ENZYMATIC POLYMERIZATION OF UDP-ACETYLMURAMYL·L-ALA·D-GLU·L-LYS·D-ALA·D-ALA AND UDP-ACETYLGLUCOSAMINE BY A PARTICULATE ENZYME FROM STAPHYLOCOCCUS AUREUS AND ITS INHIBITION BY ANTIBIOTICS

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The uridine nucleotides which accumulate in Staphylococcus aureus when growth is inhibited by penicillin are presumed to be precursors of the glycopeptide portion of the cell wall (Park and Strominger, 1957; Strominger et al., 1959). The sequence of reactions by which the most complex of these nucleotides, UDP-acetylmuramyl·L-ala·D-glu·L-lys·D-ala·D-ala, is synthesized has been described (Ito and Strominger, 1962). This report describes a particulate enzyme system obtained from S. aureus, strain H, which utilizes this nucleotide together with UDP-acetylglucosamine (UDP-GlcNAc) to form a polymer which, like the cell wall glycopeptide, can be hydrolyzed by egg white lysozyme.

Preparation of Enzyme and Substrates: The particulate enzyme was obtained after sonic disintegration of S. aureus, strain H, by centrifugation at 40,000 x g to remove cellular debris, followed by sedimentation of the small particle fraction at 100,000 x g. ^{14}C-L-Lysine and D-Ala·D-Ala were added enzymatically to UDP-acetylmuramyl·L-Ala.D-Glu with enzymes from S. aureus (Ito and Strominger,

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1962). The labeled product, UDP-acetylmuramyl·L-Ala·D-Glu·^{14}C-L-Lys·D-Ala·D-Ala had a specific activity of 60 μC/μmole. ^{32}P-Labeled UDP-acetylmuramyl-Ala-Glu-Lys-Ala-Ala and UDP-GlcNAc were isolated from S. aureus, strain Copenhagen, which had been incubated for 90 min in low phosphate media containing 15 mC of carrier-free $^{32}P_i$ and either penicillin or gentian violet (Strominger, 1959) (initial specific activities, 100 μC/μmole).

Assay of Enzyme: Reaction mixtures, containing 0.25 to 1.0 mμmole of UDP-acetylmuramyl-Ala-Glu-^{14}C-Lys-Ala-Ala, 0 to 1.2 mμmoles of UDP-GlcNAc, 5 μmoles of Tris (pH 7.8), 0.15 μmole of $MgCl_2$ and 20 μl of enzyme in a total volume of 40 μl, were applied to Whatman 3 MM filter paper and subjected to descending chromatography for 16 hours in isobutyric acid-ammonia solvent. The radioactive product remaining at the origin was counted. Other features of the assay are described below.

Properties of the Reaction: Formation of the radioactive product was dependent on the presence of UDP-GlcNAc (Figure 1). Of the other nucleotides tested, only UDP-glucose supported product formation, to the extent of about 10 % of that observed with UDP-GlcNAc (see also Nathenson and Strominger, 1963). Pretreatment of UDP-GlcNAc with venom phosphodiesterase abolished polymer formation. Attempts to demonstrate directly the incorporation of ^{14}C-acetylglucosamine from ^{14}C-UDP-GlcNAc into the polymer by a UDP-acetylmuramyl-Ala-Glu-Lys-Ala-Ala dependent system have been unsuccessful because of the very active UDP-GlcNAc:polyribitol phosphate transferase also present in the particulate enzyme (Nathenson and Strominger, 1963). When UDP-acetylmuramyl-Ala-Glu-^{14}C-Lys-Ala-Ala was hydrolyzed in 0.1 N HCl or with nucleotide pyrophosphatase, or when UDP-acetylmuramyl-Ala-Glu-^{14}C-Lys or ^{14}C-lysine was substituted for it, no product was formed.

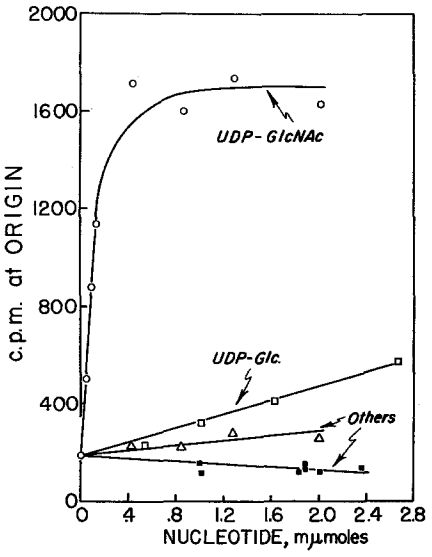

Fig. 1. Requirement for UDP-GlcNAc in formation of radioactive product from UDP-acetylmuramyl-Ala-Glu-^{14}C-Lys-Ala-Ala. The assays were carried out essentially as described in the text, except that various concentrations of nucleotides were employed as shown. The other compounds employed were UDP-GlcNAc pretreated with venom phosphodiesterase (△——△) and acetylglucosamine, UDP-acetylgalactosamine, GDP-glucose or TDP-glucose (■——■).

