[13] 5-HydroxymethyldUMP could be a hydrolytic side product of the hypothetical intermediate having a methylene bridge between the 5 position of dUMP and the no. 5 atom of tetrahydrofolate.[7] In all solvents examined by R. E. Cline, R. M. Fink, and K. Fink [*J. Am. Chem. Soc.*, **81**, 2521 (1959)], 5-hydroxymethyldeoxyuridine migrated to the same position as 5-hydroxymethyluracil or was slightly slower, and thymidine and thymine always migrated far ahead of both. We could not detect radioactivity on our chromatograms in the region between the starting line and the position of thymidine and thymine (R_F, 0.64). 5-Hydroxymethyluracil showed an R_F of 0.49. From the background level radioactivity on the chromatogram, 5-hydroxymethyldUMP could not account for more than 5% of the synthesis. 5-HydroxymethyldUMP does not appear to be an intermediate in the *E. coli* dTMP synthetase reaction.[3]

[14] Somerville, R. L., K. Ebisuzaki, and G. R. Greenberg, these PROCEEDINGS, **45**, 1240 (1959).

[15] Dirksen, M. L., J. S. Wiberg, J. F. Koerner, and J. M. Buchanan, these PROCEEDINGS, **46**, 1425 (1960).

[16] Osborn, M. J., and R. M. Huennekens, *J. Biol. Chem.*, **233**, 969 (1958).

[17] Zamenhof, S., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 3, p. 702.

[18] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[19] Fiske, C. H., and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

[20] Nash, T., *Biochem. J.*, **55**, 416 (1953).

[21] Cohen, S. S., *Federation Proc.*, **20**, 641 (1961).

[22] Flaks, J. G., and S. S. Cohen, *J. Biol. Chem.*, **234**, 1501 (1959).

[23] Koerner, J. F., M. S. Smith, and J. M. Buchanan, *J. Biol. Chem.*, **235**, 2691 (1960).

[24] Kornberg, A., S. B. Zimmerman, S. R. Kornberg, and J. Josse, these PROCEEDINGS, **45**, 772 (1959).

[25] Mathews, C. K., and S. S. Cohen, *J. Biol. Chem.*, **238**, PC853 (1963).

[26] Friedkin, M., E. J. Crawford, E. Donovan, and E. J. Pastore, *J. Biol. Chem.*, **237**, 3811 (1962).

[27] Wiberg, J. S., and J. M. Buchanan, these PROCEEDINGS, **51**, 421 (1964).

[28] Dirksen, M. L., J. C. Hutson, and J. M. Buchanan, these PROCEEDINGS, **50**, 507 (1963).

[29] Streisinger, G., F. Mukai, W. J. Dreyer, B. Miller, and S. Horiuchi, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 25. See also Anfinsen, C., *Federation Proc.*, **20**, 634 (1961).

[30] Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 375.

[31] Sarabhai, A. S., A. O. W. Stretton, S. Brenner, and A. Bolle, *Nature*, **201**, 13 (1964).

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*LIPID-PHOSPHOACETYLMURAMYL-PENTAPEPTIDE AND LIPID-PHOSPHODISACCHARIDE-PENTAPEPTIDE: PRESUMED MEMBRANE TRANSPORT INTERMEDIATES IN CELL WALL SYNTHESIS**

BY JOHN S. ANDERSON,† MICHIO MATSUHASHI, MARY A. HASKIN, AND JACK L. STROMINGER

DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF WISCONSIN MEDICAL SCHOOL, MADISON

