On the Initial Stage in Peptidoglycan Synthesis. Phospho-*N*-acetylmuramyl-pentapeptide Translocase (Uridine Monophosphate)*

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ABSTRACT: Enzyme preparations from *Staphylococcus aureus* Copenhagen catalyze the transfer of phospho-*N*-acetyl-muramyl-pentapeptide from uridine diphospho-*N*-acetylmuramyl-pentapeptide to an acceptor with the stoichiometry

UDP-NAc-muramyl-pentapeptide + acceptor $\overset{Mg^{2+}}{\rightleftharpoons}$ acceptor–phospho-NAc-muramyl-pentapeptide + UMP

Since this enzyme is involved in the transfer of phospho-NAc-muramyl-pentapeptide from the uridine monophosphate (UMP) moiety to the membrane acceptor for subsequent peptidoglycan biosynthesis, the name phospho-NAc-muramyl-pentapeptide translocase (UMP) is proposed. In addition, the enzyme catalyzes the exchange of [^{3}H]UMP with the UMP moiety of the UDP-NAc-muramyl-pentapeptide. Enzyme preparations which have been labeled with phospho-NAc-muramyl-[^{14}C]pentapeptide are delabeled by 5′-UMP. Incubation of "acceptor–phospho-NAc-muramyl-pentapeptide" with enzyme, UMP, and Mg^{2+} results in the formation of UDP-NAc-muramyl-pentapeptide. The Michaelis constants for UDP-NAc-muramyl-pentapeptide and UMP are 1.8×10^{-6} M and 2.7×10^{-5} M, respectively. The equilibrium constant for this reaction is 0.25. The addition of cell walls from *S. aureus* Copenhagen results in a marked enhancement of the transfer reaction when UMP is added to the reaction mixture. Evidence is presented for the occurrence of a 5′-nucleotidase associated with the walls which may provide the driving force for the above reaction. The antibiotics, ristocetin and vancomycin, enhance the transfer reaction and inhibit the exchange reaction.

The biosynthesis of peptidoglycan (mucopeptide) in cell-free extracts of *Staphylococcus aureus* from uridine diphospho-*N*-acetylmuramyl-pentapeptide and UDP-NAc-glucosamine[1] has been described by Chatterjee and Park (1964) and Meadow *et al.* (1964). A study of these systems (Struve and Neuhaus, 1965a) provided evidence for the participation of an acceptor for phospho-NAc-muramyl-pentapeptide in the synthesis of peptidoglycan according to the following reaction

UDP-NAc-muramyl-pentapeptide + acceptor $\rightleftharpoons$ UMP + acceptor–phospho-NAc-muramyl-pentapeptide (1)

where acceptor is associated with the enzyme. The evidence for this reaction is derived from three observations. (1) There is a transfer of phospho-NAc-muramyl-pentapeptide from UDP-NAc-muramyl-pentapeptide, with a stoichiometric release of uridine monophosphate, to the enzyme preparation which is independent of UDP-NAc-glucosamine. (2) The enzyme preparation which was labeled by phospho-NAc-muramyl-[^{14}C]pentapeptide is readily delabeled by UMP. Furthermore, the addition of UMP from zero time results in a pronounced inhibition of the transfer reaction. (3) The incubation of [^{3}H]UMP with enzyme and UDP-NAc-muramyl-pentapeptide results in the incorporation of [^{3}H]UMP into the UMP moiety of UDP-NAc-muramyl-pentapeptide.

Meadow *et al.* (1964) found that the products of peptidoglycan synthesis are UMP derived from UDP-NAc-muramyl-pentapeptide and UDP derived from UDP-NAc-glucosamine. Independently, Anderson *et al.* (1965) have recently presented evidence for the transfer of phospho-NAc-muramyl-pentapeptide to a lipid acceptor associated with the membrane. The lipid–phospho-NAc-muramyl-pentapeptide intermediate which has been extensively purified (Strominger *et al.*, 1965) is an acceptor for NAc-glucosamine from UDP-NAc-glucosamine. This lipid–phosphodisaccharide intermediate is subsequently utilized for the forma-

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[1] Abbreviations used: NAc = *N*-acetyl; UMP = uridine monophosphate; UDP = uridine diphosphate; UTP, uridine triphosphate.

tion of peptidoglycan (Anderson *et al.*, 1965). In addition, glycine from glycyl-s-RNA (soluble ribonucleic acid) is transferred to the lipid intermediates (Park, 1964, 1965; Strominger *et al.*, 1965).

It is the purpose of this communication to define the transfer, reverse, and exchange reactions involving UDP-NAc-muramyl-pentapeptide. Since this enzyme is involved in the transfer of phospho-NAc-muramyl-pentapeptide from the UMP moiety to the membrane acceptor, we propose the name phospho-NAc-muramyl-pentapeptide translocase (UMP). The addition of cell walls prepared from *S. aureus* enhances this reaction. Evidence is presented for the occurrence of a 5′-nucleotidase associated with the walls which may provide the driving force for the translocase.

Experimental Section

Materials. [^{3}H]UMP, [^{3}H]CMP, and [^{3}H]AMP were purchased from Schwarz Bio-Research, Inc. D-[^{14}C]-Alanine was the product of the California Corp. for Biochemical Research. The plastic beads (styrene–divinylbenzene copolymer, 20–50 mesh, 8% cross-linked) were a gift of Dow Chemical Co. Sephadex G-25 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Inc. Antifoam 66 and Triton X-100 are the products of General Electric (Silicone Products) and Rohm and Haas, respectively. UDP-NAc-glucosamine, UDP-glucose, and the 5′-mononucleotides are the products of Sigma Chemical Co. Purified alkaline phosphatase was purchased from Pentex Inc.

Antibiotics were kindly given by the following: vancomycin and D-cycloserine, Eli Lilly Co.; ristocetin (Spontin) and erythromycin A base, Abbott Laboratories; neomycin sulfate, The Squibb Institute for Medical Research; kanamycin sulfate, Bristol Laboratories; streptothricin sulfate, The Upjohn Co.; polymyxin B sulfate, Chas Pfizer and Co.; chloramphenicol, Parke Davis and Co.; novobiocin sodium, Merck Sharp and Dohme Research Laboratories. The following were purchased: D-penicillamine–HCl·H_2O and streptomycin sulfate, California Corp. for Biochemical Research; bacitracin, Nutritional Biochemical Corp.; gramicidin, K and K Laboratories; penicillin-G, Sigma Chemical Co.

UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys and UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]-Ala were prepared as previously described (Neuhaus and Struve, 1965). Nucleotide pyrophosphatase (specific activity = 21 nmoles of UDP–glucose cleaved/min/mg) (assay according to Schliselfeld *et al.*, 1965) was prepared from bovine seminal plasma (kindly provided by Dr. E. Goldberg) by the "simplified preparation for bull seminal 5′-nucleotidase" (Heppel and Hilmoe, 1951). A unit of pyrophosphatase is that amount of enzyme which will catalyze the formation of 1 nmole of glucose 1-phosphate/min from UDP–glucose. Brownlee and Wheat (1960) found that nucleotide pyrophosphatase is a component of this preparation. The pyrophosphatase was further purified by chromatography on a DEAE-Sephadex column (1.1 × 22 cm) which was prepared according to the instructions from the manufacturer. The column was washed with 0.01 M Tris–HCl, pH 7.8, containing 0.2 M KCl. Two pyrophosphatase fractions were eluted with this buffer (fraction A eluted at 12 ml, specific activity = 23 nmoles/min/mg; fraction B eluted at 29 ml, specific activity = 20 nmoles/min/mg). Bovine serum albumin was added to these fractions to a final concentration of 5 mg/ml.

