ISOLATION AND UTILIZATION OF PHOSPHOLIPID INTERMEDIATES IN CELL WALL GLYCOPEPTIDE SYNTHESIS*

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The utilization of uridine diphosphate acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and uridine diphosphate acetylglucosamine (UDP-GlcNAc) for biosynthesis of a linear cell wall glycopeptide involves the formation of membrane-bound lipid intermediates (Anderson, Matsuhashi, Haskin and Strominger, 1965; Matsuhashi, Dietrich and Strominger, 1965). Lipid intermediates were subsequently also shown to participate in the biosynthesis of cell wall lipopolysaccharides in Salmonella (Weiner, Higuchi, Rothfield, Saltmarsh-Andrew, Osborn and Horecker, 1965; Wright, Dankert and Robbins, 1965). In the initial studies, two lipid intermediates in glycopeptide synthesis, MurNAc-(-pentapeptide)-P-lipid and GlcNAc-MurNAc(-pentapeptide)-P-lipid, were generated in situ in the heavy particles (presumably cell membrane fragments), which catalyze glycopeptide synthesis in both S. aureus and M. lysodeikticus (Anderson et al., 1965; Matsuhashi, et al., 1965). In the present communication, the isolation of these two lipid intermediates, some analyses of them and studies of their utilization for glycopeptide synthesis will be reported.

For the isolation of GlcNAc-MurNAc(-pentapeptide)-P-lipid from M. lysodeikticus,

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a large scale reaction mixture containing 1.3 μmoles of UDP-MurNAc-pentapeptide-^{14}C (specific activity, 0.69×10^6 c.p.m./μmole, labeled in both D-alanine residues), 2.0 μmoles of UDP-GlcNAc, 2.5 mmoles of Tris-HCl, pH 8.6, 0.7 mmole of $MgCl_2$ and 9.0 ml of particulate enzyme, from M. lysodeikticus (70 mg of protein), prepared after disruption of the cells by grinding with alumina, in a total volume of 25 ml was incubated at 37° for 15 minutes. The mixture was then extracted with 3 volumes of n-butanol: 6 M acetic acid, containing sufficient pyridine to bring the pH to 4.2 (2:1). The extracts were washed with water, taken to dryness on a flash evaporator, dissolved in 10 ml of chloroform:methanol (7:1) and applied to a column of DEAE-cellulose, acetate form (Rouser, Galli, Lieber, Blank and Privett, 1964). None of the radioactive lipid intermediate was eluted by the various solvents described by Rouser et al. (1964) although most of the neutral lipids, phospholipids and colored pigments present in the butanol extract were eluted. Subsequent elution with methanol:6 M pyridinium acetate, pH 4.2 (1:1) yielded all of the radioactive material together with a small amount of organic phosphate in a single peak. This material was recovered and further purified by chromatography on a column of silicic acid from which it was eluted by chloroform:methanol (1:1) as a single symmetrical peak of radioactive phospholipid. Occasionally small amounts of other phosphate containing compounds were separated by silicic acid chromatography. Thin layer chromatography on silica gel G in three solvents demonstrated that this material migrated as a single compound with respect to its phosphate, radioactivity and fatty acid. GlcNAc-MurNAc(-pentapeptide)-P-lipid from S. aureus chromatographed similarly during its isolation. Analyses of these two compounds (Table 1) indicated that the compounds are complex glycerophosphatides related to the cardiolipins. They differed significantly from each other in the amount of glycerol and fatty acid present. The identification of glycerol diphosphate disaccharide-pentapeptide as a hydrophilic degradation product is reported in the fol-

lowing paper (Dietrich, Matsuhashi and Strominger, 1965). The structure of the hydrophobic portion of the two phospholipids is under investigation.

Table 1

Analyses of GlcNAc-MurNAc(-pentapeptide)-P-Phospholipid

	Phospholipid from:	
	M. lysodeikticus	S. aureus
GlcNAc-MurNAc(-pentapeptide)[1]	(1.0)	(1.0)
Total phosphate[2]	4.8	5.3
Glycerol[3]	2.3	3.1
Fatty acid[4]	2.2	3.3
Fatty acid[5]	1.9	2.9

[1] All data are expressed relative to the glutamic acid content of disaccharide-pentapeptide, measured in a Beckman-Spinco amino acid analyzer.

[2] Measured by the method of Lowry, Roberts, Leiner, Wu and Fair (1954).

[3] Measured after hydrolysis in 4 N HCl at 100° for 10 hours with glycerokinase and α-glycerophosphate dehydrogenase (Wieland, 1963). These values should be regarded as minimum values since some loss of glycerol may occur during preparation of the sample, and hydrolysis may not be complete.

