[3] Mayr, E., *Animal Species and Evolution* (Cambridge, Mass.: Harvard University Press, 1963).
[4] Ehrman, L., these PROCEEDINGS, **49**, 155–157 (1963).
[5] Carmody, G., A. Díaz Collazo, Th. Dobzhansky, L. Ehrman, I. S. Jaffrey, S. Kimball, S. Obrebski, S. Silagi, T. Tidwell, and R. Ullrich, *Am. Midl. Nat.*, **68**, 67–82 (1962).
[6] Dobzhansky, Th., and O. Pavlovsky, *Chromosoma*, **13**, 196–218 (1962).
[7] Malogolowkin, C., unpublished data.
[8] Ehrman, L., *Genetics*, **46**, 1025–1038 (1961).

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*BIOSYNTHESIS OF CELL WALL MUCOPEPTIDE BY A PARTICULATE FRACTION FROM STAPHYLOCOCCUS AUREUS**, †

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Mucopeptides have been defined chemically as those polymers which contain N-acetyl muramic acid, N-acetyl glucosamine, and a characteristic peptide generally composed of D-and L-alanine, D-glutamic acid, either L-lysine or diaminopimelic acid, and in some cases glycine or aspartic acid.[1, 2] These glycopeptides are essential structural components of the cell walls of bacteria and related organisms such as blue-green algae, rickettsia, the psittacosis group of viruses, and the actinomyces (see review[3]). The mucopeptide of *S. aureus* H contains a polysaccharide "backbone" composed of N-acetyl muramic acid and N-acetyl glucosamine. Attached to the polysaccharide are ester-linked acetyl groups and a peptide composed of L-alanine, D-glutamic acid, L-lysine, D-alanine, and about 5 glycine residues. There is evidence that considerable cross-linkage occurs between peptide chains.[4–6] Such extensive cross-linkage between mucopeptide chains would explain the strength these materials give to the wall.

Nucleotide-bound sugars, which are frequently precursors of simple polysaccharides, have recently been shown to be precursors of more complex polysaccharides as well.[7, 8] That synthesis of mucopeptide may be similar to polysaccharide synthesis was thus suggested some years ago by the observation that interference with the growth of staphylococci by penicillin led to accumulation within the cells of 3 uridine nucleotides.[9] The structure of the largest compound, uridine diphospho-N-acetyl-muramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine (UDP-muramyl-peptide) is shown in Figure 1.[9, 10] Since N-acetyl muramic acid and these amino acids are components of the mucopeptide, the 3 uridine compounds have been implicated as precursors of mucopeptide.[11] The composition of the smaller nucleotides suggested that they may be intermediates on a pathway leading to formation of the largest nucleotide-muramyl-peptide. Indeed, Ito and Strominger[12] have demonstrated that a pathway for the formation of UDP-muramyl-peptide from UDP-N-acetyl muramic acid exists in staphylococci. The alternate transfer of muramyl-peptide and N-acetyl glucosamine from their respective uridine nucleotides to an acceptor mucopeptide would thus make the initial polymerization of mucopeptide "backbone" comparable to synthesis of polysaccharide. Subsequent addition of glycine to the polymer and the introduc-

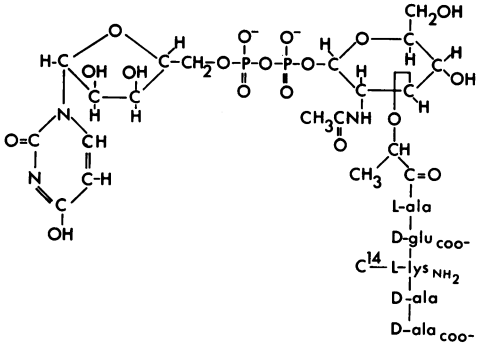

FIG. 1.—Structure of UDP-muramyl-(C^{14}-lysine)-peptide.

tion of cross-links makes the over-all synthesis considerably more complex, however.