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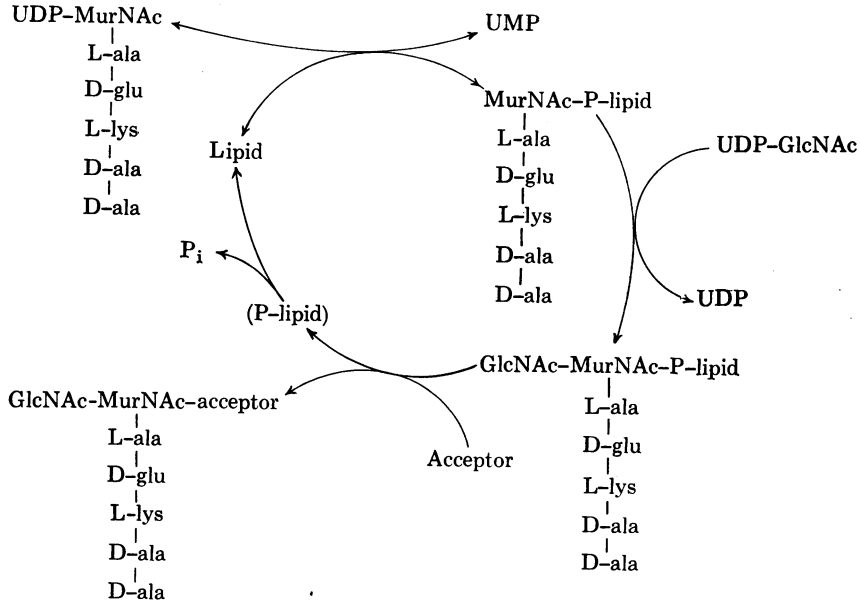
The uridine nucleotides, uridine diphospho-acetylmuramyl·L-ala·D-glu·L-lys·D-ala·D-ala (UDP-MurNAc-pentapeptide) and uridine diphospho-acetylglucosamine (UDP-GlcNAc), are substrates for a reaction catalyzed by a particulate enzyme prepared from *Staphylococcus aureus* in which a linear glycopeptide com-

posed of alternating MurNAc-pentapeptide and GlcNAc residues is formed, a precursor of the insoluble highly cross-linked glycopeptide of the cell wall.[1, 2] Previously we have reported that UMP and P_i are the other products of the reaction from UDP-MurNAc-pentapeptide while UDP is formed from UDP-GlcNAc.[2, 3] In this paper we wish to document this finding more extensively with enzymes obtained from both *S. aureus* and *Micrococcus lysodeikticus*. Moreover, it has been found that the initial reaction in glycopeptide synthesis is the transfer of phosphoacetylmuramyl-pentapeptide to a lipid fraction with formation of UMP. Then GlcNAc is transferred to the lipid fraction with formation of UDP, and finally the disaccharide, GlcNAc-MurNAc-pentapeptide, is transferred to an acceptor with the release of inorganic phosphate. Thus, the sequence can be written as follows:



The probable significance of this cycle in transport through the cell membrane will be discussed.

Methods.—Substrates: UDP-MurNAc · L-ala · D-glu · L-lys · D-ala · D-ala, labeled with either C^{14}-L-lys, H^3-L-lys, C^{14}-D-ala · C^{14}-D-ala, or H^3-D-ala · H^3-D-ala and UDP-GlcNAc-C^{14} were prepared enzymatically. P^{32}-UDP-MurNAc-pentapeptide and P^{32}-UDP-GlcNAc, labeled in both phosphates, were isolated from cells of *S. aureus*. Details of these preparations will be described in a subsequent publication.

Preparation of particulate enzyme: Cells of *Staphylococcus aureus*, strain H, and *Micrococcus lysodeikticus*, ATCC 4698, were harvested from log-phase cultures at 25% of maximum growth. After disruption of washed cells by grinding with alumina, the particulate fraction sedimenting between 20,000 and 105,000 × *g* was collected, washed twice in 0.02 *M* Tris-HCl, pH 8.2, containing 10^{-4} *M* $MgCl_2$, and resuspended in buffer containing 0.02 *M* Tris-HCl, pH 8.0, 0.01 *M* $MgCl_2$, and 0.001 *M* 2-mercaptoethanol. Particulate enzyme prepared following sonic disintegration of *S. aureus* cells[2, 3] was virtually inactive when assayed by the test tube method described below. However, disruption of cells by grinding with alumina has allowed preparation of particulate enzyme which is independent of the filter paper requirement. By contrast, particulate enzyme prepared from *M. lysodeikticus* after sonic disintegration had considerable activity in the test tube assay, but nevertheless was stimulated 2- to 6-fold by incubation on filter paper; that prepared after grinding with alumina was fully active in the test tube assay.