Enzyme Preparation. S. aureus Copenhagen (kindly provided by Dr. J. L. Strominger) was grown at 37° for 8 hr with aeration in a medium containing 0.5% glucose, 0.5% K_2HPO_4, 1% yeast extract, 1% peptone, and 0.01% antifoam. The medium was inoculated with 1 l. of an overnight culture. The yield of bacteria from 15 l. of medium was 54 g. (wet wt). The cells were washed in 0.02 M Tris–HCl, pH 7.8, and resuspended in 306 ml of 0.005 M Tris–HCl, pH 7.8, containing 1 M KCl. The bacteria were disrupted in a Bronwill mechanical cell homogenizer (Braun, Model MSK) at 4000 cycles/min for 5 min with cooling by CO_2. Disruption was carried out in a 4-oz narrow-mouth Teflon bottle on aliquots (40 ml) to which had been added 35 g of plastic beads and 2 drops of antifoam. The beads were removed by filtration through 110-mesh nylon cloth.

After removal of cell walls and unbroken cells (3200*g* for 45 min), the particulate enzyme fraction was sedimented by centrifugation at 105,000*g* for 45 min. The precipitate was suspended in 0.005 M Tris–HCl, pH 7.8, containing 1 M KCl and purified by centrifugation between 3200*g* for 45 min and 105,000*g* for 45 min. This operation was repeated four times. The final precipitate (600 mg dry wt) was resuspended in 4.7 ml of 0.02 M Tris–HCl, pH 7.8. The enzyme preparation was stored at −196°.

Cell Wall Isolation. Unbroken cells were removed from the initial precipitate (3200*g*, 45 min) by centrifugation at 3000*g* for 10 min. The cell walls were sedimented at 9800*g* for 20 min. This precipitate was suspended in 0.005 M Tris–HCl, pH 7.8, containing 1 M KCl and purified by centrifugation between 3000*g* for 10 min and 9800*g* for 20 min. This operation was repeated two additional times. The final precipitate (1.6 g dry wt) was suspended in 18.8 ml of 0.02 M Tris–HCl, pH 7.8. An aliquot (376 mg dry wt) was further purified by extraction with 4×10^{-3} M Triton X-100 for 30 min at 0–2°. After repeating the extraction, the walls were washed three times in 0.005 M Tris–HCl, pH 7.8, containing 1 M KCl and two times in 0.02 M Tris–HCl, pH 7.8. The precipitate was suspended in 4.3 ml of 0.02 M Tris–HCl, pH 7.8 (160 mg dry wt).

Assay of the Phospho-NAc-muramyl-pentapeptide Translocase (UMP). Two assays were used to measure the activity of the translocase. The tube assay involves the transfer of phospho-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala from UDP-NAc-muramyl-[^{14}C]pentapeptide to the acceptor associated with the enzyme. The exchange assay involves the exchange of [^{3}H]UMP with the UMP moiety of the UDP-NAc-muramyl-pentapeptide. The latter assay has been used in those cases where the initial velocity is required.

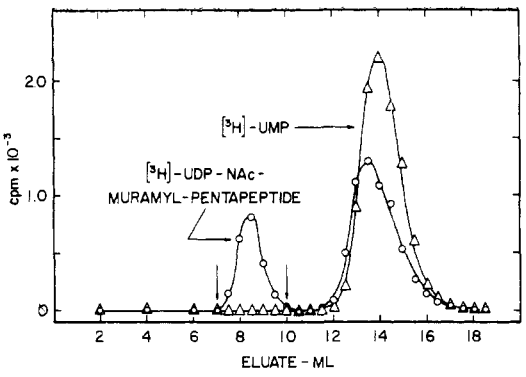

FIGURE 1: Elution profile in the exchange assay. The exchange assay was used with 0.64 mg of enzyme preparation. The complete system (O–O) was incubated for 4 min at 25° while the control (△-△) was incubated for 4 min without enzyme. The incubation was terminated and applied to the G-25 Sephadex column as described in the text. Fractions (0.5 ml) were collected and assayed for radioactivity. The fraction of the eluate between the arrows was routinely collected and assayed for radioactivity in the exchange assay.

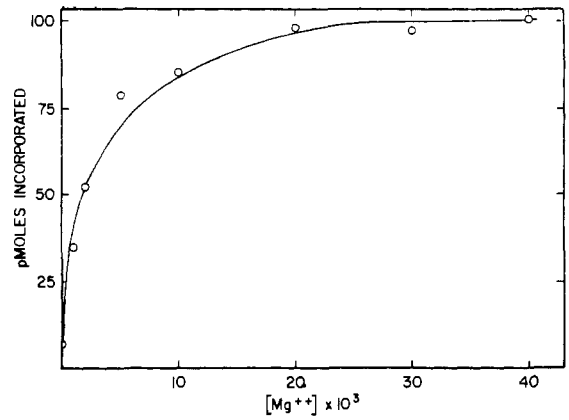

FIGURE 2: Effect of Mg^{2+} on the transfer reaction. The tube assay was used with 1.4 mg of the enzyme preparation.

In the exchange assay the standard mixture contained 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; 7.2 × 10^{-5} M UDP-NAc-muramyl-pentapeptide; 4.5 × 10^{-6} M [^{3}H]UMP (4.5 × 10^4 cpm/nmole); and enzyme preparation in a total volume of 0.10 ml. The enzyme preparation was added to the assay mixture at 25° and incubated for 10 min at this temperature. The reaction was terminated by the addition of 0.005 ml of 12 M $HClO_4$. The mixture was centrifuged at 34,800*g* for 10 min, and the supernatant fraction was quantitatively applied to a Sephadex G-25 Fine column (0.28 cm^2 × 62.5 cm). The column was developed with 0.02 M potassium phosphate buffer, pH 7.0, at a flow rate of 0.16 ml/min. The initial effluent (7 ml) was discarded. The second effluent (3 ml) which contained the [^{3}H]-UDP-NAc-muramyl-pentapeptide was collected in a polyethylene vial for the determination of radioactivity. The third effluent (20 ml) contained the [^{3}H]UMP. The separation of [^{3}H]UDP-NAc-muramyl-pentapeptide and [^{3}H]UMP is illustrated in Figure 1.

The tube assay contained 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; 1.5 × 10^{-5} M UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala (6.3 or 8.4 × 10^3 cpm/nmole); and enzyme preparation in a total volume of 0.1 ml. The enzyme preparation was added to the assay mixture at 25° and incubated for 15 min at this temperature. The reaction was terminated with 2.0 ml of 0.3 N $HClO_4$ and the precipitate was sedimented at 7700*g* for 5 min. The precipitate was washed two times with aliquots (2 ml) of 0.3 N $HClO_4$ and assayed for radioactivity. The tube assay is essentially that described by Chatterjee and Park (1964).