The purpose of this paper is to present evidence: (1) that the nucleotide-bound muramyl-peptide is a natural precursor of mucopeptide by demonstrating its utilization in a cell-free system; (2) that the system also incorporates glycine into mucopeptide (this latter incorporation being sensitive to ribonuclease and stimulated by ribosomes and conditions favorable for mucopeptide synthesis); and (3) that penicillin, vancomycin, and bacitracin—antibiotics which interfere with mucopeptide synthesis—interfere with utilization of UDP-muramyl-peptide but only under very special conditions related to growth.

Materials and Methods.—Preparation of radioactive UDP-muramyl-peptide: A washed suspension of log phase cells of *S. aureus* (cell density *ca.* 1.5 mg dry wt/ml) was shaken vigorously for 90 min at 37° in the presence of vancomycin (20 μg/ml) in a medium (pH 6.8) composed of 10^{-3} *M* glycine, 10^{-3} *M* L-glutamic acid, 5×10^{-4} *M* L-alanine, 2×10^{-4} *M* L-lysine, 10^{-3} *M* $MgCl_2$, 10^{-4} *M* $MnCl_2$, 1.7×10^{-4} *M* uracil, 3×10^{-6} *M* thiamine, 8.2×10^{-6} *M* nicotinamide, 2.85×10^{-2} *M* glucose, and 8×10^{-2} *M* K_2HPO_4. The cells were then harvested by centrifugation, washed, and extracted three times with cold 0.3 *N* $HClO_4$. The extract was neutralized with KOH, the insoluble $KClO_4$ removed, and the extract applied to a Dowex-1-formate column (200–400 mesh). A gradient elution with ammonium formate-formic acid[13] was employed, and the fractions containing the labeled UDP-muramyl-peptide were combined and lyophilized. The final product was identified as UDP-muramyl-peptide by paper chromatography and electrophoresis. For preparation of substrate labeled in the amino acid, C^{14}-L-lysine or H^3-DL-glutamate was used. The pyrimidine moiety was labeled by adding uracil-2-C^{14} to the incubation medium. The final specific activities (assuming no dilution from the cellular pool) were 3×10^3 cpm/mμmole for the UDP-muramyl-peptide labeled in lysine or glutamate and 6.6×10^3 cpm/mμmole when the label was in the uracil moiety.

Preparation of crude particulate fraction: *Staphylococcus aureus* strain H was grown at 37° with aeration to a cell density of 0.3–0.4 mg (dry wt) per ml in a medium composed of 5 gm peptone, 5 gm yeast extract, 3 gm K_2HPO_4, and 2 gm glucose, pH 7.2. The glucose was sterilized separately. The cells were disrupted by shaking 7 ml of a 3% suspension of cells in 0.05 *M* tris-HCl buffer, pH 8, with an equal volume of acid-washed Ballotini no. 12 glass beads for 10–15 minutes in a Mickle disintegrator. To obtain the crude particulate fraction, the extract, after removal of unbroken cells and cell walls, was centrifuged at $105{,}000 \times g$ for 45 min. The light yellow translucent pellet obtained from 300 mg of cells was resuspended with the aid of an homogenizer in 2–4 ml of 0.02 *M* tris-HCl buffer, pH 8.0 containing 0.01 *M* $MgCl_2$ and 0.001 *M* mercaptoethanol. This suspension represents the crude particulate fraction used in the initial studies of mucopeptide synthesis. Procedures for fractionation of the synthesizing system are described under *Results.*

Assay of activity: In a typical experiment, radioactive substrates and other needed components in 0.2 ml 0.01 *M* tris-HCl, pH 8, were incubated at 37° with 1 mg of the particulate fraction for 10 min. The reaction was stopped by addition of 4 ml of cold 0.3 *M* $HClO_4$. The insoluble residue was sedimented by centrifugation at $8{,}000 \times g$ for 5 min and washed twice with 4 ml of 0.3 *M* $HClO_4$. The sediment was then transferred with the aid of 1 ml of water to bottles containing 20 ml of a mixture consisting of 400 gm naphthalene, 14 gm 2,5 diphenyloxazole (PPO), 0.6 gm p-bis-2-(5 phenyloxazolyl)-1-benzene (POPOP), and 1,930 ml of dioxane and counted for 20 min in a liquid scintillation counter (Nuclear-Chicago).