Assay of glycopeptide synthetase activity: A typical reaction mixture for assay of glycopeptide synthesis by the particulate enzyme of *M. lysodeikticus* contained 2.0 mμmoles of C^{14}- or H^3-labeled UDP-MurNAc-pentapeptide (2–10 × 10^4 cpm), 2.0 mμmoles of UDP-GlcNAc, 0.8 μmoles of $MgCl_2$, 2.5 μmoles of Tris-HCl, pH 8.6, and 60 μg of enzyme protein in a total volume of 25 μl. Incubation was at 37°, usually for 60 min. After inactivation, reaction mixtures were subjected to paper chromatography on Whatman 3 MM filter paper in isobutyric acid–1 *N* NH_4OH (5:3). The glycopeptide remained at the origin of the chromatogram and was counted in a liquid scintillation spectrometer. Glycopeptide synthetase activity in the particulate enzyme of *S. aureus* was assayed by the above procedure with the following modifications: the amount of $MgCl_2$ added was reduced from 0.8 μmole to 0.08 μmole, 25 mμmoles of ATP was added, and the incubation was at 20°. Activity was 50% greater in the presence of ATP than in its absence for reasons which are not understood. At 20° activity was considerably greater than at 37°. The specific activities for incorporation of MurNAc-pentapeptide were 3.5 and 15 mμmoles/mg protein/hr for the particulate enzymes prepared from *S. aureus* and *M. lysodeikticus*, respectively.

Results.—UMP, inorganic phosphate, and UDP as reaction products: Reaction mixtures containing P^{32}-UDP-MurNAc-pentapeptide labeled in the peptide with either C^{14}-L-lys or H^3-L-lys and unlabeled UDP-GlcNAc, together with control reaction mixtures from which UDP-GlcNAc was omitted, were incubated with the particulate enzyme from either *S. aureus* or from *M. lysodeikticus*. In addition to the labeled glycopeptide, P^{32}-UMP and P^{32}-inorganic phosphate were formed in approximately equivalent amounts (Table 1). No P^{32}-UDP was detected. Recently, it has been demonstrated with similar enzyme preparations that UMP exchanges with UDP-MurNAc-pentapeptide, thus confirming by another method that UMP is a product of this reaction.[5] The exchange is due to reversibility of the first step in the reaction sequence (see below).

By contrast, when P^{32}-UDP-GlcNAc-C^{14} and unlabeled UDP-MurNAc-pentapeptide were the substrates with enzyme from either *S. aureus* or *M. lysodeikticus*, C^{14}-GlcNAc was incorporated into polymer and a nearly equivalent amount of P^{32}-UDP was formed (Table 1). When both substrates were labeled with P^{32}, all three compounds, UDP, UMP, and Pi, were formed in nearly equivalent amounts, thus conclusively demonstrating that UMP and Pi could not have been formed from UDP-MurNAc-pentapeptide by degradation of UDP.

TABLE 1

URIDINE NUCLEOTIDE REACTION PRODUCTS IN GLYCOPEPTIDE SYNTHESIS

Labeling of Substrates		Labeled Reaction Products (mμmoles)			
UDP-MurNAc-pentapeptide	UDP-GlcNAc	Glycopeptide	UDP	UMP	Pi
M. lysodeikticus					
P^{32}, C^{14}-L-lys	No label	0.79	0	0.80*	0.64
No label	P^{32}, C^{14}-GlcNAc	0.76	0.68	0	0.01
S. aureus					
P^{32}, H^3-L-lys	No label	1.17	0	1.19*	0.97
No label	P^{32}, C^{14}-GlcNAc	1.12†	1.04†	0	0.17

* In the absence of UDP-GlcNAc, P^{32}-UMP (but not P_i) was formed from P^{32}-UDP-MurNAc-pentapeptide by the exchange reaction. If the reaction mixture contains appreciable amounts of UMP, formation of P^{32}-UMP by this exchange can be extensive. Correction for the exchange is important in measurement of stoichiometry.

† In *S. aureus* UDP-GlcNAc-C^{14} is also a substrate for teichoic acid synthesis.[4] A relatively small correction for the teichoic acid synthesis was obtained from incubations from which UDP-MurNAc-pentapeptide was omitted.

Reactions were carried out essentially as described under *Methods*. After inactivation of enzymes, 80 mμmoles each of carrier UDP and UMP were added to each reaction mixture. Residual substrates and reaction products were separated by two-dimensional paper chromatography. The solvents were isobutyric acid–1 *N* NH_4OH (5:3) and 95% ethanol–1 *M* NH_4OAc, pH 7.0 (7.5:3). Carrier UDP and UMP were located by absorption of ultraviolet light. Glycopeptide remained at the origin. Radioautograms were prepared to locate all of the radioactive materials which were then counted on the paper in a liquid scintillation spectrometer. All values were corrected for control values obtained from reaction mixtures from which the nonlabeled substrate was omitted.

