On the Initial Stage in Peptidoglycan Synthesis. III. Kinetics and Uncoupling of Phospho-*N*-acetylmuramyl-pentapeptide Translocase (Uridine 5′-Phosphate)*

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ABSTRACT: The initial reaction in the biosynthesis of peptidoglycan is catalyzed by phospho-*N*-acetylmuramyl-pentapeptide translocase (uridine diphosphate-*N*-acetylmuramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala: C_{55}-isoprenoid alcohol phosphate phospho-*N*-acetylmuramyl-pentapeptide transferase) according to the reaction

uridine diphosphate-*N*-acetylmuramyl-pentapeptide + acceptor phosphate $\overset{Mg^{2+}}{\rightleftharpoons}$ acceptor diphosphate-*N*-acetylmuramyl-pentapeptide + uridine monophosphate

where acceptor is C_{55}-isoprenoid alcohol. The kinetics of transfer are characterized by a biphasic reaction when uridine monophosphate is measured ($k_1 = 0.018$ min^{-1}; $k_2 = 1.8 \times 10^{-8}$ M min^{-1}). The formation of acceptor diphosphate-*N*-acetylmuramyl-pentapeptide is characterized by pseudo-first-order kinetics ($k_1 = 0.018$ min^{-1}). With the liberation of uridine monophosphate in the second phase, there is a corresponding formation of phospho-*N*-acetylmuramyl-pentapeptide. On the basis of the kinetic evidence, the formation of an enzyme–phospho-*N*-acetylmuramyl-pentapeptide intermediate in the transfer to C_{55}-isoprenoid alcohol phosphate is proposed. In addition to the transfer reaction, the enzyme catalyzes the exchange of uridine monophosphate with the uridine monophosphate moiety of uridine diphosphate-*N*-acetylmuramyl-pentapeptide. The ratio of the exchange rate to the transfer rate varies from 6 to 24. Thus, the transfer of phospho-*N*-acetylmuramyl-pentapeptide from the proposed enzyme intermediate to lipid acceptor phosphate is the rate-limiting step. Dodecylamine inhibits the synthesis of lipid acceptor diphosphate-*N*-acetylmuramyl-pentapeptide ($K_i = 6.5 \times 10^{-4}$ M) and causes the release of phospho-*N*-acetylmuramyl-pentapeptide. The effects of various surfactants on the exchange and transfer reactions are described and interpreted on the basis of the proposed model for the transfer of phospho-*N*-acetylmuramyl-pentapeptide from UDP-*N*-acetylmuramyl-pentapeptide to lipid acceptor-phosphate.

The biosynthesis of peptidoglycan, the major structural polymer of bacterial cell walls, is catalyzed by a series of enzymes associated with the bacterial membrane (Anderson *et al.*, 1966, 1967; Araki *et al.*, 1966; Chatterjee and Park, 1964; Meadow *et al.*, 1964). The initial reaction in this series is catalyzed by phospho-*N*-acetylmuramyl-pentapeptide translocase (UMP) (Anderson *et al.*, 1965; Struve and Neuhaus, 1965; Struve *et al.*, 1966) according to the reaction

UDP-*N*-acetylmuramyl-pentapeptide + lipid acceptor phosphate $\overset{Mg^{2+}}{\rightleftharpoons}$ UMP + lipid acceptor diphosphate-*N*-acetylmuramyl-pentapeptide (1)

where the lipid acceptor is C_{55}-isoprenoid alcohol (Higashi *et al.*, 1967). The reaction is reversible and has a $K_{equil} = 0.25 \pm 0.04$ (Struve *et al.*, 1966). The enzyme also catalyzes the following exchange

[^{3}H]UMP + UDP-*N*-acetylmuramyl-pentapeptide $\overset{Mg^{2+}}{\rightleftharpoons}$ [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide + UMP (2)

The lipid product of the reaction contains a pyrophosphate link between the C_{55}-isoprenoid alcohol and *N*-acetylmuramyl-pentapeptide (Dietrich *et al.*, 1965; Struve *et al.*, 1966). Specificity studies have revealed at least two requirements for the translocase. The enzyme cannot utilize to a significant extent either the fluoro-substituted UDP-*N*-acetylmuramyl-pentapeptide (Stickgold and Neuhaus, 1967) or UDP-*N*-acetylmuramyl-tripeptide (Struve and Neuhaus, 1965; Anderson *et al.*, 1967).

