# EVIDENCE FOR AN INITIAL ACCEPTOR OF UDP-NAc-MURAMYL-PENTAPEPTIDE IN THE SYNTHESIS OF BACTERIAL MUCOPEPTIDE[1]

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The enzymatic polymerization of UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala and UDP-NAc-glucosamine in particulate preparations of Staphylococcus aureus H has been demonstrated by Chatterjee and Park (1964) and Meadow et al. (1964). The product formed in this reaction has been characterized as mucopeptide by the latter workers using the sequential action of muramidase and ß-acetylglucosaminidase. As a result of cross-linking by glycine, muramidase did not release dialyzable peptides from the product observed by Chatterjee and Park (1964). Meadow et al. (1964) found that the products of the polymerization are UMP derived from UDP-NAc-muramyl-pentapeptide and UDP derived from UDP-NAc-glucosamine. Park (1964) reported that UDP is the product from both nucleotides.

In our studies on the incorporation of analogs of the UDP-NAc-muramyl-pentapeptide (Neuhaus and Struve, 1964 a,b) into mucopeptide, we repeated the assays described by Chatterjee and Park (1964) and Meadow et al. (1964). Although we have confirmed these observations, certain differences between the systems made it necessary to study these reactions further. These studies

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indicate the participation of an acceptor for UDP-NAc-muramyl-pentapeptide in the biosynthesis of cell-wall mucopeptide.

## EXPERIMENTAL

UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-l-$^{14}$C-D-ala-l-$^{14}$C was prepared by the reaction catalyzed by UDP-NAc-muramyl-L-ala-D-glu-L-lys: D-ala-D-ala ligase (ADP) (Neuhaus and Struve, 1964b). D-ala-l-$^{14}$C-D-ala-l-$^{14}$C was synthesized with D-alanine: D-alanine ligase (ADP) (Neuhaus, 1962). The Tris-$MgCl_2$ preparations of S. aureus Copenhagen and S. aureus H were prepared according to the procedure described by Chatterjee and Park (1964). The cells were disrupted with glass beads in the Mickle disintegrator. After removal of cell walls and unbroken cells, the extract was centrifuged at 105,000 x g for 45 minutes. The precipitate was washed three times with a buffer that contained 0.02 M Tris-HCl, pH 7.8, 0.01 M $MgCl_2$, and 0.001 M mercaptoethanol. A second particulate fraction (Tris-KCl) was prepared in a similar manner with 0.005 M Tris-HCl, pH 7.8, containing 1 M KCl. The washing was performed five times. The "Tube Assay" contained: 0.05 M Tris-HCl, pH 7.8; 0.01 M $MgCl_2$; 3.1 x $10^{-5}$M UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-l-$^{14}$C-D-ala-l-$^{14}$C (6.3 x $10^3$ cpm/mμmole); other additions as indicated; and particulate enzyme in a total volume of 0.1 ml. The tubes were incubated for 15 minutes at 25°. The precipitation, washing of precipitate, and assay of radioactivity was carried out as described by Chatterjee and Park (1964). The "Paper Assay" was performed by streaking the above reaction mixture on Whatman 3 MM paper. The paper was partially dried for 1 minute at 37° and then incubated in a water saturated atmosphere for 1 hr at 37° (Meadow et al., 1964, and Anderson[3]). The UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-l-$^{14}$C-D-ala-l-$^{14}$C was separated from the origin by descending chromatography with isobutyric acid-concd $NH_4OH$-$H_2O$ (66:1:33). The origins containing the mucopeptide were assayed for radioactivity in the Packard Tri-Carb Liquid Scintillation Spectrometer.

## RESULTS AND DISCUSSION

In Table 1 the "Tube[4] and Paper Assay" for preparations from S. aureus Copenhagen and S. aureus H are compared. All preparations showed a dependence on UDP-NAc-glucosamine in the "Paper Assay." With the Tris-$MgCl_2$ preparation from S. aureus H the "Tube Assay" was stimulated two-fold by UDP-NAc-glucosamine. In comparison Meadow et al. (1964) observed an eight-fold stimulation in the "Paper Assay" with UDP-NAc-glucosamine while Chatterjee and Park (1964) observed a five-fold stimulation in the "Tube Assay". In an attempt to define

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[3] J. S. Anderson, personal communication.