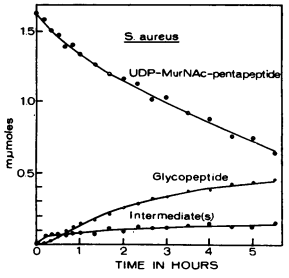


FIG. 1.—Time course of glycopeptide synthesis. The reaction mixture contained 28 mμmoles of UDP-MurNAc·L-ala·D-glu·L-lys·C^{14}-D-ala·C^{14}-D-ala, 118 mμmoles of UDP-GlcNAc, 1.6 μmoles of ATP, 1.2 μmoles of $MgCl_2$, 36 μmoles of Tris-HCl, pH 8.6, and 1.1 mg of protein as *S. aureus* particulate enzyme in a total volume of 290 μl. During incubation at 20°, 19-μl aliquots were removed at intervals, inactivated, and resolved by paper chromatography in isobutyric acid–1 *N* NH_4OH (5:3) into glycopeptide (origin), residual substrate (R_f = 0.18), and lipid intermediate(s) (R_f = 0.9). All components were located by radioautography and then counted in a liquid scintillation spectrometer.

Detection of a lipid intermediate in the reaction: The above data suggested that P-MurNAc-pentapeptide might be transferred to some carrier with subsequent transfer of MurNAc-pentapeptide from this carrier. In the chromatographic assay, the radioactive glycopeptide remained at the origin. In addition, a small amount of radioactive material was observed which migrated near the front of the solvent (R_f = 0.9). This material was formed rapidly and its amount was maximum prior to formation of a significant amount of glycopeptide (Fig. 1). In fact, the time required for the formation of this material accounts for the short lag in glycopeptide synthesis consistently observed with enzyme from *S. aureus*.

Further investigation of the radioactive material of R_f = 0.9 with various labeled substrates indicated that it contained one phosphate and both the L-lysine and D-alanine from P^{32}-UDP-MurNAc-pentapeptide-C^{14}. Struve and Neuhaus[5] quoted this result as a personal communication and suggested that the presence of this material (which they observed as acid-precipitable radioactivity) accounted for the UMP exchange which they reported. This material could be totally extracted from incubation mixtures by n-butanol in the presence of isobutyric acid and was partially extracted by a variety of organic solvents, e.g., petroleum ether, diethyl ether, and chloroform-methanol. The carrier is defined as a lipid because of the solubility of the intermediate in organic solvents.

The intermediate, bound to the particles, was prepared from a reaction mixture containing UDP-MurNAc-pentapeptide-C^{14} in the absence of UDP-GlcNAc, by centrifugation at 105,000 × *g*. When these labeled particles were reincubated with UDP-GlcNAc, glycopeptide was formed (Table 2). When UDP-GlcNAc was omitted, no glycopeptide was formed, and when UMP was added, the intermediate was "driven back" to UDP-MurNAc-pentapeptide. Although only 30–60 per cent of the lipid intermediate could be converted to glycopeptide in the presence of UDP-GlcNAc, essentially all of it could be converted to UDP-MurNAc-pentapeptide by addition of UMP. Occasionally, even in the absence of added UMP, some formation of UDP-MurNAc-pentapeptide was observed, presumably due to reversal of the reaction by endogenously formed UMP. Several experiments indicated that formation of glycopeptide proceeded directly from the lipid intermediate and did not depend on reversal to form UDP-MurNAc-pentapeptide. Addition of a 19-fold excess of unlabeled UDP-MurNAc-pentapeptide in the presence of UDP-GlcNAc only slightly diminished incorporation. Moreover, when phosphomonoesterase was added to destroy traces of UMP, utilization of the lipid intermediate was unaffected although the reversal to form UDP-MurNAc-pentapeptide was eliminated.