It is the purpose of this paper to present experiments that provide evidence for the participation of an enzyme–phospho-*N*-acetylmuramyl-pentapeptide intermediate in

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the reaction catalyzed by the translocase. The evidence for this proposal is derived from the following three types of experiments: (1) kinetics of product formation, (2) differential effects of surfactants, and (3) uncoupling by dodecylamine.

Experimental Section

Materials. [^{3}H]UMP and [^{3}H]uracil were purchased from Schwarz Bio-Research, Inc. only D-[^{14}C]Alanine was the product of the California Corp. for Biochemical Research. *Staphylococcus aureus* Copenhagen was a gift from Dr. J. L. Strominger. Bacterial alkaline phosphatase (chromatographically purified) was obtained from Worthington Biochemical Corp. Sarkosyl NL-97 (sodium lauroyl sarcosinate) was a gift from Geigy Chemical Corp. Cutscum was purchased from Fisher Scientific Co. The membrane preparation and the sources of other chemicals have been previously described (Struve *et al.*, 1966).

UDP-*N*-acetylmuramyl-L-Ala-D-γ-Glu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala was prepared according to the procedure described by Neuhaus and Struve (1965). The preparation of UDP-*N*-acetylmuramyl-pentapeptide was carried out by the procedure described by Anderson *et al.* (1966) for [^{32}P]UDP-*N*-acetylmuramyl-pentapeptide and purified by the method of Stickgold and Neuhaus (1967). [^{3}H]UDP-*N*-acetylmuramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala was prepared by the method described for [^{3}H]fluorouridine diphosphate-*N*-acetylmuramyl-pentapeptide (Stickgold and Neuhaus, 1967). A more convenient method for the preparation of this nucleotide utilizes the exchange reaction catalyzed by the translocase (reaction 2). The reaction mixture contained 1 μmole of UDP-*N*-acetylmuramyl-pentapeptide, 75 nmoles of [^{3}H]UMP (2.4 Ci/mmole), 6 mg (dry weight) of membrane preparation, 100 μmoles of Tris-HCl (pH 7.8), 20 μmoles of $MgCl_2$, and 33 μmoles of KCl in a total volume of 0.15 ml. The mixture was incubated at 25° for 1 hr. The exchange reaction was terminated by heating the mixture in a boiling-water bath for 2 min. The [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide was isolated from the supernatant fraction by the method of Stickgold and Neuhaus (1967).

Assay of Phospho-N-acetylmuramyl-pentapeptide Translocase (UMP). Two types of assays have been used to measure the activity of this enzyme. The transfer assay involves the formation of C_{55}-isoprenoid alcohol diphosphate-*N*-acetylmuramyl-pentapeptide according to reaction 1. Transfer assay A measures the synthesis of lipid diphosphate-*N*-acetylmuramyl-[^{14}C]pentapeptide from UDP-*N*-acetylmuramyl-[^{14}C]pentapeptide. The reaction mixture contained 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.17 M KCl, 3.3×10^{-5} M UDP-*N*-acetylmuramyl-[^{14}C]pentapeptide (12.3 cpm/pmole), and membranes in a total volume of 0.06 ml. The addition of KCl to the reaction mixture enhances the activity of the enzyme (M. G. Heydanek and F. C. Neuhaus, unpublished observations). The reaction mixture was incubated at 25° for 15 min and terminated by the addition of 0.5 ml of cold 0.3 M $HClO_4$. The precipitate was isolated on a Millipore filter (0.45 μ). The tube and filter were washed twice with 1.0 ml of cold 0.3 M $HClO_4$. The filter was washed with an additional 5.0 ml of cold 0.3 M $HClO_4$. The damp filter was dissolved in the Triton–toluene scintillation fluid, and the radioactivity was determined.

Transfer assay B measures the release of [^{3}H]UMP from [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide followed by the hydrolysis of [^{3}H]UMP to [^{3}H]uridine. The reaction mixture contained 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.17 M KCl, 3.8×10^{-5} M [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide (1.99 cpm/pmole), 1.5 munits of alkaline phosphatase, and membrane preparation in a total volume of 0.06 ml. The reaction mixture was incubated for 15 min at 25°. After terminating the reaction by the addition of 0.2 ml of 0.2 M acetic acid, the mixture was applied to a column (5 × 10 mm) of Dowex 1-acetate (100–200 mesh, X-8). Two aliquots (0.2 ml) of 0.2 M acetic acid were added and the effluents (0.66 ml) were collected and assayed for [^{3}H]uridine.