TABLE 1

ACTIVITY OF VARIOUS CELL FRACTIONS FOR INCORPORATION OF FREE H^3-LYSINE OR MURAMYL-(C^{14}-LYSINE)-PEPTIDE FROM UDP-MURAMYL-(C^{14}-LYSINE)-PEPTIDE

Fraction	$\mu\mu$moles incorporated*		Total $\mu\mu$moles incorporated by fraction	
	C^{14}	H^3-lysine	C^{14}	H^3-lysine
Intact cells (+H^3-lysine)	0 ± 1	278	0 ± 600	176,000
Disrupted cells	20	...	14,000	
"Unbroken cells" (sedimented by 2500 × g for 10 min)	29	...	667	
"Cell wall" fraction (sedimented by 4300 × g for 45 min)	31	...	2,450	
"Particulate enzyme" (sedimented by 105,000 × g for 45 min) (+H^3-lysine)	258	32	11,860	1,470
Supernatant	5	...	2,000	

* C^{14} incorporated is a measure of incorporation of muramyl-peptide. H^3-lysine is a measure of utilization o free lysine. Results expressed in $\mu\mu$moles per mg dry weight incorporated in 10 min (see text for description of fractions). The incubation mixture contained 100 mμmoles ATP, 10 mμmoles UDPAG, 1.8 mμmoles UDP-muramyl-(C^{14}-lysine)-peptide, 1 μmole $MgCl_2$, 500 mμmoles mercaptoethanol, 10 μg chloramphenicol, 5 μmoles tris-HCl buffer, pH 8.0, and 0.4–2 mg of the fraction under test in a final volume of 0.2 ml. 10 mμmoles of H^3-lysine (1580 cpm/mμmole) were added as indicated. All experiments were incubated for 10 min at 37°.

Results.—Table 1 shows the activity of various cell fractions that incorporate radioactivity from added UDP-muramyl-(C^{14}-lysine)-peptide. As can be seen, the particulate fraction, composed of membrane material and ribosomes, contained the bulk of the activity, whereas intact cells were unable to utilize the nucleotide-bound peptide. Conversely, though intact cells incorporated free lysine readily, the particulate fraction incorporated free H^3-lysine at one eighth the rate of incorporation of C^{14} from UDP-muramyl-(C^{14}-lysine)-peptide. The incorporation of C^{14} is believed to be a measure of muramyl-peptide transferred from UDP-muramyl-(C^{14}-lysine)-peptide. These data suggest that the nucleotide-bound muramyl-peptide is a mucopeptide precursor and that the activity of the particulate fraction is not due to the presence of intact cells. The particulate fraction used in most of this work contained membrane material (30–40%) and ribosomes (60–70%) bathed in a dilute solution of cell sap. The rate of incorporation of C^{14} from UDP-muramyl-(C^{14}-lysine)-peptide into the particulate fraction leveled off rapidly with time as seen in Figure 2. Thus, the activities recorded (based on incorporation in 10 min) are not necessarily a measure of relative initial rates.

The rapid leveling off in activity may be due to the unstable nature of this incorporation system. Incubation of the particulate fraction at 37° resulted in more than a 50% loss of activity in 1 hr. Addition of substrates, Mg, ATP, or -SH compounds, to the particulate fraction was without any stabilizing effect. Nevertheless, the crude enzyme fraction could be stored

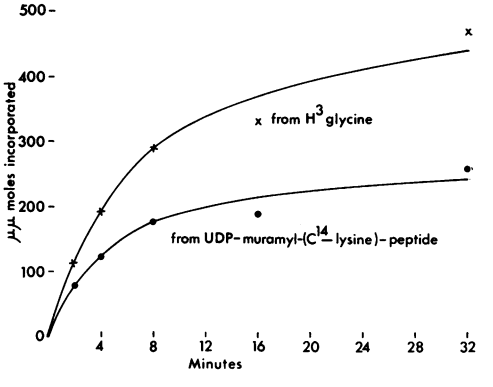

FIG. 2.—Rate of incorporation of H^3-glycine and of muramyl-(C^{14}-lysine)-peptide from UDP-muramyl-(C^{14}-lysine)-peptide by the particulate fraction. The incubation mixture contained: 10 mμmoles H^3-glycine, 1.8 mμmoles UDP-muramyl-(C^{14}-lysine)-peptide, 50 mμmoles ATP, 50 mμmoles UDPAG, 4 μmoles KCl, 2 μmoles $MgCl_2$, 5 μmoles tris-HCl pH 8.0, 200 mμmoles mercaptoethanol, 10 μg chloramphenicol, and 1.2 mg of particulate fraction in a total volume of 175 μl.

