*STRUCTURE OF A LIPID INTERMEDIATE IN CELL WALL PEPTIDOGLYCAN SYNTHESIS: A DERIVATIVE OF A C_{55} ISOPRENOID ALCOHOL**

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The peptidoglycan of bacterial cell walls is synthesized in a reaction mechanism in which a membrane-bound lipid serves as a carrier for the sugars and amino acids that eventually become part of the peptidoglycan.[1] One intermediate in the reaction cycle is represented as lipid-P-P-disaccharide-pentapeptide. This intermediate is common to a number of different bacteria. However, its disaccharide-pentapeptide moiety (GlcNAc-MurNAc-L-ala-D-glu-L-lys-D-ala-D-ala)[2] becomes modified in various ways in different organisms.[3] The modified disaccharide-peptide moiety is then transferred to an endogenous acceptor with introduction of a new unit into a growing peptidoglycan.

The purpose of the present paper is to report that lipid-P-P-disaccharide-pentapeptide has been obtained in a highly purified form from *Micrococcus lysodeikticus*, and that the nature of the lipid moiety of this intermediate has been elucidated.

Soon after the discovery of the occurrence of a lipid intermediate in peptidoglycan synthesis, a lipid carrier in the biosynthesis of the lipopolysaccharide of the cell wall of *Salmonella* was described.[4, 5] The structure of this substance is reported in a paper by Wright *et al.*[6]

Isolation of Lipid-P-P-Disaccharide-Pentapeptide from M. lysodeikticus.—Large-scale preparation of the lipid intermediate was carried out using techniques essentially the same as those described previously.[7] Particulate enzyme was prepared after lysozyme lysis of cells of *M. lysodeikticus*[8] and incubated with the uridine nucleotide substrates to generate the lipid intermediate. The incubation mixture contained 1000 ml of particulate enzyme prepared in 0.05 *M* Tris-HCl buffer, pH 7.4, and 0.01 *M* $MgCl_2$, 1.7 ml of 12 mM UDP-MurNAc-pentapeptide (containing 8.7×10^6 cpm of C^{14}-lysine),[9] 8 ml of 10 mM UDP-GlcNAc and 30 ml of 5 per cent deoxycholate. Enzyme prepared after lysozyme lysis of cells of *M. lysodeikticus* is able to synthesize the lipid intermediates, but is unable to utilize these intermediates for peptidoglycan synthesis. Deoxycholate was included in the incubation mixture in order to minimize the addition of endogenous glycine to the lipid intermediate.[8] After incubation at 20° for 30 minutes, the mixture was extracted with 1 liter of *n*-butanol:6 *M* pyridinium acetate (2:1), pH 4.2, followed by two extractions with the same volume of *n*-butanol. The combined extracts were back-washed with 2 liters of water and then taken to dryness and redissolved in 50 ml of chloroform:methanol (1:1). The yield was 3×10^6 cpm (34%, based on UDP-MurNAc-C^{14}-pentapeptide).

The extract was placed on a column of *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) (2.5 × 25 cm) and eluted as previously described.[7] The radioactive eluate obtained with methanol:6 *M* pyridinium acetate, pH 4.2 (1:1), was pooled

(900 ml). To it was then added 1.8 liters of water and 1.8 liters of butanol. The aqueous phase was extracted with 500 ml of butanol twice. The butanol phases were combined, taken to dryness, and redissolved in 4.5 ml of chloroform:methanol (7:1). The yield was 2.2×10^6 cpm.

This material was then placed on a column of silicic acid (1.5×20 cm) and eluted stepwise with mixtures of chloroform:methanol, (*a*) 7:1, (*b*) 5:1, (*c*) 3:1, (*d*) 1:1, and (*e*) methanol. The radioactive lipid was eluted by the last solvent. Fractions containing the radioactivity were pooled and placed again on the same column that was now eluted with a gradient of methanol in chloroform. The radioactive lipid was eluted when the chloroform:methanol mixture was approximately 30:70. It was apparent that the material obtained in this way was not homogeneous, since the specific activity (cpm per phosphate) was lower both in the leading edge of the radioactive peak and in its trailing edge than in the peak tubes.