Transfer assay A is a measure of the equilibrium position and depends on the lipid acceptor phosphate concentration, whereas transfer assay B is a measure of both the enzyme and lipid acceptor phosphate concentration. Since bacterial alkaline phosphatase is present in transfer assay B, only the forward direction is observed.

The exchange assay involves the exchange of UMP with the [^{3}H]UMP moiety of [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide according to the reaction

$$\text{UMP} + [^3\text{H}]\text{UDP-}N\text{-acetylmuramyl-pentapeptide} \overset{Mg^{2+}}{\rightleftharpoons} \text{UDP-}N\text{-acetylmuramyl-pentapeptide} + [^3\text{H}]\text{UMP} \quad (3)$$

After terminating the reaction, the UMP was hydrolyzed and the [^{3}H]uridine was separated from [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide and assayed for radioactivity. This assay differs from the exchange assays previously reported (Struve *et al.*, 1966; Stickgold and Neuhaus, 1967). With this modification the separation of the radioactive products is easier to perform. The reaction mixture contained 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.17 M KCl, 3.3×10^{-5} M [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide (1.2–8.4 cpm/pmole), 3.3×10^{-4} M UMP, and membrane preparation in a total volume of 0.06 ml. The mixture was incubated at 25° for 10 min and the reaction was terminated by placing the tube in a boiling-water bath for 3 min. After cooling to 25°, 0.5 unit of alkaline phosphatase was added to the reaction mixture, and the mixture was incubated for 10 min at 25°. The reaction was terminated by the addition of 0.2 ml of 0.2 M acetic acid and the [^{3}H]-uridine was separated from [^{3}H]UDP-*N*-acetylmuramyl-pentapeptide as described for transfer assay B. The rate of exchange, *R*, was calculated from the first-order rate equation described by Duffield and Calvin (1946). *R* is presented in moles per liter per minute.

Preparation of Lipid-Free Enzyme. All operations involving organic solvents were carried out at −15°. The solvents were carefully dried and water was avoided in all subsequent steps. A portion of the particulate enzyme (30–50 mg) was dried by lyophilization. The material was suspended in 4 ml of ethanol–heptane (1:1, v/v), and the suspension was stirred with a

motorized stirring rod for 2 min. The suspension was centrifuged for 15 min at 34,000*g*. The clear supernatant liquid was removed and the precipitate was suspended in ethyl ether (4 ml). The suspension was stirred for 2 min and centrifuged as described above. The supernatant fraction was removed and the precipitate was again suspended in ethanol–heptane. After centrifuging, the supernatant fraction was removed and a second ether extraction was performed. The resulting precipitate was resuspended in 4 ml of ethanol–heptane and allowed to stand at −15° for 1 hr with occasional stirring. The supernatant fraction was removed by centrifugation and a third ether extraction was performed. The resulting precipitate was cooled to −68° and dried in *vacuo*. The residue was suspended in 0.05 M Tris-HCl (pH 7.8; 0.2 ml) and will be referred to as "E–H enzyme."

The extracts from the organic phase were evaporated to dryness on a rotary evaporator. The residue was suspended in 0.05 M Tris-HCl (pH 7.8, 0.2 ml) and will be referred to as crude lipid acceptor.

Analytical Procedures. Measurements of radioactivity were made in polyethylene vials using the Packard Tri-Carb liquid scintillation spectrometer (Model 314-EX). For transfer assay A, B, and the exchange assay, 15 ml of Triton–toluene (2:1, v/v) scintillation fluid described by Patterson and Green (1965) and evaluated by Benson (1966) were used. Under these conditions the ^{3}H and ^{14}C efficiencies were 16 and 75%, respectively. For the analysis of radioactivity on paper chromatograms, the scintillation fluid was toluene containing 0.3% 2,5-diphenyloxazole (^{14}C efficiency = 42%). The nucleotides and sugar intermediates were chromatographed in the following descending solvent systems: (A) isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v) (P-L Biochemicals, 1967) and (B) 1-butanol–acetic acid–H_2O (2:1:1, v/v) (Ito and Strominger, 1962). All other procedures have been previously described (Struve *et al.*, 1966; Stickgold and Neuhaus, 1967).