TABLE 2

REQUIREMENTS FOR INCORPORATION FROM UDP-MURAMYL-(C^{14}-LYSINE)-PEPTIDE AND H^3-GLYCINE

	Additions and omissions	Amount Incorporated From UDP-muramyl-(C^{14}-lysine)-peptide $\mu\mu$moles/mg	Amount Incorporated From H^3-glycine $\mu\mu$moles/mg
Expt. 1.	Complete	123	218
	−UDPAG	23	70
	−UDP-muramyl-(C^{14}-lysine)-peptide	—	85
	−glycine	138	—
	−ATP	92	102
	−KCl	126	140
	−chloramphenicol	116	223
Expt. 2.	Complete (2.5 μmoles $MgCl_2$)	172	442
	−Mg	80	316
	−Mg + 2.5 μmoles $MnCl_2$	98	340
	−Mg + 0.25 μmole EDTA	22	40
	+ 0.25 μmole EDTA	116	318

The incubation mixture in Expt. 1 contained 50 mμmoles ATP, 1.8 mμmoles UDP-muramyl-(C^{14}-lysine)-peptide, 5 mμmoles H^3-glycine, 5 mμmoles UDPAG, 4 μmoles KCl, 2 μmoles $MgCl_2$, 4 μmoles tris-HCl pH 8.0, and 10 μg chloramphenicol with additions as indicated and 1.5 mg of once-washed "membrane particles" in a volume of 0.2 ml. In Expt. 2, the incubation mixture was the same except that 2.5 μmoles $MgCl_2$ were used. 1.3 mg of the crude particulate fraction was used. Incubation was for 10 min at 37°.

frozen for two weeks or repeatedly thawed and refrozen without appreciable loss of activity. The amount of incorporation of C^{14}-lysine from UDP-muramyl-(C^{14}-lysine)-peptide in 10 min was proportional to the concentration of particulate fraction up to about 20 mg dry wt/ml. The rate of incorporation of label from UDP-muramyl-(C^{14}-lysine)-peptide was maximal in the pH range 8.0–8.2. Therefore, the pH of the incorporation system was maintained at 8.0 unless otherwise stated.

The requirements for maximum incorporation are shown in Table 2. As shown, maximum incorporation was dependent on UDPAG and Mg and to a lesser extent on ATP, but was not dependent on glycine or KCl. The incorporation of glycine (another component of *S. aureus* mucopeptide) was dependent on both UDPAG and UDP-muramyl-(C^{14}-lysine)-peptide. Glycine incorporation showed a more marked stimulation by ATP and was stimulated by KCl and Mg. Manganese did not replace Mg at equivalent concentration for either incorporation. Chloramphenicol was present in the incubation mixture to prevent incorporation of glycine into protein, but omission of chloramphenicol did not greatly increase the amount of incorporation. As will be shown later, the properties of the product are consistent with the view that glycine is being incorporated into mucopeptide rather than into protein. The pH optimum for glycine incorporation was also about 8.0. The system for glycine incorporation was found to be more unstable than that for incorporation of muramyl-peptide. The rate of incorporation of glycine also leveled off rapidly with time as shown in Figure 1. Radioactivity from UDP-muramyl-(H^3-glutamate)-peptide was also found to be incorporated by the enzyme. This incorporation had the same characteristics as that described for UDP-muramyl-(C^{14}-lysine)-peptide, i.e., stimulation by UDPAG and ATP. We also used UDP-muramyl-peptide labeled in the uracil moiety. The results are shown in Table 3: experiment 1 shows that C^{14} from UDP-muramyl-peptide (C^{14}-uracil) was poorly incorporated; experiment 2 shows that the uracil-labeled UDP-muramyl-peptide was in fact UDP-muramyl-peptide, since it stimulated incorporation of glycine as effectively as authentic C^{12}-UDP-muramyl-