Nucleotidase Assay. The incubation mixture contained 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; [^{3}H]-UMP (6.1 × 10^4 cpm/nmole); and wall, enzyme preparation, or supernatant fraction in a total volume of 0.1 ml. The mixture was incubated for 10 min at 25°. The reaction was terminated by the addition of 0.5 ml of 0.2 M acetic acid, and the mixture was quantitatively applied to a Dowex 1 (acetate) X-8 (200–400 mesh) column (5 × 20 mm). Two aliquots (0.5 ml) of 0.2 M acetic acid which were used to wash the reaction tube were applied to the column. The effluents containing

TABLE I: Stoichiometry of Product Formation in the Forward Reaction.[a]

Time (min)	Ac-PMp (pmoles)	UMP (pmoles)	Uridine (pmoles)	UMP + Uridine (pmoles)
5.5	19	16	2	18
15.0	25	18	9	27

[a] The complete incubation contained 511 pmoles of [^{3}H]UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-[^{14}C]-Ala-D-[^{14}C]Ala (^{3}H = 2.7 × 10^4 cpm/nmole and ^{14}C = 9.0 × 10^3 cpm/nmole); 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; and 0.6 mg of enzyme preparation in a total volume of 0.1 ml. The reaction was terminated with 0.6 ml of 0.3 N $HClO_4$. The precipitate was washed successively with 0.5 ml and 0.4 ml of 0.3 N $HClO_4$. Uridine, uracil, UMP, UDP, and UDP-NAc-muramyl-pentapeptide were added as carriers to the washings. The supernatant fractions were applied to a Sephadex G-25 column (2 cm^2 × 115 cm) (fine grade) and developed with 0.02 M NH_4HCO_3 at a flow rate of 1.16 ml/min. The following elution volumes (ml) were observed: bovine serum albumin, 102; UDP-NAc-muramyl-pentapeptide, 123; UDP, 170; UMP, 182; uridine, 229; uracil, 249. No radioactivity was observed in the uracil or UDP fractions.

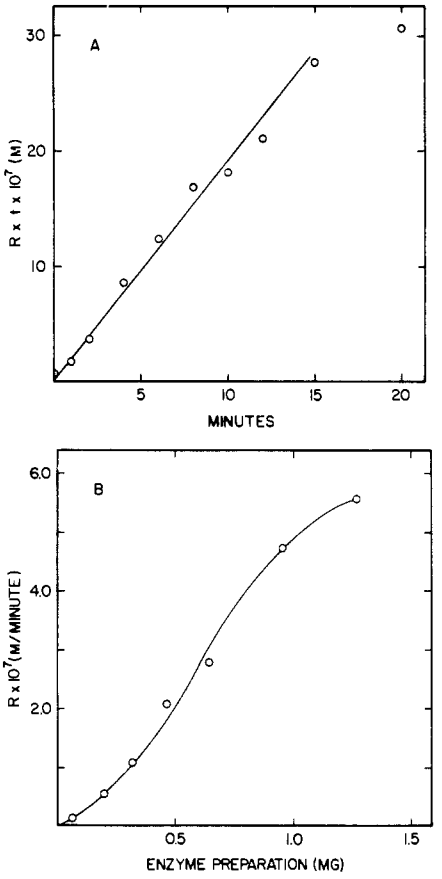

FIGURE 3: The amount of [^{3}H]UMP exchanged as a function of time (A) and the effect of enzyme concentration on the rate of exchange (B). In A, the exchange assay was used with 0.3 mg of enzyme preparation.

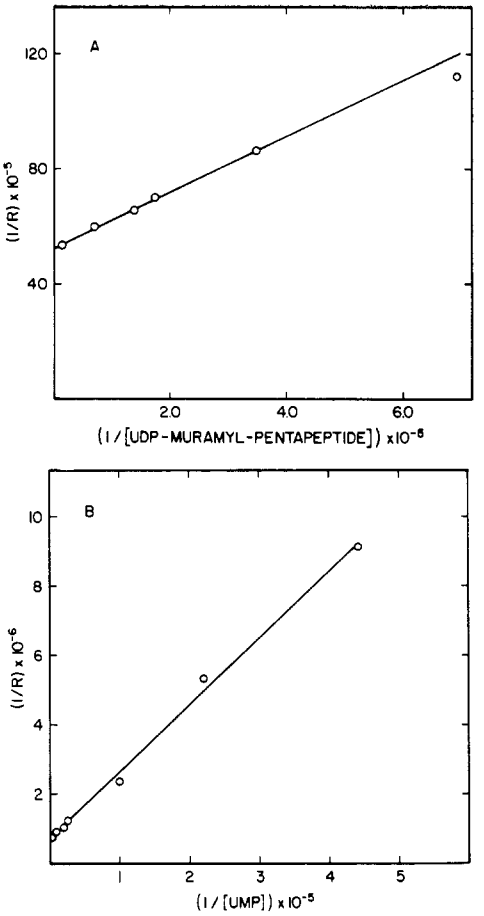

FIGURE 4: Effect of UDP-NAc-muramyl-pentapeptide (A) and UMP (B) on the rate of exchange. The exchange assay was used with 0.3 mg of enzyme preparation. In A, the rate (R) was measured with varying concentrations of UDP-NAc-muramyl-pentapeptide and in B with varying concentrations of UMP. The concentrations of UMP in A and UDP-NAc-muramyl-pentapeptide in B are 4.5×10^{-6} M and 7.2×10^{-5} M, respectively.

the [^{3}H]uridine were collected in a polyethylene scintillation vial for the determination of radioactivity.

Chromatography and Analytical Procedures. The nucleotides and sugar intermediates were chromatographed in the following descending solvent systems: A, isobutyric acid–concentrated NH_4OH–H_2O (66:1:33) (Pabst, 1961); B, ethanol–1 M ammonium acetate, pH 7.5 (7.5:3) (Ito and Strominger, 1962); C, ethanol–1 M ammonium acetate, pH 3.8 (7:3) (Pabst, 1961); D, isopropyl alcohol–concentrated NH_4OH–H_2O (7:1:2) (Khorana and Vizsolyi, 1959); E, 1-butanol–acetic acid–H_2O (2:1:1) (Ito and Strominger, 1962). The other analytical procedures are identical with those previously described (Neuhaus and Struve, 1965). Measurements of radioactivity were made in polyethylene vials using the Packard Tri-Carb liquid scintillation spectrometer (Model 314-EX). For the tube assay, exchange assay, and nucleotidase assay the scintillation fluid (15 ml) was that described by Bray (1960). For the analysis of radioactivity on chromatograms, the scintillation fluid was toluene containing 0.3% 2,5-diphenyloxazole.

Results

Forward Reaction. When UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala was incubated with the enzyme preparation, transfer of the phospho-NAc-muramyl-[^{14}C]pentapeptide to the particulate enzyme resulted (Struve and Neuhaus, 1965a). A rapid decrease with time in the rate of labeling was observed. The addition of 5′-UMP at 15 min resulted in a rapid release of radioactivity from the labeled enzyme preparation. The following nucleotides (2.5×10^{-5} M) were not effective in the delabeling of this preparation: UTP, UDP, 2′(3′)-UMP, 5′-AMP, 5′-CMP, and 5′-GMP. In addition, UDP-glucose, UDP-NAc-glucosamine, and uridine had no effect on the delabeling of these particles in the time interval tested (1 min). If 5′-UMP were added at zero time, only 11% of the