TABLE 2

UTILIZATION OF PARTICLE-BOUND MURNAC(-PENTAPEPTIDE)-P-LIPID FOR GLYCOPEPTIDE SYNTHESIS

Additions	Compounds Formed (μμmoles)		
	Glycopeptide	UDP-MurNAc-pentapeptide	Intermediate
Zero-time control	1	4	102
No additions	1	56	67
UDP-GlcNAc	54	4	63
UMP	1	101	7
UDP-GlcNAc + UDP-MurNAc-pentapeptide	46	29	61

The initial reaction mixture contained 25 mμmoles of UDP-MurNAc·L-ala·D-glu·L-lys·C^{14}-D-ala·C^{14}-D-ala, 10 μmoles of $MgCl_2$, 32 μmoles of Tris-HCl, pH 8.6, and 1.7 mg of protein as *M. lysodeikticus* particulate enzyme (prepared from sonically disrupted cells) in a total volume of 250 μl. After 30 min incubation at 37°, 12 ml of cold 0.02 *M* Tris-HCl, pH 7.8, containing 10^{-4} *M* $MgCl_2$, was added to dilute the reaction mixture which was then centrifuged at 105,000 × *g* for 1 hr. The pellet was washed once in buffer of the same composition, centrifuged again at 105,000 × *g* for 1 hr, and resuspended in 0.1 ml of buffer containing 0.02 *M* Tris-HCl, pH 8.0, 0.01 *M* $MgCl_2$, and 0.001 *M* 2-mercaptoethanol.

New reaction mixtures were established which contained 0.8 μmole of $MgCl_2$, 2.6 μmole of Tris-HCl, pH 8.6, and a 10-μl aliquot of the resuspended labeled *M. lysodeikticus* particulate enzyme in a total volume of 20 μl. Other components, added as indicated, were 1.9 mμmoles of UDP-MurNAc-pentapeptide, 3.3 mμmoles of UDP-GlcNAc, or 2.1 mμmoles of UMP. All incubations were for 1 hr at 37°. Separation and quantitation of radioactive components were the same as described for Fig. 1.

In a separate experiment, P^{32}-UDP-MurNAc-pentapeptide-C^{14} was employed to label the lipid intermediate. On subsequent incubation of such labeled particles with UDP-GlcNAc, approximately equal amounts of inorganic phosphate and glycopeptide were formed.

Mode of utilization of UDP-GlcNAc for glycopeptide synthesis: If UDP-GlcNAc-C^{14} was utilized as substrate in the absence of UDP-MurNAc-pentapeptide, no labeling of the lipid fraction occurred. However, in the presence of UDP-MurNAc-pentapeptide, C^{14}-GlcNAc entered the lipid fraction in an amount equal to the amount of MurNAc-pentapeptide in this fraction. Moreover, in experiments with P^{32}-UDP-MurNAc·L-ala·D-glu·H^3-L-lys·D-ala·D-ala and UDP-GlcNAc-C^{14}, the lipid fraction contained approximately equal amounts of P^{32}-phosphate, H^3-lysine, and C^{14}-GlcNAc. When P^{32}-UDP GlcNAc and unlabeled UDP-MurNAc-pentapeptide were employed as substrates, no P^{32} entered the lipid intermediate.

Particles were prepared containing this intermediate labeled with C^{14}-GlcNAc by incubation of the complete system for 5 min (see Fig. 1). At this time, formation of the intermediate was maximum while a minimum of glycopeptide product had been formed. These particles were washed free of residual subtrates and then reincubated in the assay system. Glycopeptide was formed from such particles containing this labeled lipid intermediate without other additions of uridine nucleotides (Table 3, expt. *A*). Incubation of UMP with the particle-bound lipid intermediate containing both P-MurNAc-pentapeptide and GlcNAc did not yield either nucleotide substrate. Only when both UDP and UMP were added was it possible to detect minimal amounts of the nucleotides formed by reversal of the reaction sequence.

Nature of the lipid intermediate: Preliminary experiments on the nature of this material have been concerned with the linkage of P-MurNAc-pentapeptide and GlcNAc to the lipid. This lipid intermediate was not affected by treatment with *E. coli* phosphomonoesterase or venom phosphodiesterase. A major question was whether P-MurNAc-pentapeptide-H^3 and C^{14}-GlcNAc were linked to each other or to separate lipids. After hydrolysis of the intermediate in 0.1 *N* HCl at 100° for 15 min, GlcNAc-MurNAc-pentapeptide-H^3,C^{14} was formed in 70 per cent yield.