Results

Kinetics of Product Formation. In Figure 1A, the kinetics of transfer and UMP formation are shown. In agreement with our previous report (Struve *et al.*, 1966), there is a stoichiometric release of [^{3}H]UMP coupled with the formation of lipid diphosphate-*N*-acetylmuramyl-[^{14}C]pentapeptide for the first 15 min. However, in experiments designed to titrate the lipid acceptor concentration of the membrane preparation, it was observed that UMP continued to be formed at a zero-order rate. In contrast, the lipid product attained an apparent steady-state level. In Figure 1B the first-order plot for the formation of lipid product is shown. The data from Figure 1A are plotted according to the equation

$$\log (C_\infty - C_t) = -\frac{k}{2.303}t + \log C_\infty \qquad (4)$$

where C_∞ is the concentration of lipid product extrapolated from an extended time period and C_t is the concentration of lipid product at time, t. Since alkaline phosphatase was included in the reaction mixture, there is no reverse reaction, and therefore, treatment of the data according to eq 4 is justified. The pseudo-first-order rate constant for lipid-product formation is 0.018 min^{-1}. The data[1] for UMP formation were analyzed by the method of Bender *et al.* (1967) for the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl trimethylacetate. In this treatment the amount of free acceptor ($C_\infty - C_t$) is equal to the difference between the extrapolated zero-order rate curve (*cf.* Figure 1A) and the observed value of UMP formed. From the first-order plot for UMP formation in Figure 1B, derived from the determination of uridine, the rate constant for UMP formation is 0.018 min^{-1}. The difference in intercept on the ordinate may be either the result of difficulties in establishing the extrapolated zero-order rate of UMP formation or establishing C_∞ for lipid product. The pseudo-zero-order rate constant for UMP formation is 1.8×10^{-8} M min^{-1}.

In addition to the lipid product and UMP, an additional ^{14}C-labeled compound was detected in the reaction mixture. In order to identify this product, incubations were performed that lacked bacterial alkaline phosphatase. In Figure 2 radioactivity profiles of chromatographic separations in solvent system A are shown for incubations at 0, 60, and 240 min. In addition to UDP-*N*-acetylmuramyl-pentapeptide and lipid product, a ^{14}C-labeled compound is observed at R_F 0.32. If bacterial alkaline phosphatase is added to the reaction mixture at zero time, a compound is observed at R_F 0.5 (Figure 2D). Comparable results were obtained with solvent system B. Incubation of UDP-*N*-acetylmuramyl-[^{14}C]pentapeptide with nucleotide pyrophosphatase results in the formation of UMP and phospho-*N*-acetylmuramyl-[^{14}C]pentapeptide (Struve *et al.*, 1966). The position of phospho-*N*-acetylmuramyl-pentapeptide is shown in the top profile of Figure 2. If bacterial alkaline phosphatase is incorporated into the incubation mixture containing UDP-*N*-acetylmuramyl-pentapeptide and nucleotide pyrophosphatase, uridine and *N*-acetylmuramyl-pentapeptide (R_F 0.5) are detected. From a comparison of these standards with the products of the transfer reaction (Figure 1A), it is concluded that the additional compound in the reaction mixture is *N*-acetylmuramyl-[^{14}C]pentapeptide. In the absence of alkaline phosphatase (Figure 2B, C) phospho-*N*-acetylmuramyl-[^{14}C]pentapeptide is formed in addition to the lipid product and UMP. Thus, the results in Figure 1A may be represented by the following reactions.

[^{3}H]UDP-*N*-acetylmuramyl-[^{14}C]pentapeptide + lipid acceptor phosphate $\rightleftarrows$ lipid acceptor diphosphate-*N*-acetylmuramyl-[^{14}C]pentapeptide + [^{3}H]UMP (5)

[^{3}H]UDP-*N*-acetylmuramyl-[^{14}C]pentapeptide $\longrightarrow$ [^{3}H]UMP + phospho-*N*-acetylmuramyl-[^{14}C]pentapeptide (6)

[1] In the reaction mixtures that contain alkaline phosphatase, the amount of UMP is derived from a determination of uridine and the amount of phospho-*N*-acetylmuramyl-pentapeptide is derived from a determination of *N*-acetylmuramyl-pentapeptide.