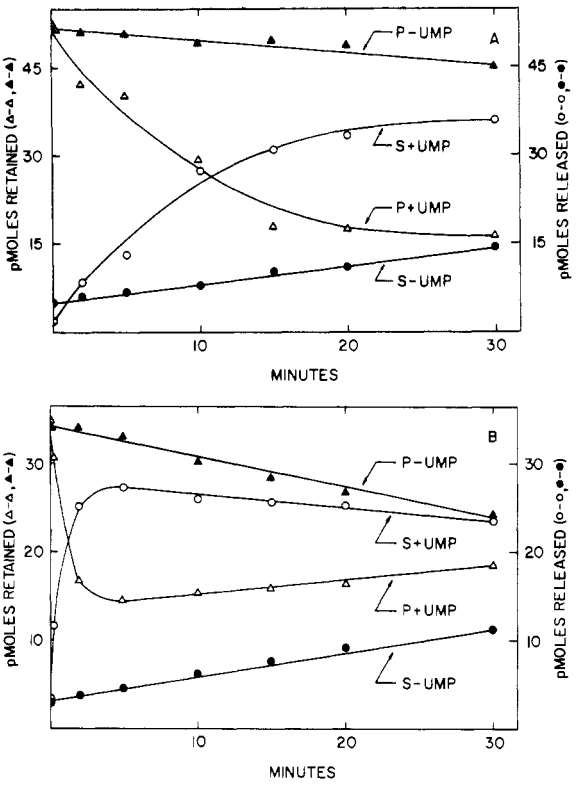

FIGURE 5: Release of radioactivity from acceptor-phospho-NAc-muramyl-[^{14}C]pentapeptide in the absence (A) and in the presence of enzyme preparation (B). In A, the complete reaction mixture contained 50 pmoles of acceptor-phospho-NAc-muramyl-[^{14}C]pentapeptide (8.43×10^3 cpm/nmole), partially purified by precipitation with $HClO_4$ (see Table II); 1.0×10^{-3} M UMP; 0.05 M Tris-HCl, pH 7.8; and 0.01 M $MgCl_2$ in a total volume of 0.1 ml. The mixture was incubated at 25° for the indicated time interval. The reaction was terminated by the addition of 1.5 ml of 0.3 N $HClO_4$. The precipitate was washed two times and assayed for the amount of acceptor-phospho-NAc-muramyl-[^{14}C]-pentapeptide as described for the tube assay. The supernatant fraction was neutralized with KOH and the potassium perchlorate was removed by centrifugation. The supernatant fluid was assayed for the released radioactivity. For B, the reaction mixture contained in addition to the above components 1.4 mg of enzyme preparation. The abbreviations are P, precipitate; S, supernatant fraction.

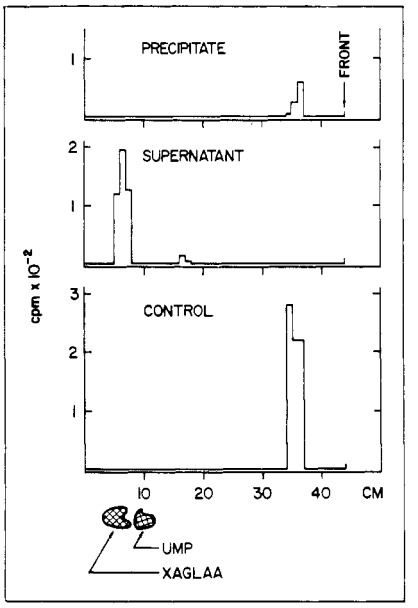

FIGURE 6: Identification of the released radioactivity as UDP-NAc-muramyl-[^{14}C]pentapeptide. A reaction mixture identical with that described for Figure 5B was carried out with 1.0×10^{-3} M UMP. At 15 min the reaction was terminated with 0.3 N $HClO_4$ and the precipitate was removed by centrifugation. The supernatant fraction was neutralized with KOH and the potassium perchlorate was discarded. This fraction and the precipitate were chromatographed in solvent A. Sections of the chromatogram were assayed for radioactivity. The chromatogram labeled "control" was performed by streaking an aliquot of the perchloric acid precipitated acceptor-phospho-NAc-muramyl-[^{14}C]pentapeptide at the origin followed by chromatography in solvent A. The abbreviation is: XAGLAA, UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-L-Ala.

transfer was observed at 15 min (Struve and Neuhaus, 1965a).

A labeled intermediate equivalent to the amount of phospho-NAc-muramyl-pentapeptide transferred was observed after chromatography in solvent A (see Figure 6). This intermediate was assumed to be acceptor-phospho-NAc-muramyl-pentapeptide or a derivative. The transfer of 1 mole of phospho-NAc-muramyl-[^{14}C]pentapeptide from UDP-NAc-muramyl-[^{14}C]pentapeptide resulted in the formation of 1 mole of UMP (Table I). The small amount of uridine was derived from UMP by 5′-nucleotidase activity (*cf.* Table IV) which was associated with the enzyme preparation. Uracil and UDP were not products of the forward reaction. The sum of UMP and uridine was equivalent to the amount of phospho-NAc-muramyl-[^{14}C]pentapeptide transferred. Therefore, the forward reaction may be formulated according to the following equation:

$$\text{UDP-NAc-muramyl-pentapeptide} + \text{acceptor} \longrightarrow \text{UMP} + \text{acceptor-phospho-NAc-muramyl-pentapeptide} \quad (2)$$

The forward reaction required the presence of Mg^{2+} (Figure 2). From data obtained with the tube assay, one-half the maximum activity was observed with 2×10^{-3} M Mg^{2+}.

Exchange of [^{3}H]UMP with the UMP Moiety of UDP-NAc-muramyl-pentapeptide. The rapid delabeling

by UMP suggested that [^{3}H]UMP would exchange with the UMP moiety of UDP-NAc-muramyl-pentapeptide. This exchange was observed and was found to be specific for UDP-NAc-muramyl-pentapeptide (Struve and Neuhaus, 1965a). The [^{3}H][^{14}C]nucleotide co-chromatographed with UDP-NAc-muramyl-pentapeptide in solvents A and B. Acid hydrolysis (0.1 N HCl, 3 min, 100°) of the nucleotide gave [^{3}H]UDP.

In order to characterize the exchange reaction, the amount of [^{3}H]UMP exchanged was calculated from the rate equation for an exchange reaction of the following type

$$UX + U' \rightleftharpoons U'X + U \qquad (3)$$

where U′ is [^{3}H]UMP and UX is UDP-NAc-muramyl-pentapeptide. The first-order rate equation (Duffield and Calvin, 1946) is

$$-\ln\left(1 - \frac{[U'X]}{[U'X]_\infty}\right) = Rt\frac{(a+b)}{ab} \qquad (4)$$

where $a = [UX] + [U'X]$ and $b = [U'] + [U]$, Rt is the amount exchanged, and $[U'X]/[U'X]_\infty$ is the fraction exchanged. The amount of [^{3}H]UMP exchanged is proportional to time for 15 min (Figure 3A). The effect of enzyme concentration on the exchange reaction is shown in Figure 3B. The rate of exchange (R) will be presented in moles exchanged/liter per minute. The pH optimum for the exchange reaction is 7.6.

Effect of UDP-NAc-muramyl-pentapeptide and UMP on the Exchange Reaction. The rates of exchange at varying concentrations of UDP-NAc-muramyl-pentapeptide and UMP were measured. From the Lineweaver–Burk plots (Lineweaver and Burk, 1934) shown in Figure 4A and 4B, the Michaelis constants (K_m) for UDP-NAc-muramyl-pentapeptide (1.8×10^{-6} M) and UMP (2.7×10^{-5} M) have been established. In the calculation of these constants from exchange data it was assumed that the concentrations of acceptor and acceptor–phospho-NAc-muramyl-pentapeptide were saturating. The K_m for Mg^{2+} established from exchange data is identical with the concentration of Mg^{2+} required for one-half of the maximum activity in the tube assay (Figure 2).