TABLE 3

UTILIZATION OF C^{14}-URACIL-LABELED UDP-MURAMYL-PEPTIDE IN THE PARTICULATE SYSTEM

	Additions	$\mu\mu$moles incorporated of C^{14}
Expt. 1.	UDP-muramyl-peptide (C^{14}-uracil) (2mμmoles)	7
	UDP-muramyl-(C^{14}-lysine)-peptide (1.8 mμmoles)	310
		of H^3-glycine
Expt. 2.	5 mμmoles H^3-glycine	104
	UDP-muramyl-peptide (C^{14}-uracil) (2 mμmoles) + 5 mμmoles H^3-glycine	442
	C^{12}-UDP-muramyl-peptide (5mμmoles) + 5 mμmoles H^3-glycine	452

The incubation mixture contained in a total volume of 0.2 ml, 50 mμmoles ATP, 5 mμmoles UDPAG, 200 mμmoles $MgCl_2$, and 5 μM tris-HCl (pH 8.0) plus the additions as shown plus 1.2 mg of the particulate fraction. Incubation was for 20 min at 37°. Results are expressed as $\mu\mu$moles incorporated per mg dry wt per 20 min.

TABLE 4

REQUIREMENTS FOR MAXIMUM INCORPORATION FROM UDP-MURAMYL-(C^{14}-LYSINE)-PEPTIDE AND FROM FREE H^3-GLYCINE

Additions or omissions	Amount Incorporated from UDP-muramyl-(C^{14}-lysine)-peptide $\mu\mu$moles/mg	H^3-glycine $\mu\mu$moles/mg
Complete	142	343
−"membrane particles"	18	81
−ribosomes	96	131
−supernatant	163	123
+10 μg RNAase	144	14

The incubation mixtures contained 50 mμmoles ATP, 5 mμmoles UDPAG, 10 mμmoles H^3-glycine (1920 cpm/mμmole), 1.8 mμmoles UDP-muramyl-(C^{14}-lysine)-peptide, 500 mμmoles $MgCl_2$, 4 μM KCl, 500 mμM mercaptoethanol, 10 μg chloramphenicol, 5 μmoles tris-HCl buffer, pH 8.0, 0.5 mg "membrane particles" (washed three times at 38,000 × g for 20 min), 1.5 mg "ribosomes" (supernatant from "membrane particles" sedimented at 105,000 × g for 90 min and resuspended), and 10 μl of a 10× concentrated solution of dialyzed supernatant in a final volume of 0.25 ml. All experiments were incubated for 10 min at 37°. Results are expressed in $\mu\mu$moles incorporated in 10 min per mg (dry wt) of "membrane particles."

TABLE 5

SPECIFIC REQUIREMENT OF UDP-MURAMYL-PEPTIDE FOR MAXIMUM INCORPORATION OF GLYCINE

Additions and omissions	Amount incorporated $\mu\mu$moles glycine/mg dry wt
Complete	343
−UDP-muramyl-peptide	86
−UDP-muramyl-peptide +UDP-muramic acid	71
−UDP-muramyl-peptide +UDP-muramyl-L-alanine	108
−UDP-muramyl-peptide +UDP-glucose	85

Incubation mixtures and conditions were the same as for Table 4.

peptide. Since label from UDP-muramyl-peptide labeled with glutamate or lysine was incorporated but label from (C^{14}-uracil)-UDP-muramyl-peptide was not, it is likely that the incorporation of label indicates incorporation of the muramyl-peptide fragment as a unit.

The particulate material was further fractionated by differential centrifugation to obtain "membrane particles" and ribosomes. As can be seen from Table 4, the "membrane particles," the ribosomes, and a soluble nondialyzable material in the cell extract were needed for maximum incorporation of glycine. The soluble material was not inactivated by heating at 100° for 10 min. RNAase added to the incubation mixture destroyed its ability to incorporate glycine but did not interfere with the incorporation of C^{14} from UDP-muramyl-(C^{14}-lysine)-peptide.