[4] Our "Tube Assay" represents the perchloric acid precipitable radioactivity and may be either mucopeptide or covalently linked intermediates or a combination of both.

Table I

Effect of UDP-NAc-glucosamine on incorporation of UDP-NAc-muramyl-pentapeptide-$^{14}C$

| S. aureus | Copenhagen | | H | | Assay |
|---|---|---|---|---|---|
| Preparation; Tris- | $MgCl_2$ | KCl | $MgCl_2$ | KCl | |
| -UDPAG | 57 | 153 | 41 | 46 | Tube |
| +UDPAG | 67 | 120 | 92 | 61 | |
| -UDPAG | 0 | 3.1 | 0.6 | 0.7 | Paper |
| +UDPAG | 26 | 32 | 61 | 13 | |

The velocity is given as µmoles/mg per 15 minutes in the "Tube Assay" and µmoles/mg per hour in the "Paper Assay". The "Tube Assay" contained $1 \times 10^{-4}$M UDP-NAc-glucosamine while the "Paper Assay" contained 10 mµmoles of UDP-NAc-glucosamine. A zero time control was used to correct all values. The amount of particle preparation is expressed as mg dry weight.

the system for kinetic and specificity studies, a preliminary fractionation was attempted. The particulate preparation from S. aureus Copenhagen which had been washed five times with 1 M KCl showed no stimulation with UDP-NAc-glucosamine in the "Tube Assay". This preparation was chosen for further study.

A rapid decrease with time in the rate of labeling was observed with the Tris-KCl preparation (Fig 1). It is suggested that an inhibitor was being formed during the reaction. Specificity studies with various nucleotides demonstrated that UMP was the most effective inhibitor. If UMP was added at the beginning of the incubation, at 15 minutes only 11% of the incorporation was observed.

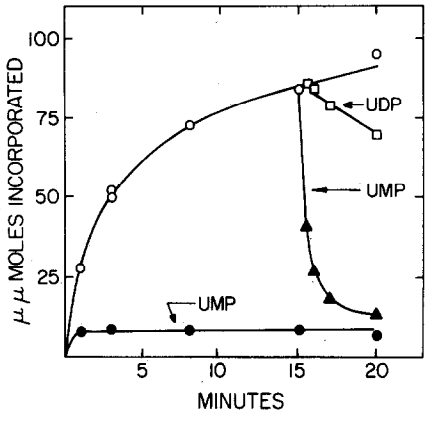


Fig. 1 Effect of UMP on Incorporation.

The "Tube Assay" was used with 0.53 mg of Tris-KCl preparation from S. aureus Copenhagen. The additions are: O-O, none; ●-●, $2.5 \times 10^{-5}$M UMP at zero time; ▲-▲, $2.5 \times 10^{-5}$M UMP at 15 minutes; □-□, $2.5 \times 10^{-5}$M UDP at 15 minutes.

On the other hand, if UMP was added at 15 minutes, a rapid delabeling of the particles resulted. The small effect with UDP may be the result of a contamination of the UDP with UMP.

Meadow et al. (1964) and Anderson and Meadow (1964) reported that UMP was derived from the UDP-NAc-muramyl-pentapeptide in the synthesis of mucopeptide. In addition, Anderson and Strominger[5] observed that a small fraction of the incubation mixture became labeled when $^{32}$P-labeled UDP-NAc-muramyl-pentapeptide was the substrate. These results together with those presented in this communication suggest the following reaction:

$$\text{UDP-NAc-muramyl-pentapeptide} + \text{Acceptor} \rightleftharpoons \text{Acceptor-O-}\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}\text{-O-NAc-muramyl-pentapeptide} + \text{UMP}.$$

The addition of UMP appears to shift the equilibrium concentrations with the formation of acceptor and UDP-NAc-muramyl-pentapeptide.

This reaction predicts that UMP-$^3$H will exchange with the UMP moiety of UDP-NAc-muramyl-pentapeptide. The results shown in Table II demonstrate the exchange of UMP-$^3$H with this nucleotide. The exchange is specific for the complete nucleotide.

Meadow et al. (1964) observed in the "Paper Assay" that ristocetin and vancomycin were the most effective inhibitors of mucopeptide synthesis.