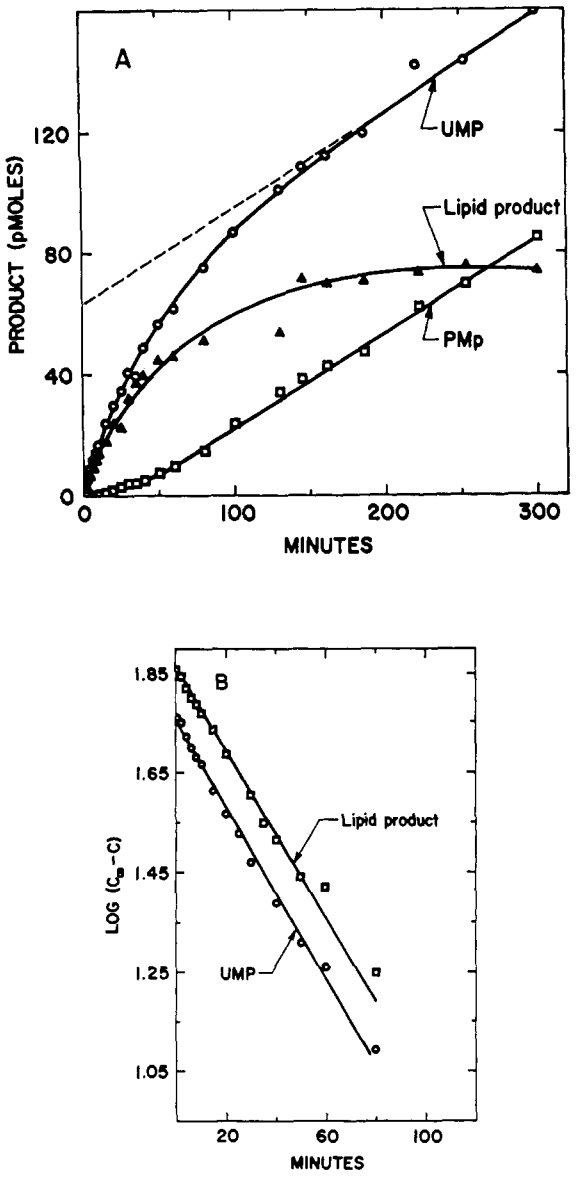

FIGURE 1: Formation of UMP, lipid product, and phospho-*N*-acetylmuramyl-pentapeptide as a function of time (A) and first-order analysis of these data (B). The reaction mixture contained 14.4 mg of membranes, 5.0×10^{-5} M [^{3}H]-UDP-*N*-acetylmuramyl-[^{14}C]pentapeptide (16.9 cpm/pmole of [^{3}H], 6 cpm/pmole of [^{14}C]), 0.01 M $MgCl_2$, 0.05 M Tris-HCl (pH 7.8), 0.17 M KCl, and 2.5 units of alkaline phosphatase in a total volume of 0.90 ml. Aliquots (25 μl) were withdrawn at the indicated time and diluted with 0.50 ml of 0.2 M acetic acid at 3° and allowed to stand at 3° for 15 min. The samples were centrifuged at 15,000*g* for 15 min and the supernatant fraction was removed. The precipitate was washed twice with 0.50 ml of cold 0.2 M acetic acid and centrifuged. The precipitate was suspended in 1.5 ml of 0.2 M acetic acid and assayed for radioactivity. The supernatant fractions were pooled and the products were separated on Dowex 1-acetate. The column effluents were collected and assayed for ^{14}C and ^{3}H. In B, the data were analyzed as described in the text. Although the actual products were dephosphorylated, they are referred to, both in the text and graph, as the direct enzymatic products, *i.e.*, UMP and phospho-*N*-acetylmuramyl-pentapeptide. PMp = phospho-*N*-acetylmuramyl-pentapeptide.

$$[^3\text{H}]\text{UMP} \longrightarrow P_i + [^3\text{H}]\text{uridine} \quad (7)$$

$$\text{phospho-}N\text{-acetylmura myl-[}^{14}\text{C]pentapeptide} \longrightarrow P_i + N\text{-acetylmuramyl-[}^{14}\text{C]pentapptide} \quad (8)$$