The specificity of the system for UDP-muramyl-peptide is shown in Table 5. The incorporation of glycine was reduced almost 75 per cent by omitting UDP-muramyl-peptide. UDP-N-acetyl muramic acid and UDP-N-acetyl muramyl-L-alanine (two other nucleotides normally present in *S. aureus* H) did not substitute for UDP-muramyl-peptide. This strongly suggests that UDP-muramyl-peptide is

specifically required for mucopeptide synthesis and that the other nucleotide-bound muramic acid derivatives are merely precursors.

Effect of cell wall fractions as primers for the reaction: Attempts were made to demonstrate a requirement for an exogenous acceptor or a primer in the above system. Addition of *S. aureus* cell walls or fractions of wall made soluble by alkali or autolysis had no effect on the incorporation system. It is likely that mucopeptide present in the particulate enzyme fraction served as acceptor.

Characterization of products: Only a few mμmoles of product have been prepared for study. Consequently, indirect methods were used in order to compare it with mucopeptide. Paper chromatography after acid hydrolysis demonstrated that the C^{14}-lysine and H^3-glycine were incorporated as such and had not undergone conversion to other compounds. The product was extractable with hot acid. In a typical experiment 70 per cent of the radioactive product extracted with hot acid was nondialyzable, and the remainder was readily separable from H^3-glycine on Sephadex G25. The nondialyzable material was treated in various ways as shown in Table 6 and redialyzed to determine the extent of breakdown caused by the various treatments. As can be seen, trypsin, RNAase, and alkali did not break down this product. Since the product was soluble in hot acid and was not made dialyzable by trypsin, it is not protein in nature. Since the radioactivity was not rendered dialyzable by the action of RNAase or alkali, it was not present as activated amino acids on transfer RNA. Resistance to all these treatments is characteristic of mucopeptide. Even though lysozyme attacks mucopeptide, it apparently does not release dialyzable peptides because of cross-linkage between peptides.[6] Hence, the test with lysozyme may indicate considerable cross-linkage.

Effect of antibiotics: Antibiotics known to interfere with mucopeptide synthesis in intact staphylococci, namely, penicillin,[16a, 16b] vancomycin,[14, 15] and bacitracin,[16a, 16b] did not interfere with mucopeptide synthesis by this cell-free system. However, as shown in Table 7, the particulate fraction from staphylococci grown in the presence of any of these antibiotics for 20 min lost much of its ability to utilize UDP-muramyl-(C^{14}-lysine)-peptide, although it still could incorporate glycine.

TABLE 6

THE EFFECT OF VARIOUS TREATMENTS ON THE NONDIALYZABLE SOLUBLE PRODUCT

Conditions during treatment	Amount nondialyzable after treatment: μμmoles lysine	μμmoles glycine
Water	150	1,130
+Trypsin 50 μg/ml	169	1,471
+Ribonuclease 25 μg/ml	156	1,250
+NaOH 0.2 *M*	222	1,985
+Lysozyme 50 μg/ml	156	1,515

The nondialyzable fraction employed was obtained by a large-scale incubation of the complete incorporation system. After washing with cold acid, the labeled material was extracted with 0.3 *M* $HClO_4$ in 10 min at 90° and dialyzed overnight. Separate aliquots were treated as indicated in the table at 37° for 1 hr, redialyzed for 16 hr against 100 volumes of water, and the amount remaining in the nondialyzable fraction was determined. The sample incubated with lysozyme was first treated with 0.1 *N* NaOH to remove O-acetyl groups. (*S. aureus* mucopeptide is susceptible to lysozyme after this treatment.)