TABLE 3

UTILIZATION OF PARTICLE-BOUND GLCNAC-MURNAC(-PENTAPEPTIDE)-P-LIPID FOR GLYCOPEPTIDE SYNTHESIS

Additions	Intermediate Prepared in: (A) Absence of Ristocetin Glycopeptide ($\mu\mu$moles)	Δ	(B) Presence of Ristocetin Glycopeptide ($\mu\mu$moles)	Δ
Zero-time control	41	—	4	—
No addition	84	43	33	29
UDP-GlcNAc	83	42	32	28
UDP-MurNAc-pentapeptide	87	46	48	44
Ristocetin	48	7	7	3

The initial reaction mixtures *A* and *B* contained 22 mμmoles of UDP-MurNAc-pentapeptide, 20 mμmoles of UDP-GlcNAc-C^{14}, 16 μmoles of $MgCl_2$, 50 μmoles of Tris-HCl, pH 8.6, and 3.0 mg protein as *M. lysodeikticus* particulate enzyme in a total volume of 400 μl. *B* also contained 20 μg of ristocetin. Both mixtures were incubated 5 min at 37°. Labeled particles were prepared as described in Table 2. Particles prepared in the presence of ristocetin contained only the labeled intermediate, whereas those prepared in its absence also contained some glycopeptide.

New reaction mixtures which contained 70–80 $\mu\mu$moles of labeled intermediate bound to the particulate enzyme (0.1 mg of protein) in a total of 25 μl were established as described in Table 2, and incubated for 1 hr at 37°. When indicated, 1.0 μg of ristocetin was added.

After treatment with β-acetylglucosaminidase this compound yielded MurNAc-pentapeptide-H^3 and C^{14}-GlcNAc, and after treatment with acetylmuramyl-L-alanine amidase it yielded C^{14}-disaccharide and H^3-pentapeptide. All of these compounds were identified by paper chromatography in three solvents. These results indicate that GlcNAc is glycosidically linked to MurNAc-pentapeptide in the lipid.

The fate of D-ala·D-ala of the pentapeptide moiety during the reaction: UDP-MurNAc·L-ala·D-glu·L-lys·D-ala·D-ala contains a pentapeptide moiety. However, degradation of the cell wall has revealed that 90 per cent of the peptides linked to acetylmuramic acid in the cell wall glycopeptide are tetrapeptides, L-ala·D-glu·L-lys·D-ala, and only 10 per cent are pentapeptides.[6, 7] To establish whether or not both D-alanine residues were incorporated into the product from UDP-MurNAc-pentapeptide, double labeling experiments were carried out with unlabeled UDP-GlcNAc and either UDP-MurNAc·L-ala·D-glu·C^{14}-L-lys·H^3-D-ala·H^3-D-ala (ratio of H^3 to C^{14} = 2.96) or UDP-MurNAc·L-ala·D-glu·H^3-L-lys·C^{14}-D-ala·C^{14}-D-ala (ratio of H^3 to C^{14} = 89.5) and enzyme from *M. lysodeikticus*. If an alanine residue were lost in the reaction, in the former case the ratio of H^3 to C^{14} in the product should have been half of that in the substrate, while in the latter case it should have doubled. No change was found in the ratio in the glycopeptide in either case (ratio of H^3 to C^{14} = 3.14 and 84.8, respectively), demonstrating that both D-alanine residues were incorporated into the product. The terminal D-alanine residue must be lost in some subsequent reaction in cell wall assembly.

Effects of antibiotics on the glycopeptide synthetase: Five antibiotics (penicillins, vancomycin, bacitracin, ristocetin, and novobiocin) induce accumulation of UDP-MurNAc-pentapeptide in *S. aureus*. Ristocetin and vancomycin, which are selective inhibitors of cell wall synthesis, appear to be specific inhibitors of glycopeptide synthetase. At ristocetin or vancomycin concentrations which inhibited growth by 50 per cent, formation of glycopeptide by enzyme prepared from these treated cells was also inhibited to the same extent (Table 4). At a variety of antibiotic concentrations growth inhibition was paralleled by inhibition of the synthetase. *In vitro* the enzyme prepared from cells ground with alumina was also inhibited at the same concentrations required to inhibit growth.