Reverse Reaction. The isolation of acceptor–phospho-NAc-muramyl-pentapeptide for the study of the reverse reaction was accomplished by precipitation with 0.3 N $HClO_4$ at 0–4°. When this material was incubated with 1×10^{-3} M UMP and Mg^{2+}, the radioactivity associated with the acceptor–phospho-NAc-muramyl-pentapeptide-[^{14}C] was released (half-life = 6 min) (Figure 5A). With the addition of enzyme preparation the half-life was 0.5 min (Figure 5B). The released radioactivity was identified as UDP-NAc-muramyl-pentapeptide by cochromatography with authentic material in solvents A, B, and C. The results for solvent A are shown in Figure 6. The requirements for the reverse reaction are illustrated in Table II. If the perchloric acid treated acceptor–phospho-NAc-muramyl-pentapeptide is treated with Triton X-100, a fraction is obtained which shows a dependence on enzyme, Mg^{2+}, and UMP for the formation of UDP-NAc-muramyl-pentapeptide. These results define the reverse reaction in the following manner:

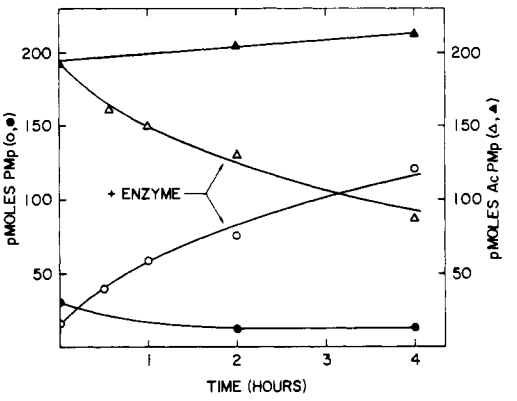

FIGURE 7. Release of phospho-NAc-muramyl-[^{14}C]-pentapeptide. The reaction mixture contained 200 pmoles of acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide (9.3×10^3 cpm/nmole) (perchloric acid precipitated fraction); 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; and 0.83 unit of nucleotide pyrophosphatase (fraction A) in a total volume of 0.1 ml. The reaction mixture was incubated at 37° for the indicated time interval, and the reaction was terminated by drying the mixture on Whatman 3MM paper. The amounts of phospho-NAc-muramyl-[^{14}C]pentapeptide and acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide were determined as described in Table III. In ▲ and ● the pyrophosphatase was omitted.

acceptor–phospho-NAc-muramyl-pentapeptide + UMP ⟶ UDP-NAc-muramyl-pentapeptide + acceptor (5)

On the Nature of the Covalent Bond between Acceptor and Phospho-NAc-muramyl-pentapeptide. Treatment of the isolated acceptor–phospho-NAc-muramyl-pentapeptide with preparations of nucleotide pyrophosphatase purified from bovine seminal plasma resulted in the release of phospho-NAc-muramyl-pentapeptide (Table III). Phosphodiesterase (venom) and purified alkaline phosphatase did not release radioactivity from the acceptor–[^{14}C]intermediate. Incubation of UDP-NAc-muramyl-[^{14}C]pentapeptide with nucleotide pyrophosphatase resulted in the formation of phospho-NAc-muramyl-[^{14}C]pentapeptide. This fragment was identified by acid-labile phosphorus (0.1 M HCl, 3 min, 100°) and acid-labile reducing sugar (Table III). The fragment released from UDP-NAc-muramyl-[^{14}C]pentapeptide chromatographed with the fragment that was released from acceptor–phospho-NAc-muramyl-pentapeptide treated with nucleotide pyrophosphatase. The [^{14}C]labeled fragment that was released from acceptor–phospho-NAc-muramyl-penta-

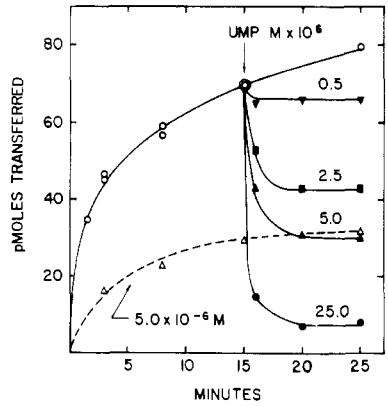

FIGURE 8: Effect of UMP on the concentration of acceptor–phospho-NAc-muramyl-pentapeptide. The tube assay was used with 0.88 mg of enzyme preparation. Increasing concentrations of UMP were added to a separate series at 15 min. The concentrations of UMP are: ▼—▼, 5.0×10^{-7} M; ■—■, 2.5×10^{-6} M; ▲—▲, 5.0×10^{-6} M; ●—●, 2.5×10^{-5} M. With △—△, 5.0×10^{-6} M UMP was added at zero time to the reaction mixtures.

peptide was isolated and hydrolyzed with acid (Table III). The product chromatographed with NAc-muramyl-pentapeptide isolated from UDP-NAc-muramyl-pentapeptide. The release of phospho-NAc-muramyl-pentapeptide from acceptor–phospho-NAc-muramyl-pentapeptide is illustrated in Figure 7.

Schliselfeld *et al.* (1965) observed that ADP and ATP are noncompetitive inhibitors of the hydrolysis of UDP-glucose catalyzed by nucleotide pyrophosphatase from nuclei. With the enzyme from bovine seminal plasma, 5′-AMP was found to inhibit the hydrolysis of UDP-NAc-muramyl-pentapeptide and acceptor–phospho-NAc-muramyl-pentapeptide.

Incubation of acceptor–phospho-NAc-muramyl-pentapeptide (perchloric acid precipitated fraction) in the presence of 1 M H_2NOH, pH 7.8, for 15 min at 37° did not result in the release of radioactivity. After treatment with 0.10 M NaOH for 2 min at 100°, 88% of the radioactivity had the same R_F as acceptor–phospho-NAc-muramyl-pentapeptide in solvent A. Under the same conditions 81% of the UDP-NAc-muramyl-pentapeptide was recovered.

Equilibrium Constant. The forward, reverse, and exchange reactions support the following equation.

$$\text{UDP-NAc-muramyl-pentapeptide} + \text{acceptor} \overset{Mg^{2+}}{\rightleftharpoons} \text{acceptor–phospho-NAc-muramyl-pentapeptide} + \text{UMP} \quad (6)$$

The addition of increasing concentrations of 5′-UMP resulted in the increased delabeling of acceptor–phospho-NAc-muramyl-pentapeptide (Figure 8). On the other hand, if 5′-UMP is added from zero time, the amount transferred was equivalent to the amount of acceptor–phospho-NAc-muramyl-pentapeptide which remained after delabeling. Thus, equilibrium conditions may be reached by either route. The equilibrium constant for reaction 6 is formulated as follows

$$K_{eq} = \frac{[\text{AcPMp}][\text{UMP}]}{[\text{UMPPMp}][\text{Ac}_0 - \text{AcPMp}]} \quad (7)$$

where AcPMp is acceptor–phospho-NAc-muramyl-pentapeptide, Ac_0 is the initial acceptor concentration, UMPPMp is UDP-NAc-muramyl-pentapeptide, and $[Ac_0 - AcPMp]$ is the free acceptor concentration. From the results in Figure 8, Ac_0 and K_{eq} were calculated to be 7.3×10^{-7} M and 0.25 ± 0.04, respectively.