Since there is a lag in the formation of *N*-acetylmuramyl-pentapeptide derived from phospho-*N*-acetylmuramyl-pentapeptide, we suggest that phospho-*N*-acetylmuramyl-pentapeptide arises from the hydrolysis of an enzyme intermediate or the lipid product rather than UDP-*N*-acetylmuramyl-pentapeptide. Thus, the membranes contain an apparent "pyrophosphatase" for UDP-*N*-acetylmuramyl-pentapeptide and it would ap-

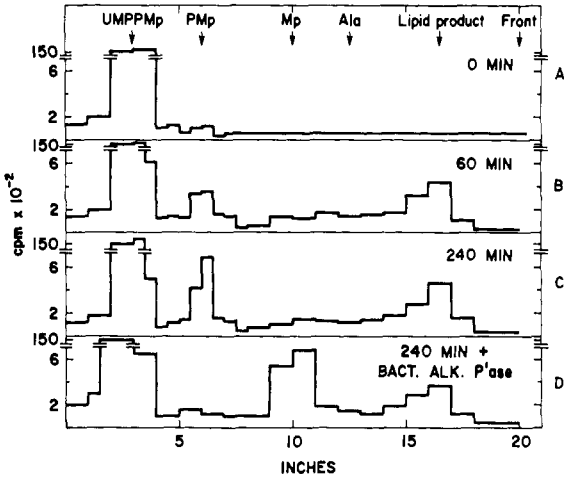

FIGURE 2: Analysis of reaction products as a function of time in the absence of alkaline phosphatase (A–C) and in the presence of alkaline phosphatase (D). The reaction mixtures for A (0 time), B (60 min), and C (240 min) are identical with those described for transfer assay A with 0.6 mg of enzyme. The reaction was terminated by placing the tube in a boiling-water bath for 2 min. The products were analyzed by descending chromatography in solvent system A. The incubation mixture in D contained 0.5 munit of alkaline phosphatase. The positions of authentic standards are designated by the arrows at the top of Figure 2A.

TABLE I: Reconstitution of Transfer Assay and Exchange Assay.[a]

Additions	Activity: Transfer (pmoles/ 15 min)	Activity: Exchange (pmoles/ 10 min)
None	9	51
0.17% deoxycholate	14	3
0.17% cutscum	63	110
Lipid	27	117
Lipid + 0.17% deoxycholate	62	27
Lipid + 0.17% cutscum	80	193

[a] Transfer assay B and the exchange assay were used with 0.34 mg of E–H enzyme. Crude lipid (60 μg) was added where indicated.

TABLE II: Effect of Dodecylamine on the Reverse Reaction.[a]

Additions	Products of Reverse Reaction (pmoles/min): Phospho-*N*-acetylmuramylpentapeptide	Products of Reverse Reaction (pmoles/min): UDP-*N*-acetylmuramylpentapeptide
None	10	15
3.3×10^{-5} M UMP	5	103
3×10^{-3} M dodecylamine	8	16
3.3×10^{-5} M UMP + 3×10^{-3} M dodecylamine	6	88

[a] The reaction mixture contained 2.3×10^{-6} M lipid diphosphate-*N*-acetylmuramyl-[^{14}C]pentapeptide (11.1 cpm/pmole), 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, and 0.41 mg of membranes in 0.06 ml. The reaction mixtures were preincubated at 25° for 10 min with dodecylamine before initiating the reaction with UMP. The products, UDP-*N*-acetylmuramyl-pentapeptide and phospho-*N*-acetylmuramyl-pentapeptide, were separated by paper chromatography in solvent system A, and the amounts were determined by measuring the radioactivity. For these experiments the lipid diphosphate-*N*-acetylmuramyl-[^{14}C]pentapeptide was prepared in the following incubation: 4.5×10^{-5} M UDP-*N*-acetylmuramyl-[^{14}C]pentapeptide (11.1 cpm/pmole), 0.05 M Tris-HCl (pH 7.8), 0.01 M $MgCl_2$, 0.5 unit of alkaline phosphatase, and 4.4 mg of enzyme in 0.30 ml. The mixture was incubated for 2 hr at 25°. The lipid diphosphate-*N*-acetylmuramyl-[^{14}C]pentapeptide–membrane complex was isolated by centrifugation at 100,000*g* for 30 min at 4°. The precipitate was resuspended in 0.30 ml of 0.005 M Tris-HCl (pH 7.8) containing 1 M KCl and recentrifuged. The washed precipitate was suspended in 0.3 ml of the same buffer and used as the source of enzyme and lipid diphosphate-*N*-acetylmuramyl-[^{14}C]pentapeptide.