A new purification step, not employed previously, was therefore sought. The pooled fractions from the silicic acid column were placed on a column of Sephadex LH-20 (1×34 cm) and followed by 99 per cent methanol. The radioactive lipid intermediate was obtained from the column together with a peak of organic phosphate at about 12 ml followed by two or three nonradioactive phosphate-containing lipids at 15 and 20 ml. After several runs through the Sephadex LH-20 column, the material appeared to be homogeneous and was then rechromatographed on the silicic acid column described above. It was apparent, however, that the leading edge of the material eluted from the silicic acid column still contained a small amount of an organic phosphate impurity. This leading edge was removed and the material was then rechromatographed again on the silicic acid column. After two more separations in this way, finally, a preparation was obtained which chromatographed on the silicic acid column as a peak of radioactivity in which the specific activity (cpm per phosphate) was constant throughout the peak. The final yield was 1.6×10^6 cpm (4.0 μmoles) or about 18 per cent of the UDP-MurNAc-C^{14}-pentapeptide initially employed as the substrate.

Biological Activity of the Isolated Lipid Intermediate.—The biological activity of the isolated lipid intermediate was examined in two ways. The particulate enzyme of *M. lysodeikticus* catalyzes an adenosine 5′-triphosphate-dependent (ATP-dependent) incorporation of C^{14}-glycine into lipid-P-P-disaccharide-pentapeptide.[8] The glycine residue becomes attached to the α-carboxyl group of glutamic acid in the pentapeptide. The isolated lipid intermediate was an active acceptor in this reaction (Table 1, expt. 1). This reaction was carried out in the presence of a low concentration of *n*-octanol that inhibits the utilization of the glycine-containing lipid intermediate for peptidoglycan synthesis.[8] In the absence of added *n*-octanol, glycine was incorporated into lipid intermediate and the glycine-containing intermediate was then utilized for peptidoglycan synthesis (Table 1, expt. 2). Thus the isolated intermediate was also a substrate for peptidoglycan synthetase, the enzyme which catalyzes the transfer of disaccharide-hexapeptide from the lipid intermediate to peptidoglycan. These observations established that no degradation essential to biological activity had occurred during the isolation.

Analyses of the Isolated Lipid Intermediate.—The purified lipid intermediate contained (molar ratios to glutamic acid in parentheses): glutamic acid (1.0), alanine (2.91), lysine (0.86), glycine (0.2), MurNAc (0.98), GlcNAc (0.84), and organic

TABLE 1

UTILIZATION OF ISOLATED LIPID INTERMEDIATE BY PARTICULATE ENZYME

Expt.	Cpm incorporated into: Lipid intermediate	Peptidoglycan
1. Octanol added	4,382	—
2. No octanol added	10,050	1,080

For the assay, 20 μl of lipid-P-P-disaccharide-pentapeptide (2.16 mμmoles) was added to the tubes and the solvent was removed by evaporation. Then 20 μl of 0.05 *M* Tris-HCl buffer, pH 7.4, containing 0.01 *M* $MgCl_2$, 2 μl of 0.43 mM C^{14}-glycine (116 μc/μmole), 2 μl of 20 mM ATP, 2 μl of 60 mM 2-mercaptoethanol, and 5 μl of particulate enzyme prepared after alumina grinding of cells of *M. lysodeikticus* were added and the tubes incubated at 37° for 20 min prior to assay by thin-layer chromatography.[10] Where indicated, 2 μl of *n*-octanol:ether (1:28) was added first to the tubes. The ether was then removed by warming prior to addition of the other components of the reaction mixture. Note that the radioactivity of the low specific activity lipid intermediate does not interfere with assay of addition to it of high specific activity C^{14}-glycine; blanks have been subtracted from the data recorded. The two experiments cannot be directly compared, since they were carried out at separate times.