TABLE 7

ACTIVITY OF THE PARTICULATE FRACTION FROM ANTIBIOTIC PRETREATED CELLS

Pretreatment with	Amount Incorporated From UDP-muramyl-(C^{14}-lysine)-peptide μμmoles/mg/4 min	H^3-glycine μμmoles/mg/4 min
None	68	74
Penicillin	11	74
Vancomycin	2	60
Bacitracin	4	31
Chloramphenicol	15	31

The antibiotics (penicillin, vancomycin, and chloramphenicol 10 μg/ml; and bacitracin 25 μg/ml) were added to *S. aureus* cells growing in complete medium. The cells were incubated an additional 20 min and then harvested, and the particulate fractions were obtained as described under *Methods*. The incubation mixture for determination of the amount of radioactivity incorporated contained 10 mμmoles H^3-glycine, 1.8 mμmoles UDP-muramyl-(C^{14}-lysine)-peptide, 5 mμmoles ATP, 50 mμmoles UDPAG, 4 μmoles KCl, 2 μmoles $MgCl_2$, 10μg chloramphenicol, 5 μmoles tris-HCl pH 8.0, and particulate fraction in a volume of 0.2 ml. Incubation was at 32° for 4 min.

In a separate study we investigated the relationship between the irreversible binding of penicillin to cells and their inability to synthesize mucopeptide. It was found that treatment with antibiotic must occur while the cells are growing rapidly in order to be effective.[17] Thus, the conditions for inactivation of the cell-free system are comparable to those for intact cells. "Membrane particles" from cells grown in the presence of chloramphenicol or tetracycline also incorporate C^{14} from UDP-muramyl(C^{14}-lysine)-peptide at a reduced rate. This will be considered below.

Discussion.—We believe that the incorporation of amino acids observed in this crude system is a reflection of the synthesis of the backbone polysaccharide of mucopeptide from UDPAG and UDP-muramyl-(C^{14}-lysine)-peptide by transfer of AG and muramyl-peptide to an acceptor; this is followed by addition of a small glycine peptide to the newly formed polymer. The polymerization reaction is mediated by a particulate fraction that is sedimented in 20 min at 39,000 x *g*. This particulate fraction is referred to as "membrane particles" to indicate its principal component. The polymerization reaction requires magnesium and is stimulated by ATP. It is insensitive to ribonuclease. The incorporation of glycine is stimulated by ribosomes, KCl, and a nondialyzable, heat-stable fraction of cell sap, as well as by the components of the complete system for the polymerization reaction. Since the incorporation of glycine is greatly reduced or in fact often prevented by the presence of ribonuclease, and since ribosomes and KCl stimulate incorporation, part of the system resembles the protein-synthesizing system of bacteria. It has been suggested that the glycine of *S. aureus* mucopeptide may be present as a polyglycine of 5 or 6 residues which serves as a cross-linking bridge between adjacent muramyl-peptide units.[6] If a polymer of 5 or 6 residues requires messenger RNA and ribosomes for its formation, the situation would be analogous to protein synthesis. The addition of the polyglycine to the mucopeptide would then be a single reaction requiring newly formed mucopeptide backbone (i.e., mucopeptide lacking glycine) as acceptor. Hence, the role of UDPAG and UDP-muramyl-(C^{14}-lysine)-peptide would be to permit synthesis of this acceptor. The ratio of the amount of glycine to lysine incorporated has been variable, from as little as 1:1 to more than 10:1. This variation may be a reflection of the amount of active glycine-incorporating components present. The dependence of the glycine-incorporating activity upon UDP-muramyl-(C^{14}-lysine)-peptide rather than UDP-muramyl-L-alanine shows a remarkable specificity. It is this dependence that leads us to believe that glycine is being incorporated into mucopeptide in this system. The stability characteristics of the product formed support this view.

The antibiotics—penicillin, vancomycin, and bacitracin—are believed to inhibit mucopeptide synthesis in whole cells by interfering with the polymerization reaction. Nevertheless, the antibiotics had no effect when added directly to the cell-free system. On the other hand, the particulate enzyme obtained from cells which had grown in the presence of antibiotic for 20 min showed specific damage. These particles were inhibited 84–97 per cent in their ability to utilize UDP-muramyl-(C^{14}-lysine)-peptide, whereas they could utilize glycine normally. This is a very peculiar phenomenon. It suggests to us that the polymerization reaction was specifically inactivated and that a small amount of incomplete mucopeptide was available to accept glycine. The fact that inactivation of the system occurred only under growth conditions may be related to the fact that penicillin kills only growing

bacteria,[18] and it may indicate that the sensitive enzyme is only available to react with antibiotics while the cells are actively growing. Inactivation of the ability of *intact cells* to synthesize mucopeptide by pretreatment with penicillin, vancomycin, or bacitracin also occurs only under growth conditions.[17]

