

Douglas *et al.*, have detected accumulation of a lipid intermediate containing *N*-acetylglucosamine-^{14}C during biosynthesis of *Staphylococcus lactis* I3 teichoic acid which consists of *N*-acetylglucosamine and glycerol linked via phosphodiester bridges.[38] In the absence of CDP-glycerol, *N*-acetylglucosaminyl lipid accumulation was stimulated and the sugar was transferred to teichoic acid upon further incubation of the

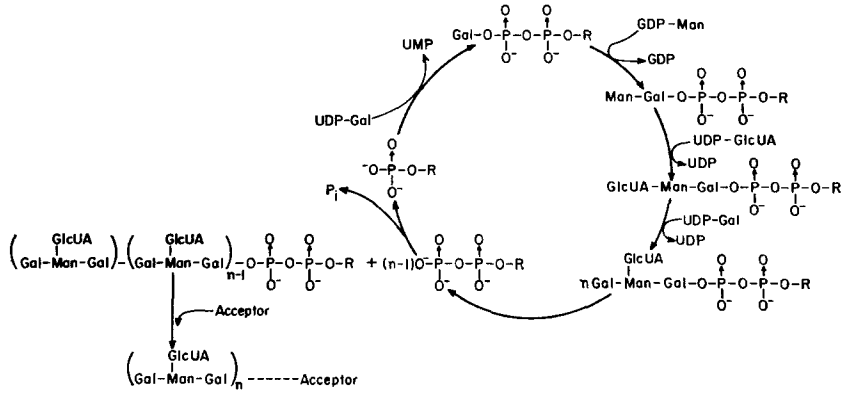

Figure 6. Proposed sequence for capsular polysaccharide synthesis. The abbreviations used here are those in Fig. 3. GlcUA designates a glucuronic acid residue.

washed particulate preparation with CDP-glycerol. Neither the structure of this lipid nor the structure of a similar *N*-acetylglucosaminyl lipid which has been detected during synthesis of the *S. lactis* 2102 wall polymer[39,40] has been determined. The acid lability of these pyrophosphate containing intermediates suggests the presence of a double bond β to a phosphate group and, therefore, the authors suggest that the lipid may be of the polyisoprenoid type.

If the intermediates in teichoic acid biosynthesis were found to have the same structures as the other isoprenoid lipid carriers, this system and the peptidoglycan synthesizing system would compete for the isoprenoid acceptor lipid. Watkinson *et al.* have investigated the competition between the two systems in *S. lactis* I3 by measuring teichoic acid synthesis alone and teichoic acid synthesis in the presence of peptidoglycan synthesis.[41] Teichoic acid synthesis was measured in the presence of CDP-glycerol-^{14}C, UDP-*N*-acetylglucosamine, particulate enzyme preparation and appropriate cofactors. Upon the addition of UDP-*N*-acetylmuramyl pentapeptide, which is required to form the lipid intermediate for peptidoglycan synthesis, the percentage of

substrate incorporated into teichoic acid decreased from 83 to 65%, suggesting a possible competition of the teichoic acid and peptidoglycan system for the lipid. Upon the addition of bacitracin or vancomycin as well as UDP-*N*-acetylmuramyl peptapeptide to the reaction mixture synthesizing teichoic acid, the percentage of substrate incorporated into teichoic acid was further reduced. These antibiotics do not modify the extent of substrate incorporation into teichoic acid in the absence of UDP-*N*-acetylmuramyl pentapeptide. However, they are known to inhibit peptidoglycan synthesis by preventing the dephosphorylation of undecaprenyl pyrophosphate (bacitracin) and by inhibiting the sugar transfer from lipid intermediates to acceptor (vancomycin).[7] The net effect of either antibiotic, therefore, is a depletion of the supply of acceptor lipid, undecaprenyl phosphate, necessary to maintain the cyclical nature of peptidoglycan synthesis. The observed inhibition in teichoic acid synthesis in the presence of these antibiotics and UDP-*N*-acetylmuramyl pentapeptide thus is consistent with the theory of competition between the two systems, since the inhibition of teichoic acid synthesis may be viewed as a consequence of the reduced quantity of undecaprenyl phosphate in the presence of the antibiotics.

In another report it was demonstrated that chloramphenicol can exert inhibition directly on synthesis of teichoic acids, and this effect was independent of any effect on protein synthesis.[42] Chloramphenicol effectively inhibited only those enzymatic systems which produced polymers containing glucose in the main chain and which had been shown in unpublished experiments to involve lipid intermediates. The authors concluded that the site of inhibition in these cases, therefore, was at the stage of transfer of glucose from the nucleotide precursor to the lipid. However, it is clear that further studies must be made before the mechanism of the chloramphenicol inhibition is understood.

Concluding Comments

The concept which unifies these independent investigations on undecaprenol intermediates is the participation of a lipid, effectively serving as a coenzyme, that is associated with the cell membrane. The location of the lipid in the membrane permits it to mediate the transfer of low molecular weight, hydrophilic compounds that serve as the building blocks of macromolecules localized within or beyond the hydrophobic cell membrane.

The most distinct difference among the lipid intermediates formed in the different systems described here is the two types of linkages formed between the sugar moieties and the lipid moiety. A pyrophosphate group forms the bridge between sugars and lipid in the intermediates for peptidoglycan, *O*-antigen, and capsular polysaccharide synthesis,[24,25,35] but a phosphodiester linkage is present between

sugar and lipid in the intermediate for mannan synthesis and glucosylation of *O*-antigen (Fig. 7).[27,28,33] Nevertheless, the first step in all of the cycles of polysaccharide biosynthesis is the same: that is, a freely reversible reaction resulting in the synthesis of a new glycosyl-1-phosphoryl bond that serves to link the sugar to the hydrophobic lipid. The phosphodiester and pyrophosphate linked intermediates thus

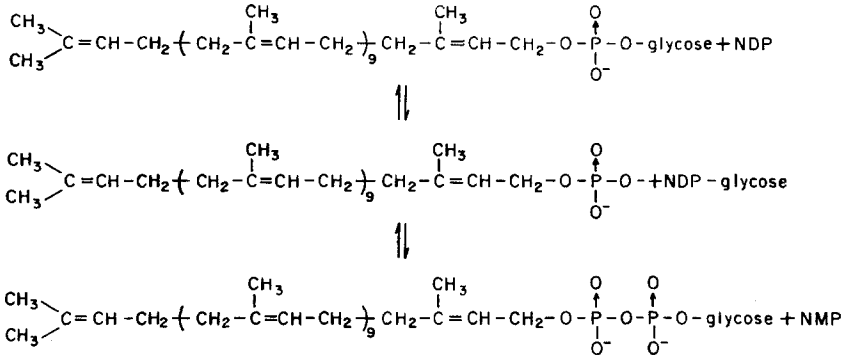

Figure 7. Structure of phosphate and pyrophosphate containing intermediates and the transfer reactions leading to their synthesis. NDP-glycose, NDP and NMP represent a sugar nucleotide, nucleoside diphosphate, and nucleoside monophosphate, respectively.

retain essentially the same reactivity as the sugar nucleotides and therefore, with regard to energetics, are just as effective as the sugar nucleotides in the biosynthesis of glycans.

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