phosphate (2.14). Thus this material contained 2 moles of organic phosphate per mole of disaccharide-pentapeptide. Both phosphate residues were extremely labile to acid hydrolysis; 88 per cent of the organic phosphate was released as inorganic phosphate after two minutes, or 100 per cent after five minutes of hydrolysis in 1 *N* HCl in 50 per cent methanol at 100°. After acid hydrolysis to release all the organic phosphate, no glycerol (measured with glycerokinase and glycerophosphate dehydrogenase) could be detected and no fatty acids were found in this purified material. These latter data are in contrast to data previously reported for less pure preparations of the lipid intermediate.[7] After treatment at 37° for 30 minutes in 0.03 *N* NaOH in 90 per cent methanol, the lipid intermediate remained in the butanol phase of a butanol-water mixture. Some of these properties were similar to properties reported for the lipid intermediate in lipopolysaccharide synthesis in *Salmonella*.[11]

It has previously been reported that the lipid intermediate from *M. lysodeikticus* is decomposed to a water-soluble fragment by heating at 100° and pH 4 for 20 minutes.[12] A sample of the purified lipid intermediate was treated in this way and a water-soluble fragment was produced that had approximately the same chromatographic mobility in isobutyric acid:1 *N* NH_4OH (5:3) and electrophoretic mobility in pyridinium acetate buffer, pH 4.2, as the fragment obtained previously. This degradation product had a ratio of phosphate to peptide of 2, and released all of its phosphate on mild acid hydrolysis. Analysis revealed that it did not contain glycerol. Treatment of this fragment with 1 *N* acetic acid at 100° resulted in the release of inorganic pyrophosphate (Table 2). By contrast, the fragment obtained in earlier studies[12] contained glycerol, released only one of its two phosphate residues on acid hydrolysis, and released inorganic pyrophosphate only after mild acid

TABLE 2

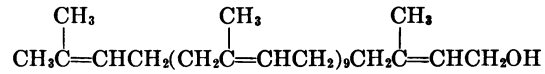
RELEASE OF INORGANIC PYROPHOSPHATE BY MILD ACID HYDROLYSIS OF THE WATER-SOLUBLE DEGRADATION PRODUCT OF THE LIPID INTERMEDIATE

Min	Pi	Pi after PPase	Δ = PPi
0	0.11	0.17	0.06
10	0.35	1.39	1.04
20	0.72	2.04	1.33
30	0.93	2.42	1.49
60	1.38	2.54	1.16
90	1.79	2.54	0.75

The reaction mixture contained the degradation product (74 mμmoles as total phosphate) in 126 μl of 1 *N* acetic acid and was heated at 100°. Aliquots of 5 μl were removed at the times indicated. Inorganic phosphate was measured before and after treatment with crystalline yeast inorganic pyrophosphatase (Sigma). Data are recorded as mμmoles.

hydrolysis followed by periodate oxidation and amine elimination. The most plausible interpretation of these early results is that glycerol diphosphate disaccharide-pentapeptide arose by an intermolecular phosphate transesterification to an impurity in the preparation of the lipid intermediate, similar to the intermolecular phosphate transesterification that has been reported by Brundish, Shaw, and Baddiley.[13] The fragment produced on pH 4 hydrolysis of the purified lipid intermediate obtained in the present study is clearly disaccharide-pentapeptide pyrophosphate.

Identification of the Lipid Moiety by Mass Spectrometry.—The mass spectrum of the lipid identified it conclusively as an undecaprenyl alcohol.



The polyisoprenoid nature was immediately suggested by a regular series of fragment ions (Table 3) separated by 68 mass units, representing random cleavage between individual isoprene units in a chain.[14] The weak molecular ion at m/e 766, accompanied by a relatively strong ion at m/e 748, equivalent to (M minus 18)$^+$, indicated that the major isoprenoid constituent of the lipid was composed of 11 isoprene units and an alcoholic functional group. There was no evidence in the mass spectrum for a terminal ring structure in the molecule of the type found in ubiquinone, plastoquinone, and vitamin K.