[5] J. S. Anderson and J. L. Strominger, personal communication.

Chatterjee and Park (1964) found no inhibition of mucopeptide synthesis in the "Tube Assay" by vancomycin. However, their particulate preparation

Table II

Exchange of UMP-$^3$H with UDP-NAc-muramyl-pentapeptide

| Addition | Exchange |
|---|---|
| | % |
| Complete | 58 |
| boiled enzyme | 0 |
| -UDP-NAc-muramyl-pentapeptide | 1 |
| -UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-D-ala +UDP-NAc-muramyl-L-ala-D-glu-L-lys | 2[a] |

The "Exchange Assay" contained: $2.5 \times 10^{-5}$M UMP-$^3$H ($5.6 \times 10^3$cpm/mμmole); $3.1 \times 10^{-5}$M UDP-NAc-muramyl-L-ala-D-glu-L-lys-D-ala-$^{14}$C-D-ala-$^{14}$C; 1.8 mg of Tris-KCl particles; 0.05M Tris-HCl, pH 7.8; 0.01M $MgCl_2$ in a total volume of 0.10 ml. The enzyme preparation was added to the assay mixture and incubated for 15 minutes at 25°. The reaction was terminated by placing the tube in a boiling water bath for 3 minutes. The UDP-NAc-muramyl-pentapeptide was separated from UMP-$^3$H by descending chromatography on Whatman 3 MM with isobutyric acid-concd $NH_4OH$-$H_2O$ (66:1:33) for 16 hours. The nucleotide containing the pentapeptide was eluted and assayed for $^{14}$C and $^3$H. The amount of nucleotide was calculated from the $^{14}$C. The $^3$H-labeled UDP-NAc-muramyl-pentapeptide-$^{14}$C was identified by chromatography in two solvent systems. Mild acid hydrolysis (0.1 N HCl, 3 minutes, 100°) of the nucleotide gave UDP-$^3$H (Neuhaus and Struve, 1964b).

[a]UDP-NAc-muramyl-tripeptide was assayed for $^3$H.

from S. aureus H which had been grown in the presence of vancomycin showed an inhibition of mucopeptide synthesis. We tested these antibiotics in the three assay systems (Table III). The results with the "Paper Assay" confirm the inhibition of mucopeptide synthesis observed by Meadow et al. (1964). In addition, the antibiotics have definite effects on the "Tube and Exchange Assay." Both ristocetin and vancomycin at a concentration of 50 μg/ml enhance the perchloric acid precipitable radioactivity. At higher

Table III

Inhibition by Ristocetin and Vancomycin

| Addition | Tube | | Exchange | Paper |
|---|---|---|---|---|
| | µµmoles/15 minutes | | % | µµmoles/hr |
| | I | II | | |
| Control | 63 | 63 | 58 | 67 |
| Ristocetin[a] | 130 | 118 | 42 | 42 |
| Vancomycin[b] | 106 | 21 | 4 | 25 |

The "Tube, Exchange, and Paper Assay" contained 1.0, 1.8, and 1.1 mg of the Tris-KCl preparation from S. aureus Copenhagen respectively. The concentrations of ristocetin and vancomycin are: "Tube Assay" I, 50 µg/ml and II, 500 µg/ml; "Exchange Assay", 200 µg/ml; and the "Paper Assay", 1 µg. The "Paper Assay" contained 10 mµmoles of UDP-NAc-glucosamine.

a. Gift of Dr. J. R. Schenck, Abbott Laboratories.
b. Gift of Dr. O. K. Behrens, Eli Lilly Co.

concentrations (500 µg/ml) ristocetin enhances while vancomycin inhibits the incorporation from UDP-NAc-muramyl-pentapeptide-$^{14}$C. In the "Exchange Assay" both antibiotics are inhibitors. Work is in progress to define further the effects by these antibiotics in the three systems.

The synthesis of mucopeptide may be visualized as occurring in two stages. The first would involve the formation of acceptor-phosphomuramyl-pentapeptide while the second would involve a polymerization with UDP-NAc-glucosamine with the elimination of $P_i$ and UDP. Ristocetin and vancomycin affect either the first or both of these stages in the in vitro systems.

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