TABLE II: Requirements for the Synthesis of UDP-NAc-muramyl-pentapeptide from Acceptor–phospho-NAc-muramyl-pentapeptide.[a]

Addition	UDP-NAc-muramyl-pentapeptide Formed (pmoles/15 min)
Complete	110
Boiled enzyme	5.6
$-Mg^{2+}$	13.5
−UMP	1.2
−Enzyme preparation	0

[a] The complete incubation contained 125 pmoles of purified acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide; 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; 1×10^{-4} M UMP; and 1.1 mg of enzyme preparation in 0.1 ml. The reaction mixtures were incubated for 15 min at 25°. The amount of UDP-NAc-muramyl-pentapeptide was determined by paper chromatography with solvent A followed by assay of the radioactivity. For these experiments acceptor–phospho-NAc-muramyl-pentapeptide was prepared in the following incubation: 1.5×10^{-5} M UDP-NAc-muramyl-L-Ala-D-Glu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala (8.43×10^3 cpm/nmole); 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; 100 μg/ml ristocetin; and 2.2 mg of enzyme preparation in 1 ml. The mixture was incubated for 1 hr at 25°. The reaction was terminated with 2 ml of 0.3 N $HClO_4$ and maintained for 10 min at 0–4°. The precipitate was removed by centrifugation at 4300*g* for 5 min. The precipitate was washed three times with three aliquots of water (2 ml). For many of the experiments (Figures 5–7 and Table III) the precipitate was suspended in 0.01 M Tris–HCl, pH 7.8, and used as such. For the experiments presented in this table the precipitate was extracted with 0.01 M Triton X-100 for 2–3 min with vigorous stirring. The precipitate was removed by centrifugation at 4300*g* for 5 min. The supernatant fraction was used as source of purified acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide.

TABLE III: Release of Phospho-NAc-muramyl-pentapeptide from UDP-NAc-muramyl-pentapeptide and Acceptor–phospho-NAc-muramyl-pentapeptide.[a]

	Incubation	UDP-NAc-muramyl-pentapeptide (pmoles)	Phospho-NAc-muramyl-pentapeptide (pmoles)	NAc-muramyl-pentapeptide (pmoles)	Acceptor–phospho-NAc-muramyl-pentapeptide (pmoles)
1a	UDP-NAc-muramyl-pentapeptide	298	3	0	—
b	Nucleotide pyrophosphatase + 1a	1	268	0	—
c	Phospho-NAc-muramyl-pentapeptide eluted from 1b + 0.1 N HCl, 3 min, 100°	0	0	189	—
2a	Acceptor–phospho-NAc-muramyl-pentapeptide	9	16	29	215
b	Nucleotide pyrophosphatase + 2a	6	136	11	140
c	Phospho-NAc-muramyl-pentapeptide eluted from 2b + 0.1 N HCl, 3 min, 100°	0	0	93	0
	R_F (solvent A)	0.16	0.29	0.51	0.82

[a] The reaction mixture contained either 300 pmoles of UDP-NAc-muramyl-[^{14}C]pentapeptide (9.0 × 10^3 cpm/nmole) or 416 pmoles of acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide (9.3 × 10^3 cpm/nmole) (perchloric acid precipitated fraction); 0.05 M Tris–HCl, pH 7.8; 0.01 M $MgCl_2$; and 2.3 units of purified nucleotide pyrophosphatase (fraction A) from bovine seminal plasma in a total volume of 0.2 ml. In 1a and 2a the reaction mixtures were maintained at 37° for 4 hr in the absence of pyrophosphatase, while in 1b and 2b the reaction mixtures were incubated in the presence of pyrophosphatase for 4 hr. In 1c and 2c the phospho-NAc-muramyl-pentapeptide was eluted and hydrolyzed with 0.1 N HCl for 3 min at 100°. The reaction mixtures were chromatographed in solvent A. The standard of muramyl-[^{14}C]pentapeptide was prepared by acid hydrolysis of UDP-NAc-muramyl-[^{14}C]pentapeptide (0.1 M HCl, 3 min, 100°) followed by chromatography with solvent A. The analysis (*cf.* Neuhaus and Struve, 1965) is as follows: alanine:glutamic acid:lysine:muramic acid, 3.0:1.0:1.1:0.78. Phospho-NAc-muramyl-[^{14}C]-pentapeptide was prepared from UDP-NAc-muramyl-[^{14}C]pentapeptide with the pyrophosphatase and isolated by chromatography in solvent A. The analysis is as follows: D-[^{14}C]Ala-D-[^{14}C]Ala:acid-labile phosphorus:acid-labile reducing sugar, 1.2:1.0:0.8. The phospho-NAc-muramyl-[^{14}C]pentapeptide and radioactive fragment released from acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide cochromatograph in solvents A, B, D, and E. Similar results were obtained with nucleotide pyrophosphatase purified from potatoes (Kornberg and Pricer, 1950).

This corresponds to a $\Delta G^{\circ\prime}$ of 0.98 kcal (Mg^{2+} = 0.01 M, pH 7.8). The enzyme concentration in the reaction mixtures described in Figure 8 is 16 mg/ml.

Effect of Cell Walls from S. aureus Copenhagen on the Translocase. The addition of walls which have been washed in 1 M KCl to the reaction mixture (tube assay) resulted in a marked enhancement of the formation of acceptor–phospho-NAc-muramyl-pentapeptide. Part of this enhancement was demonstrated to be the result of the translocase system; however, essentially all of this activity was removed by treating the walls with Triton X-100 (see cell-wall isolation). This preparation of walls was essentially free of cytoplasmic constituents as judged by electron microscopy. The addition of this preparation of walls gave only a 10% enhancement in the tube assay (Figure 9). However, in contrast to the reaction mixtures which contained only the enzyme preparation, there was a rapid relabeling after the addition of UMP in those incubations which contained the preparation of walls and enzyme (Figure 9).

The walls, the enzyme preparation for translocase, and supernatant fraction (105,000*g*) were tested for 5′-nucleotidase activity. With [^{3}H]UMP as the substrate, the initial rates of uridine liberation at varying concentrations of UMP were measured. From the Lineweaver–Burk plots (Lineweaver and Burk, 1934), the values for K_m and V_{max} were established. As shown in Table IV, the Michaelis constants for UMP are 2.7 × 10^{-6} M and 2.2 × 10^{-5} M for the wall and enzyme preparation, respectively. The specific activities for the wall enzyme, particulate enzyme, and supernatant fraction are 5.3, 0.39, and 33, respectively. Thus, in contrast to the translocase fraction, the preparation of walls has a higher specific activity and requires a lower concentration of UMP for $V_{max}/2$. Since both the walls and membranes are primarily composed of nonprotein material, a comparison based on nonwall amino acids (Table IV) may be more realistic. On this basis there is a 70-fold difference between the specific activities of the nucleotidase associated with the walls and that associated with the particulate fraction. The nucleotidase associated with the walls was compared with that found

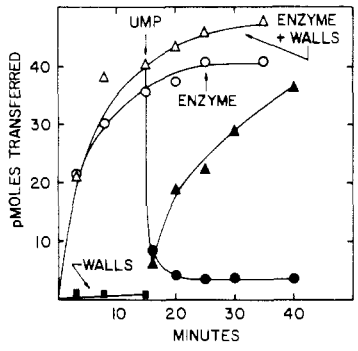

FIGURE 9: Effect of UMP on the formation of acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide in the presence of walls. The tube assay was used with 1.36 mg of Triton-treated walls and 0.44 mg of enzyme preparation. UMP (2.0×10^{-5} M) was added at 15 min to the following incubations: ●—●, enzyme preparation minus walls; ▲—▲, enzyme preparation plus walls. The control (■—■) contained only walls.