TABLE 3

MAJOR MASS SPECTRAL IONS ABOVE M/E 200 FROM THE LIPID MOIETY

m/e	Isoprene unit equiv.	Relative intensity*
203		8.1
204	$(C_5H_8)_3$	2.5
205		3.6
271		4.5
272	$(C_5H_8)_4$	2.2
273		2.2
339		3.6
340	$(C_5H_8)_5$	2.2
341		2.2
351		2.2
353		2.2
365		2.2
368		3.8
407		2.9
408	$(C_5H_8)_6$	2.2
409		1.6
475		1.8
476	$(C_5H_8)_7$	1.1
477		1.3
543		1.6
544	$(C_5H_8)_8$	1.3
545		1.1
611		1.1
612	$(C_5H_8)_9$	1.1
679		0.8
680	$(C_5H_8)_{10}$	0.9
748	$(C_5H_8)_{11}$	0.2
766	M^+	0.3

Mass spectra were obtained with a direct probe inlet using the LKB 9000 mass spectrometer. The temperature of the probe was approximately 150°C, and the spectra were recorded at 70 ev with an accelerating voltage of 3500 v. The electron current was 60 ma. The lipid intermediate was prepared by heating it in 0.1 *N* HCl in 50% methanol for 5 min to free the lipid moiety.

* Normalized to percentage of m/e 69, the most intense ion in the mass spectrum.

The positions of the major fragment ions obtained on cleavage between isoprene units were exactly analogous to those observed in the mass spectrum of solanesol.[15, 16] This observation, along with the finding of M^+ and (M minus 18)$^+$ at m/e 766 and 748, indicated that the lipid contained one double bond for each isoprene unit. In view of the isolation of a similar alcohol, called bactoprenol, from lactobacilli by Thorne and Kodicek,[17] it is particularly noteworthy that no evidence of a saturated isoprene unit was seen in the mass spectrum.

Although the major lipid present is the C_{55} isoprenoid alcohol, the mass spectral data do not eliminate the possibility that small amounts of lower isoprenologues might be present in the sample. A close comparison of the relative intensities of the major groups of ions (Table 3) with those from solanesol suggest, in fact, that the peaks at m/e 680 and 612 might consist partially of the (Minus 18)$^+$ ions derived from C_{50} and C_{45} alcohols, respectively. Normally, the peak at $(C_5H_8)_n$ is always somewhat lower than the companion peak at one lower m/e value, whereas the peaks at m/e 680 and 612 were found to be slightly higher than the peaks at m/e 679 and 611. If present, these lower homologues could not account for more than about 5 per cent of the total lipid, however.

Thin-Layer Chromatography of the Lipid Moiety and Bactoprenol.—The lipid moiety obtained after hydrolysis of the lipid intermediate at pH 4 contained two major and one minor component on thin-layer chromatography on silica gel G in heptane:ethyl acetate (90:10).[17] One of the major components (R_f = 0.25) had a chromatographic mobility identical to a sample of C^{14}-bactoprenol (kindly provided by Drs. Thorne and Kodicek). The other components (R_f = 0.46 and 1.0) are presumably rearrangement and/or dehydration products which are known to be produced from isoprenoid alcohol pyrophosphates under acidic conditions. The first major component also had a chromatographic mobility identical to bactoprenol on thin-layer chromatography on silica gel G in methanol:benzene (1:99) (R_f = 0.56).