Bacitracin also inhibited glycopeptide synthesis at concentrations near the

TABLE 4

ANTIBIOTIC SENSITIVITY OF CELL GROWTH AND OF GLYCOPEPTIDE SYNTHETASE IN *S. aureus* AND *M. lysodeikticus*

	S. aureus Antibiotic Concentration Required for 50% Inhibition of			*M. lysodeikticus* Antibiotic Concentration Required for 50% Inhibition of		
		Glycopeptide Synthetase Antibiotic Added			Glycopeptide Synthetase Antibiotic Added	
Antibiotic	Growth (μg/ml)	*In vivo* (μg/ml)	*In vitro* (μg/ml)	Growth (μg/ml)	*In vivo* (μg/ml)	*In vitro* (μg/ml)
Ristocetin	12	12	8	7	15	15
Vancomycin	6	6	10	7	15	10
Bacitracin	35	25	50	3	50	30
Novobiocin	0.03	10	500	0.3	>100	150
Penicillin G	0.04	>250	>4000	0.2	>750	4000

Inhibition of growth: Antibiotics were added at 25% of maximum growth. Results are expressed as concentration of antibiotic in the medium which resulted in 50% inhibition of growth rate. Inhibition of glycopeptide synthetase: (*A*) Antibiotic added *in vivo:* Antibiotics were added to cultures at 25% of maximum growth, and incubation was continued for 15 min. Particulate fractions were prepared from antibiotic-treated and from control cells after disruption by sonic oscillation. Particulate enzyme prepared from *S. aureus* was assayed for glycopeptide synthetase activity by incubation on filter paper.[2] Particulate enzyme from *M. lysodeikticus* was measured by both the test tube and filter paper assays, and yielded similar data by both methods. Results are expressed as antibiotic concentration (in the culture medium from which cells were obtained) which yielded particulate enzyme in which activity was 50% of that in controls. (*B*) Antibiotic added *in vitro:* Particulate enzyme prepared from cells ground with alumina was assayed as described in *Methods*. Results are expressed as the concentration of antibiotic in the assay mixture which reduced activity by 50%.

growth inhibitory level in *S. aureus*, but in *M. lysodeikticus* the concentration required to inhibit the enzyme system was significantly higher than that required to inhibit growth (Table 4). Moreover, *in vitro* only partial inhibition of the system from either organism could be obtained with bacitracin although complete inhibition could be obtained with either vancomycin or ristocetin.

The glycopeptide synthetase was not inhibited by penicillin or novobiocin *in vivo* or *in vitro* even at exceedingly high concentrations of antibiotic. Penicillin and novobiocin cannot be specific inhibitors of this reaction.

In the presence of vancomycin or ristocetin at concentrations which completely inhibited growth and glycopeptide synthesis (20 μg/ml), both of the lipid intermediates were formed rapidly. With some preparations of enzyme, the amount of the intermediates reached a concentration nearly twice that found in the absence of antibiotics. The specific inhibition of the reaction by these two antibiotics is at a point subsequent to the formation of GlcNAc-MurNAc(-pentapeptide)-P-lipid (Table 3, expt. *B*). Moreover, at concentrations of 20 μg/ml neither of these antibiotics inhibited the exchange reaction of UMP with UDP-MurNAc-pentapeptide. At concentrations of vancomycin or ristocetin 10 times higher than that required to inhibit glycopeptide synthesis, inhibition of formation of the lipid intermediates was observed.

Discussion.—The data presented support the mechanism for synthesis of glycosidic linkages in the cell wall presented in the introduction. The lipid acts catalytically as a carrier of phosphodisaccharide-pentapeptide units for polymer synthesis. Lipid-P has not yet been isolated. The chemical nature of the lipid and of the acceptor of disaccharide-pentapeptide units (presumably an incomplete glycopeptide chain present in the particulate enzyme) is being investigated.