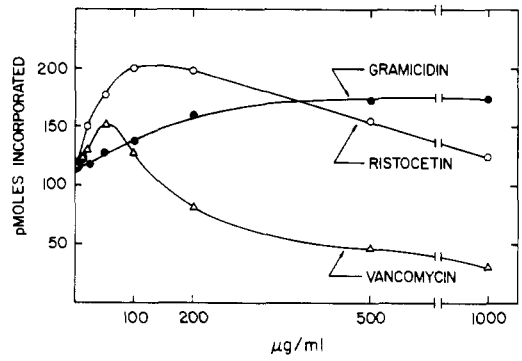

FIGURE 10: Effect of vancomycin, ristocetin, and gramicidin on the formation of acceptor–phospho-NAc-muramyl-[^{14}C]pentapeptide. The tube assay was used with 0.9 mg of enzyme preparation.

in bovine seminal plasma. The Michaelis constant for the nucleotidase from the latter source is 2.0×10^{-5} M (5×10^{-5} M; Park, 1952), in contrast to that observed with the walls. The wall-nucleotidase activity has a pH optimum of 6.7–7.5 and hydrolyzes CMP, AMP, and UMP at the following rates when tested at 5×10^{-6} M: 6.6, 6.1, 5.6 (nmoles cleaved/10 min per mg of wall).

TABLE IV: 5′-Nucleotidase Activity of Walls and Enzyme Preparation.[a]

Preparation	Specific Activity	K_m (M)
Walls (Triton-treated)	5.3[b] (220)[c]	2.7×10^{-6}
Enzyme preparation for translocase	0.39[b] (3)[c]	22×10^{-6}
Supernatant (105,000 *g*)	33[d] (91)[c]	20×10^{-6}

[a] The nucleotidase assay was used. The specific activities were calculated from V_{max} with 5′-UMP as the substrate. [b] nmoles/10 min per mg of dry weight. [c] nmoles/10 min per μmole of (threonine + leucine + isoleucine)/3. [d] nmoles/10 min per mg of protein.

Effect of Antibiotics on the Phospho-NAc-muramyl-pentapeptide Translocase. Meadow *et al.* (1964) and Anderson *et al.* (1965) reported that vancomycin and ristocetin inhibited the synthesis of peptidoglycan. These results were confirmed and extended to the reaction catalyzed by the translocase (Struve and Neuhaus, 1965a). In Table V a wide variety of antibiotics were tested in the tube assay, exchange assay, and paper assay (see Struve and Neuhaus, 1965a). In the paper assay only vancomycin, ristocetin, and bacitracin significantly inhibited the formation of peptidoglycan at a level of 5 μg (Table V). It was observed that vancomycin, ristocetin, and gramicidin enhanced the formation of acceptor–phospho-NAc-muramyl-pentapeptide in the tube assay. This stimulation has been confirmed by chromatography in solvent A. On the other hand, ristocetin and vancomycin inhibited the exchange of [^{3}H]UMP with UDP-NAc-muramyl-pentapeptide.

In Figure 10 the effect of three antibiotics on the tube assay is demonstrated. With ristocetin and vancomycin an enhancement at low concentrations (100 μg/ml) was observed while at higher concentrations (200 μg/ml) vancomycin inhibited in contrast to ristocetin.

Discussion

The results of these studies, as well as those previously reported (Struve and Neuhaus, 1965a,b), have established the presence of an enzyme which transfers phospho-NAc-muramyl-pentapeptide from the uridylic acid moiety to an acceptor which is associated with the enzyme preparation. The transfer of phospho-NAc-muramyl-pentapeptide, the inhibition and delabeling by UMP, and the exchange of UMP with the UMP moiety of UDP-NAc-muramyl-pentapeptide are consistent with the following sequence of reactions.

$$E + UMPPMp \rightleftharpoons EUMPPMp \quad (8)$$

$$EUMPPMp + Ac \rightleftharpoons AcPMp + EUMP \quad (9)$$

$$EUMP \rightleftharpoons E + UMP \quad (10)$$

where EUMPPMp is a binary complex of enzyme (E) and the substrate UDP-NAc-muramyl-pentapeptide (UMPPMp). EUMP is the binary complex of enzyme and UMP. Ac is the acceptor associated with the enzyme.

The change in free energy between acceptor–phos-

TABLE V: Specificity of Antibiotics in the Tube, Exchange, and Paper Assay.[a]

Addition	Assay				
	Tube (pmoles/15 min)			Exchange (R) $\times 10^7$ (M/min)	Paper (pmoles/hr)
	I	II	III		
Control		65		1.74	39
Bacitracin	66	54	65	1.65	13
Chloramphenicol	70	75	77	1.93[b]	39
Erythromycin A	57	64	66	1.84[b]	43
Gramicidin	65	82	106	1.77[b]	38
Kanamycin	61	65	61	1.68	43
Neomycin	67	70	68	2.18 (1.81)	57
Novobiocin	64	56	64	1.60[b]	39
D-Penicillamine	76	76	64	1.69	42
Penicillin G	65	63	67	1.67 (1.68)	36
Polymyxin B	71	67	69	1.38 (1.72)	46
Ristocetin	128	174	167	0.26 (0.83)	4
Streptomycin	69	67	73	1.71 (1.57)	34
Streptothricin	69	68	70	1.71 (1.52)	38
Vancomycin	109	45	26	0.24 (0.53)	3

[a] The tube assay, exchange assay, and paper assay contained 0.80, 0.64, and 0.80 mg of enzyme preparation, respectively. The concentrations of antibiotics are: tube assay, I, 50 μg/ml; II, 200 μg/ml; III, 500 μg/ml; exchange assay, 2000 μg/ml (200 μg/ml); paper assay, 5 μg. The paper assay was performed by streaking the reaction mixture described for the tube assay which contained in addition 10 nmoles of UDP-NAc-glucosamine on Whatman 3MM paper. The paper was partially dried for 1 min at 37° and then incubated in a water-saturated atmosphere for 1 hr at 37° (Meadow *et al.*, 1964). The UDP-NAc-muramyl-[^{14}C]pentapeptide was separated from the origin by descending chromatography with solvent A. The origins containing the peptidoglycan were assayed for radioactivity as described in the Experimental Section. The final concentrations of the components in the paper assay are difficult to establish. [b] Since the stock solutions of these antibiotics contained 50% ethanol, the final concentration of ethanol in the incubation was 10%. The control with 10% ethanol gave 2.28×10^{-7} M/min. In order to compare the rates of exchange with those performed in the absence of ethanol, the values were normalized to the rate observed with the control. No effect of ethanol (10%) was observed in the tube assay or the paper assay.

pho-NAc-muramyl-pentapeptide and UDP-NAc-muramyl-pentapeptide establishes the acceptor–intermediate as a high energy compound. The liberation of phospho-NAc-muramyl-pentapeptide from the intermediate by nucleotide pyrophosphatase is consistent with a pyrophosphate linkage between NAc-muramyl-pentapeptide and the acceptor. Thus, one phosphorus would be derived from UDP-NAc-muramyl-pentapeptide while the other would be derived from acceptor, *e.g.*, a compound similar to phosphatidic acid. The proof of this linkage must await the elucidation of the structure of the acceptor.