Discussion.—A variety of isoprenoid alcohols have been isolated from various sources in recent years. The pyrophosphates of the C_5, C_{10}, and C_{15} alcohols are intermediates in the biosynthesis of cholesterol.[18] The C_{45} alcohol (solanesol) was isolated from tobacco leaves,[15, 16] and a series of isoprenoid alcohols ranging from C_{30} to C_{80} (6–16 isoprenoid units), including a C_{55} alcohol, have been isolated from other plant tissues.[19–21] Even higher homologues, C_{100} and C_{110}, have been reported to occur in animal tissues and a fungus, respectively.[22, 23]

Two recent investigations are of particular relevance to the present work. On the one hand, it has been found that enzymes from *M. lysodeikticus* are capable of utilizing isopentenyl pyrophosphate for the elongation of farnesyl pyrophosphate to C_{20} and C_{40} isoprenoid alcohol pyrophosphates.[24, 25] These metabolic steps may be intermediate in the biosynthesis of the isoprenoid alcohol under study here. Moreover, Thorne and Kodicek have isolated a C_{55} isoprenoid alcohol, which they termed bactoprenol, from cells of several lactobacilli.[17] Nuclear magnetic resonance and mass spectra of the material that they obtained indicated the presence of ten unsaturated and one saturated isoprenoid groups in this alcohol. The mass spectrum of the C_{55} isoprenoid alcohol obtained here from *M. lysodeikticus* showed no evidence of the presence of a saturated isoprenoid residue in the molecule, and although the isoprenoid alcohol obtained from *M. lysodeikticus* and from lactobacilli were identi-

cal on thin-layer chromatography, a separation of such closely related compounds by this technique would not be expected. Bactoprenol is apparently incorporated into a lipoprotein of the bacterial cell,[26] possibly the enzyme complex which catalyzes cell wall synthesis. In keeping with the nomenclature adopted in a related field, it may be desirable to employ the name bactoprenol for the class of C_{55} isoprenoid alcohols that occur in bacterial cells. The term "bactoprenol" might thus apply to the fully unsaturated compound which is found in *M. lysodeikticus*, while "dihydrobactoprenol" might be employed for the compound obtained from lactobacilli. The occurrence of mevalonic acid as a growth factor for some bacteria,[27] which are not known to contain sterols, is probably accounted for by its utilization to form these essential isoprenoid alcohols.

Wright *et al.*[6] have shown that the lipid carrier in lipopolysaccharide synthesis in *Salmonella* is similarly a C_{55} isoprenoid alcohol to which an oligosaccharide fragment is attached through a pyrophosphate bridge. Further studies will be required to establish the identity of the two isoprenoid alcohols. In particular the *cis-trans* configurations of the olefinic groups have not been established. The related isoprenoid compounds, squalene, solanesol, and geranyl geraniol, have the all-*trans* configurations, but materials recently isolated from both plant and animal tissues apparently have mixed *cis* and *trans* double bonds.[19-23] Further studies by infrared and nuclear magnetic resonance spectroscopy and gas liquid chromatography should help to clarify these questions.

Lipid phosphate fragments can be obtained from each of the lipid intermediates by enzymatic hydrolysis. A test of the activity of lipid phosphate isolated from *Salmonella* in peptidoglycan synthesis with *M. lysodeikticus* enzyme and of lipid phosphate from *M. lysodeikticus* in lipipolysaccharide synthesis with *Salmonella* enzyme will be extremely interesting. Also, it has recently been shown that lipid pyrophosphate accumulates as the consequence of inhibition of peptidoglycan synthesis by bacitracin.[28] Bacitracin is a specific inhibitor of the dephosphorylation of lipid pyrophosphate to lipid phosphate, and may interact with lipid pyrophosphate. If indeed the lipid moieties in the two systems are identical, bacitracin should also inhibit lipopolysaccharide synthesis and lead to the accumulation of lipid pyrophosphate.

Summary.—Lipid-P-P-disaccharide-pentapeptide, the lipid intermediate in the biosynthesis of the peptidoglycan of bacterial cell walls, has been obtained in a highly purified form from enzyme preparations of *M. lysodeikticus*. The lipid moiety of the intermediate has been shown to be a C_{55} isoprenoid alcohol.

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[2] Abbreviations: GlcNac, N-acetylglucosamine; MurNAc, N-acetyl-muramic acid; UDP, uridine diphosphate.

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