There have been many other studies of biosynthesis of heteropolysaccharides, but no other example has been thoroughly studied with respect to the nature of the nucleotide products of the sugar transfer reactions. Conceivably, the synthesis of cell wall glycopeptide is not a unique example of the mechanism described. It is

possible that our observations may be related to the lipid-amino acid complexes observed in other organisms. A lipid cofactor has been identified in the synthesis of Salmonella polysaccharides, but it is believed that this substance acts by forming a complex with the lipopolysaccharide.[8]

The reaction sequence described is presumably a definitive example of a membrane transport mechanism. The cell wall of bacteria is external to the cell membrane, which contains most of the lipid in the cell. The latter is the permeability barrier of the cell, and the nucleotide intermediates employed in the synthesis of the wall are themselves synthesized internal to a membrane which is impermeable to these nucleotides. The attachment of the phosphodisaccharide-pentapeptide to a lipid at the inside of the membrane would serve to convert the cell wall precursors to a form which could be transported to the outside of the membrane and then utilized for cell wall synthesis. The problem of orientation and transport through the membrane needs to be studied in more organized systems than the disrupted particles employed here. It may be noted that if the lipid is a diglyceride, P-lipid would be a phosphatidic acid. The proposed cycle would then be similar to the phosphatidic acid cycle which has been proposed to be operative in sodium transport.[9]

The state of organization of the particles is important in the catalysis of the over-all reaction. For example, in *S. aureus*, particles prepared after sonic disintegration are relatively inactive unless spread in a film on filter paper, while particles prepared after grinding with alumina have adequate levels of enzyme activity when assayed by conventional methods. Moreover, a fraction of the lipid which is labeled by UDP-MurNAc-pentapeptide cannot be utilized for glycopeptide synthesis. This observation suggests that it has been in some way damaged, possibly by separation from other essential components of the system or by oxidation.

The two antibiotics which inhibit glycopeptide synthesis, vancomycin and ristocetin, contain both carbohydrates and amino acids, i.e., they are themselves "glycopeptides." Their structures have not yet been fully elucidated. Conceivably they are acting as analogues of the natural glycopeptide acceptor in the reaction, thereby inhibiting transfer of disaccharide-pentapeptide.

Summary —Cell wall glycopeptide in *S. aureus* or in *M. lysodeikticus* is synthesized in a reaction employing UDP-acetylmuramyl-pentapeptide and UDP-acetylglucosamine as substrates. Inorganic phosphate and UMP are the other products of the reaction arising from the former substrate while UDP is formed from the latter. The mechanism of the reaction is (1) transfer of phosphoacetylmuramyl-pentapeptide to lipid, (2) formation of acetylglucosaminyl-acetylmuramyl(-pentapeptide)-P-lipid, (3) transfer of the disaccharide-pentapeptide to an acceptor (presumably an incomplete glycopeptide) with release of inorganic phosphate and presumably of lipid. This sequence represents a novel mechanism of heteropolysaccharide synthesis. It is probably a definitive example of a cellular transport mechanism.

Vancomycin and ristocetin are specific inhibitors of glycopeptide synthesis. They do not interfere with formation of the lipid intermediates. Their locus of action is in the utilization of lipid-phosphodisaccharide-pentapeptide for glycopeptide synthesis.

Note added in proof: Lipid-phosphodisaccharide-pentapetide-C^{14} has been purified from incubation mixtures by solvent extraction followed by chromatography on DEAE-cellulose and silicic acid columns. At least two-thirds of the radioactivity of the isolated material was utilized by the particulate enzyme from *M. lysodeikticus* to form glycopeptide.

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[1] Chatterjee, A. N., and J. T. Park, these PROCEEDINGS, **51**, 9 (1964).

[2] Meadow, P. M., J. S. Anderson, and J. L. Strominger, *Biochem. Biophys. Res. Commun.*, **14**, 382 (1964).

[3] Anderson, J. S., and P. M. Meadow, *Federation Proc.*, **23**, 380 (1964).

[4] Nathenson, S. G., and J. L. Strominger, *J. Biol. Chem.*, **238**, 3161 (1963).

[5] Struve, W. G., and F. C. Neuhaus, *Biochem. Biophys. Res. Commun.*, **18**, 6 (1965).

[6] Ghuysen, J.-M., *Biochim. Biophys. Acta*, **47**, 561 (1961).

[7] Ghuysen, J.-M., D. J. Tipper, and J. L. Strominger, in *Abstracts*, Sixth International Congress of Biochemistry, New York, 1964, p. 508.

[8] Rothfield, L., and B. L. Horecker, these PROCEEDINGS, **52**, 939 (1964).

[9] Hokin, L. E., and M. R. Hokin, *Federation Proc.*, **22**, 8 (1963).