The particulate fraction from cells pretreated with chloramphenicol (which specifically inhibits protein synthesis while mucopeptide synthesis seemingly continues in whole cells)[19, 20] can utilize neither glycine nor UDP-muramyl-(C^{14}-lysine)-peptide at a normal rate. This unexpected result leads us to speculate that only newly synthesized polymerizing enzyme participates in mucopeptide synthesis. Cole and Hahn have shown that cell wall synthesis is restricted to a narrow equatorial region in *Streptococcus pyogenes*.[21] If staphylococci are similar, then polymerizing enzyme fixed to the membrane would be physically at the site of synthesis of wall for a limited period of time. Thus, in effect, growth in the presence of chloramphenicol would have depleted the site of polymerizing enzyme because no new enzyme was formed and the site of synthesis moved as mucopeptide synthesis proceeded.

As this report shows, we have demonstrated only stimulatory effects rather than absolute requirements. We have not made extensive attempts to free the "membrane particles" or ribosomes of substrates, primers, or cofactors. It is quite likely that the "membrane particles" fraction is contaminated with ribosomes and vice versa. Work is under way to resolve the components of the system.

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† The following abbreviations are used: UDPAG, uridine diphospho-N-acetyl glucosamine; AG, N-acetyl glucosamine; RNAase, ribonuclease.

[1] Perkins, H. R., and H. J. Rogers, *Biochem. J.*, **72**, 647 (1959).

[2] Park, J. T., in *Immunochemical Approaches to Microbiology* (Rutgers Univ. Press, 1960), p. 123.

[3] Perkins, H. R., *Bact. Rev.*, **27**, 18 (1963).

[4] Rogers, H. J., in *The Structure and Function of the Membranes and Surfaces of Cells*, Biochemical Society Symposia No. 22, ed. D. J. Bell and J. K. Grant (Cambridge University Press, 1963), p. 55.

[5] Salton, M. R. J., *Biochim. Biophys. Acta*, **52**, 329 (1961).

[6] Mandlestam, M. H., and J. L. Strominger, *Biochem. Biophys. Res. Comm.*, **5**, 446 (1961).

[7] Markowitz, A., and A. Dorfman, *J. Biol. Chem.*, **237**, 273 (1962).

[8a] Nikaido, H., these PROCEEDINGS, **48**, 1337, 1542 (1962).

[8b] Osborn, M., S. Rosen, L. Rothfield, and B. Horecker, these PROCEEDINGS, **48**, 1831 (1962).

[9] Park, J. T., *J. Biol. Chem.*, **194**, 877, 885, 897 (1952).

[10] Strominger, J. L., *Physiol. Rev.*, **40**, 55 (1960).

[11] Park, J. T., and J. L. Strominger, *Science*, **129**, 99 (1957).

[12] Ito, E., and J. L. Strominger, *J. Biol. Chem.*, **235**, PC5, PC7 (1960).

[13] Park, J. T., *Fed. Proc.*, **13**, 271 (1954).

[14] Jordan, D. C., *Biochem. Biophys. Res. Comm.*, **6**, 167 (1961).

[15] Reynolds, P. E., *Biochim. Biophys. Acta*, **52**, 403 (1961).

[16a] Park, J. T., *Biochem. J.*, **70**, 2P (1958).

[16b] Mandlestam, J., and H. J. Rogers, *Biochem. J.*, **72**, 654 (1959).

[17] Park, J. T., M. Griffith, and I. Stevenson, unpublished manuscript.

[18] Garrod, L. P., *Lancet*, **247**, 673 (1944).

[19] Mandlestam, J., and H. J. Rogers, *Nature*, **181**, 956 (1958).

[20] Hancock, R., and J. T. Park, *Nature*, **181**, 1050 (1958).

[21] Cole, R. M., and J. J. Hahn, *Science*, **135**, 722 (1962).