The addition of cell walls prepared from *S. aureus* resulted in a small stimulation of the tube assay. However, when UMP was added to the reaction mixtures that contained walls, there was a delabeling followed by a rapid relabeling of the enzyme preparation. In the absence of walls there was no relabeling by UDP-NAc-muramyl-[^{14}C]pentapeptide. The wall preparation contains a 5′-nucleotidase activity that has a high affinity for UMP. Since the equilibrium constant of the translocase favors the reverse reaction, the driving force for this system can be attained by coupling the formation of acceptor–phospho-NAc-muramyl-pentapeptide with the hydrolysis of UMP catalyzed by the nucleotidase associated with the wall. Although there is nucleotidase activity in the cytoplasmic (105,000*g* supernate) and membrane fraction (105,000*g* precipitate), the difference in K_m suggests that the wall enzyme is not identical with the nucleotidase in these fractions.

With *E. coli*, formation of spheroplasts results in the release of alkaline phosphatase (Malamy and Horecker, 1964), acid phosphatase (Neu and Heppel, 1964), and a Co^{2+} stimulated 5′-nucleotidase (Neu and Heppel, 1964). Malamy and Horecker (1964) concluded that the alkaline phosphatase is not an integral component of the cell wall, and both groups concluded that these enzymes are associated with a compartment between the cell wall and cell membrane.

The nucleotidase associated with the walls from *S. aureus* is not removed by extensive washing with 1 M KCl or 4×10^{-3} M Triton X-100. The latter treatment removes the translocase from the wall preparation (see Figure 9). These data suggest that the nucleotidase

is an integral component of the cell wall in *S. aureus*. Wall preparations which have been treated with trypsin do not contain nucleotidase activity.

An alternative model for the transport of phospho-NAc-muramyl-pentapeptide has been proposed by Strominger *et al.* (1965) and Anderson *et al.* (1965). This involves the formation of the lipid(acceptor)–phosphodisaccharide which can be transported through the cell membrane to the site of wall synthesis. Thus, NAc-glucosamine is transported with the NAc-muramyl-pentapeptide. This model would require an additional system for the transport of NAc-glucosamine for teichoic acid biosynthesis. Furthermore, this model does not utilize the free energy of hydrolysis of UMP for the translocation.

Meadow *et al.* (1964) observed that vancomycin and ristocetin were the most effective inhibitors of peptidoglycan synthesis. These antibiotics have been tested with the phospho-NAc-muramyl-pentapeptide translocase in the exchange assay and tube assay. In the tube assay at low concentrations of ristocetin and vancomycin there is an enhancement of acceptor–phospho-NAc-muramyl-pentapeptide formation. On the other hand, with both antibiotics there is an inhibition of the exchange assay. This dichotomy of results is not readily explained.

Several sites of vancomycin action are under investigation in other laboratories. (1) The inhibition of teichoic acid biosynthesis has been demonstrated by Burger and Glaser (1964) and Glaser (1964). (2) Adsorption of vancomycin to acidic groups on the wall could inhibit the addition of new wall components to the existing peptidoglycan (Best and Durham, 1964, 1965). (3) Jordan (1965) observed that inhibition of membrane synthesis by vancomycin "in growing cells begins sometime *after* the cessation of wall production." (4) Hancock and Fitz-James (1964) and Shockman and Lampen (1962) observed that vancomycin inhibits the growth of protoplasts. (5) Anderson *et al.* (1965) observed that the glycopeptide synthetase (peptidoglycan synthetase) is inhibited by low concentrations of vancomycin. (6) The work described in this paper presents evidence for effects on the reaction catalyzed by the translocase. Thus, there is a multiplicity of action sites for this antibiotic. Inhibition at one or more of these sites could lead to the observed accumulation of cell-wall precursors (Reynolds, 1961).

Strominger *et al.* (1965) and Anderson *et al.* (1965) have shown that the acceptor–phospho-NAc-muramyl-pentapeptide is utilized for acceptor–phosphodisaccharide synthesis. Glycine from glycyl-s-RNA is transferred to this lipid intermediate (Park, 1964, 1965; Strominger *et al.*, 1965). Peptidoglycan synthetase utilizes this intermediate to form the peptidoglycan of the wall. Thus, the high-energy intermediate described in this paper and by Anderson *et al.* (1965) occupies a key role in the biosynthesis of one of the heteropolymers found in the walls of *S. aureus*.

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Structural Studies of Ribonuclease. XX. Acrylonitrile. A Reagent for Blocking the Amino Groups of Lysine Residues in Ribonuclease*

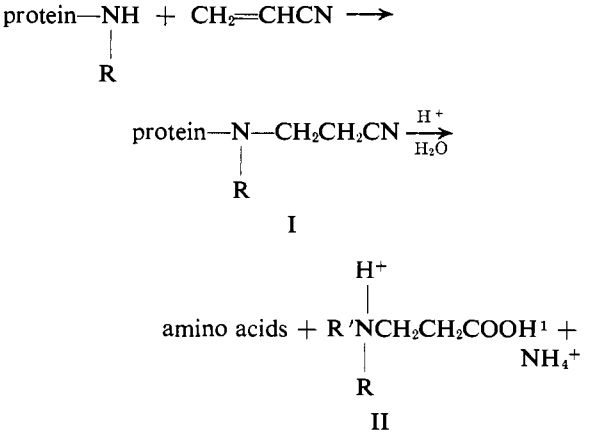
John P. Riehm and Harold A. Scheraga

ABSTRACT: The reaction between ribonuclease and acrylonitrile is described. It has been possible to demonstrate that acrylonitrile reacted with the ϵ-amino groups of lysine residues in this protein.

In addition, a derivative was prepared in which all ten lysine residues had reacted with this reagent. Although this derivative was shown to be enzymatically inactive, it did possess physicochemical properties which were similar to those of ribonuclease.

Acrylonitrile has been utilized by Weil and Seibles (1961) to specifically block the thiol groups of reduced β-lactoglobulin and reduced α-lactalbumin, and by Plummer and Hirs (1964) to specifically block the thiol groups of reduced ribonuclease A and reduced ribonuclease B. Although the studies of Weil and Seibles showed that acrylonitrile reacted only with thiol groups, Plummer and Hirs noted a slow reaction with the ϵ-amino groups of lysine residues when the reaction was allowed to proceed above pH 9.5. Subsequently, Kalan *et al.* (1965) reported that the cyanoethylation of β-lactoglobulin and κ-casein resulted in a 25 and 40%, respectively, loss in lysine content. Additional information (McKinney *et al.*, 1950, 1951, 1952) also suggests that the side chains of lysyl and/or histidyl residues could react under certain experimental conditions.

As shown in the accompanying scheme, the reaction between a protein and acrylonitrile is visualized as yielding the cyanoethyl derivatives of active hydrogen compounds, such as those of lysine and histidine residues (I). Since it contains two hydrogens on its primary ϵ-amino group, lysine could react to produce the mono- and/or the dicyanoethyl derivative, while histidine could yield the 1- and/or the 3-cyanoethyl derivative. Subsequent acid hydrolysis (McKinney *et al.*, 1952) would undoubtedly yield the corresponding carboxyethyl compounds (II). These carboxyethyl compounds should chromatograph (on amino acid analysis according to the procedure of Moore *et al.*, 1958, as adapted by Piez and Morris, 1960) at a faster rate than do the parent compounds. Furthermore, since these derivatives are primary α-amino acids, they should produce ninhydrin-positive zones. Therefore, any decrease in the lysine or histidine content of a protein, after acrylonitrile treatment and acid hydrolysis, should be accounted for by the appearance of the respective carboxyethyl derivatives.



This paper reports on the reaction between acrylonitrile and ribonuclease, a protein which contains no thiol groups. It was possible to demonstrate that

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[1] R and R′ would be H and $HOOCCH(NH_3^+)(CH_2)_4$, respectively, if the nitrogen-containing group were lysine.