[4] Measured after reaction with alkaline hydroxylamine (Hestrin, 1949).

[5] Measured by gas chromatography. Qualitatively the distribution of fatty acids was similar to that found in the total lipids of these gram-positive bacteria. We are grateful to Drs. Paul Thomas and John Law for carrying out these analyses.

In order to study the utilization of the isolated lipid intermediates, GlcNAc-MurNAc(-pentapeptide)-P-phospholipid and MurNAc(-pentapeptide)-P-phospholipid with high specific radioactivity (70×10^6 c.p.m./μmole, labeled with ^{14}C-L-lysine) were isolated by the same methods from similar small scale incubation mixtures con taining undiluted radioactive nucleotide precursors. Both of the intermediates iso-

lated from M. lysodeikticus could be utilized for glycopeptide synthesis with M. lysodeikticus particles (Table 2). Utilization of GlcNAc-MurNAc(-pentapeptide)-

Table 2

Utilization of MurNAc(-pentapeptide)-P-Phospholipid and GlcNAc-MurNAc(-pentapeptide)-P-Phospholipid from M. lysodeikticus for Glycopeptide Synthesis

The reaction mixtures contained, in a total volume of 10 µl, 1 µmole of Tris-HCl, pH 8.6, 0.35 µmole of $MgCl_2$, 3.8 µl of particulate enzyme from M. lysodeikticus (57 µg of protein), 2.3 µmoles of $(NH_4)_2SO_4$, 0.85 µg of duponol and 85 µµmoles of isolated MurNAc(-pentapeptide-^{14}C)-P-phospholipid. The incubation with GlcNAc-MurNAc(-pentapeptide-^{14}C)-P-phospholipid was similar except that 100 µµmoles of the isolated lipid were used and $(NH_4)_2SO_4$ and duponol were not added. Where indicated, 1.3 mµmoles of UDP-GlcNAc was added. The reaction mixtures were incubated at 37° for 1 hour, and assayed as described previously (Anderson et al., 1965).

Conditions:	µµmoles of glycopeptide formed from: MurNAc (-pentapeptide)-P-Phospholipid	GlcNAc-MurNAc (-pentapeptide)-P-Phospholipid
Zero-time control	0.1	0.4
Complete system	1.3	34.8
+ UDP-GlcNAc	34.6	34.7

P-phospholipid required no further addition of uridine nucleotides, while utilization of MurNAc(-pentapeptide)-P-phospholipid required addition of UDP-GlcNAc. Moreover, on addition of UMP, formation of UDP-MurNAc-pentapeptide from MurNAc(-pentapeptide)-P-phospholipid could be demonstrated in the reverse reaction. The reversibility of the initial step in glycopeptide synthesis was first demonstrated by Struve and Neuhaus (1965). The apparent Michaelis constants for the isolated intermediates were exceedingly high and it was not possible to saturate the particulate system. Correspondingly, the observed velocity of the reactions with the isolated intermed-

iates was only 4% of that of the overall reaction from the uridine nucleotides. Utilization was not improved by addition of a number of detergents to the systems, but efforts to find better conditions for utilization of the isolated intermediates are continuing.

These two intermediates were also isolated from S. aureus at high specific activity. A number of observations have suggested that the intermediates from S. aureus are more labile during isolation than those from M. lysodeikticus. It has not been possible to demonstrate appreciable utilization of the S. aureus intermediates either by the S. aureus particulate enzyme or by the M. lysodeikticus particulate enzyme. The intermediates isolated from M. lysodeikticus also could not be utilized by the S. aureus particles.

Although a number of observations have suggested that the phospholipid carriers in M. lysodeikticus and S. aureus may not be identical, the present data should not be construed as having conclusively demonstrated that they are different. This question requires careful investigation. More complex forms of the intermediates occur since glycine is added to the phospholipids of both organisms in the form of a pentaglycine chain with a free amino end substituted on the ϵ-amino group of lysine in the case of S. aureus (Matsuhashi, Dietrich and Strominger, 1965), or as a single glycine residue with a free carboxyl group substituted on the α-carboxyl group of glutamic acid in the case of M. lysodeikticus (Matsuhashi, Dietrich and Gilbert, 1965). The phospholipid intermediates in these forms have also been isolated, and studies of them are currently in progress.

SUMMARY

Phospholipid intermediates in cell wall glycopeptide synthesis, MurNAc(-pentapeptide)-P-phospholipid and GlcNAcMurNAc(-pentapeptide)-P-phospholipid, have been

isolated from both M. lysodeikticus and S. aureus. Some analyses and studies of utilization of the isolated compounds for glycopeptide synthesis have been reported.

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