When ^{32}P-labeled UDP-acetylmuramyl-Ala-Glu-Lys-Ala-Ala or ^{32}P-labeled UDP-GlcNAc was employed as substrate, the observed radioactivity at the origin was equivalent to not more than 5 % of the amount of radioactive product formed under similar conditions with the ^{14}C-substrate, indicating that the UDP moiety of the nucleotides was not included in the polymeric product. Two dimensional paper chromatography of reaction mixtures containing both ^{32}P- and ^{14}C-UDP-acetylmuramyl-Ala-Glu-Lys-Ala-Ala revealed a UDP-GlcNAc dependent increase in ^{32}P-UMP plus $^{32}P_i$ which was approximately equivalent to the amount of ^{14}C-polymer formed. It has not so far been possible to trap UDP as a product of the reaction.

A most unusual feature of the reaction is the fact that it occurred only when the incubation mixture was spread on filter paper. No product was formed during incubation in a test tube. Moreover, the maximum observed reaction occurred when the incubation mixture was partially dried on the filter paper. For optimum activity the reaction mixture was spotted at the origin of a paper chromatogram, allowed to air dry for 10 min and then incubated in a humid atmosphere for 60 min. Under these conditions 50 % of the added sub-

strate was converted to product. Whether free hydroxyl groups of cellulose serve as an artificial acceptor for transglycosylation or whether the paper serves as an inert support for the reaction has not yet been determined. Cell wall, glycopeptide, teichoic acid, chitin, diacetylchitobiose and a variety of degradation products obtained during studies of cell wall structure (Ghuysen and Strominger, 1963) did not serve as acceptors. Cellulose is, however, a β-1,4-glycan, similar to the polysaccharide of the cell wall, and its physical state may be similar to that of the natural acceptor for this reaction. Experiments to clarify the nature of the requirement for filter paper are currently in progress.

Effect of antibiotics: Inhibition of bacterial growth by five antibacterial substances is known to result in accumulation of UDP-acetylmuramyl-Ala-Glu-Lys-Ala-Ala. The concentrations, in μg/ml, of these antibiotics required for 50 % inhibition of the reactions under study were: penicillin, 20,000; bacitracin, 4,000; novobiocin, 750; vancomycin, 200 and ristocetin, 200. The effects of penicillin and bacitracin are almost certainly without physiological significance and the significance of the effect of novobiocin is questionable.* Moreover, particles prepared from cells pretreated with penicillin had normal enzymatic activity. These substances, may, therefore, induce nucleotide accumulation in some other way, e.g. by interfering with synthesis or competency of the acceptor, with access of the substrate to the enzyme or the acceptor, or even with replication of a cell wall synthesizing particle. Cycloserine (which inhibits at an earlier step in cell wall synthesis) and chloramphenicol (which has no effect on cell wall synthesis) also inhibited only at exceedingly high concentrations (20,000 μg/ml or

*This statement is made with the reservation that the system under study is an artificial one in that an artificial acceptor, cellulose, may be involved. The interaction of drugs with these enzymes employing their normal acceptor could conceivably be different.

higher in each case). The concentrations of vancomycin and ristocetin, however, are not far from the concentrations of these substances required for inhibition of bacterial growth (10-20 μg/ml, measured with about 10^7 cells/ml), particularly when the fact is considered that cultures containing 3 x 10^8 cells/ml are employed as a source of enzyme and the particulate enzyme prepared from them represents a 2000-fold concentration from the original culture (0.5 ml of enzyme from 1 liter of culture).*

Nature of the Reaction Product: That glycosidic bonds have actually been formed has been demonstrated by the lysozyme sensitivity of the product. Although the product could be partially extracted from the chromatogram origins by neutral phosphate buffer, addition of egg white lysozyme not only enhanced the extent of extraction but cleaved the extracted polymer to yield a low-molecular weight fragment having a chromatographic mobility in the isobutyric acid-ammonia solvent intermediate between authentic phospho-acetylmuramyl-Ala-Glu-Lys-Ala-Ala and acetylmuramyl-Ala-Glu-Lys-Ala-Ala. When lysozyme treatment of the ^{14}C-polymer was coupled with β-acetylglucosaminidase, the radioactive fragment formed had a mobility identical with that of acetylmuramyl-Ala-Glu-Lys-Ala-Ala. It has been previously shown that lysozyme is able to effect hydrolysis of glycosidic bonds in the glycopeptide portion of S. aureus cell walls and that additional treatment with β-acetylglucosaminidase released acetylglucosamine. With the natural glycopeptide the polymer remained intact, presumably because of polypeptide cross-bridges (Mandelstam and Strominger, 1961).

*These particles also contain a polyribitol phosphate synthetase which utilizes CDP-ribitol as substrate (Ishimoto and Strominger, 1963). Among the antibiotics mentioned, only novobiocin inhibits this synthetase (50 % inhibition at about 750 μg/ml). Since this enzyme is inhibited only by high concentrations of vancomycin or ristocetin (greater than 2500 μg/ml), the inhibition of the uridine nucleotide glycosyltransferases by these substances appears to be selective.

It appears, therefore, that UDP-acetylmuramyl-Ala-Glu-Lys-Ala-Ala and UDP-GlcNAc are substrates for a reaction catalyzed by a particulate enzyme system (presumably containing UDP-acetylmuramyl-Ala-Glu-Lys-Ala-Ala: and UDP-GlcNAc:glycopeptide glycosyltransferases) in which a polymer similar to cell wall glycopeptide is synthesized. Many features of these reactions (including the possible acceptor requirement, their interaction with antibiotics and the precise nature of the product) remain to be investigated.

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