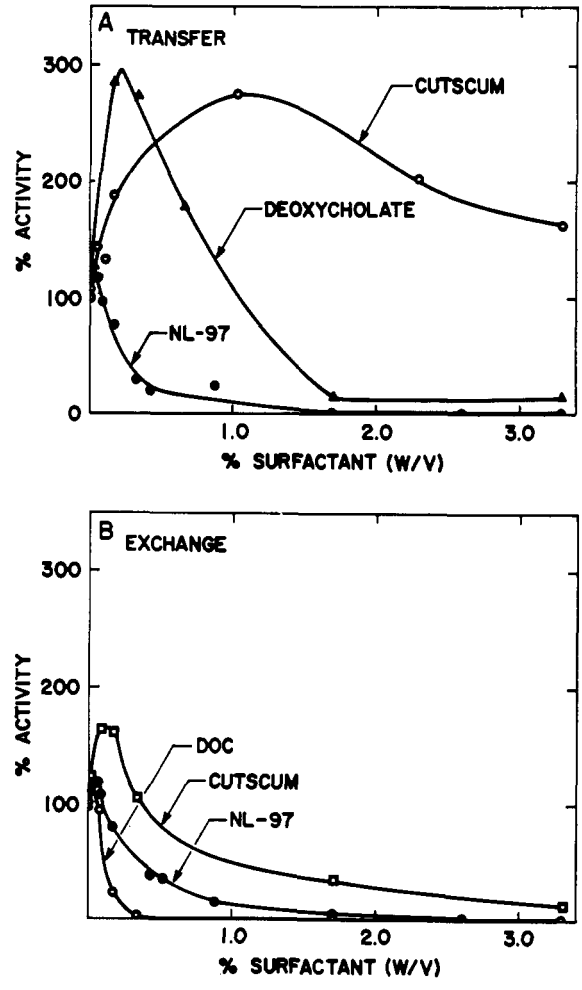

FIGURE 3: Effect of surfactants on the transfer reaction (A) and exchange reaction (B). Transfer assay B and the exchange assay were used with 0.6 mg of membranes in A and B, respectively. The surfactants were preincubated for 15 min with the membrane preparation before initiating the reaction.

pear that reaction 6 occurs *via* intermediates inherent in reaction 5.

Reconstitution of Phospho-N-acetylmuramyl-pentapeptide Translocase. Reconstitution of the lipid acceptor with the enzyme requires the addition of a surfactant, *e.g.*, deoxycholate (Dietrich *et al.*, 1967; Struve, 1966). The choice of surfactant has proven to be the most difficult aspect of this project. A wide variety of surfactants were tested for reconstitution of the transfer reaction. Only deoxycholate and cutscum were effective in reconstituting the transfer system. Sodium dodecyl sulfate, Triton X-100, Brij 35, Tween 20, 40, 80, sodium cholate, and ethyldimethylhexadecylammonium bromide were not effective in reconstituting the lipid acceptor and E–H enzyme (*cf.* Experimental Section). One criterion for a good surfactant in this system is that it affect all assays in an identical manner. In Table I cutscum and deoxycholate are compared in transfer assay B and the exchange assay. In the transfer assay deoxycholate is effective for reconstitution whereas in the exchange assay the same surfactant at the identical concentration is an effective inhibitor. Cutscum, on the other hand, stimulates both the transfer and exchange assays. In contrast to deoxycholate, the stimulation observed with cutscum and lipid is the summation of the individual enhancements. It would appear that cutscum has unmasked or activated additional lipid acceptor in the "E–H enzyme."

In Figure 3A,B an analysis of the effect of concentration of surfactant on the translocase is shown. Three detergents are compared in the transfer assay (Figure 3A) and exchange assay (Figure 3B). Deoxycholate and cutscum enhance the transfer reaction, whereas sodium lauroyl sarcosinate (NL-97) does not. Additional deoxycholate (>1%) markedly inhibits the transfer reaction. In the exchange assay, only cutscum significantly stimulates, whereas deoxycholate at comparable concentra-

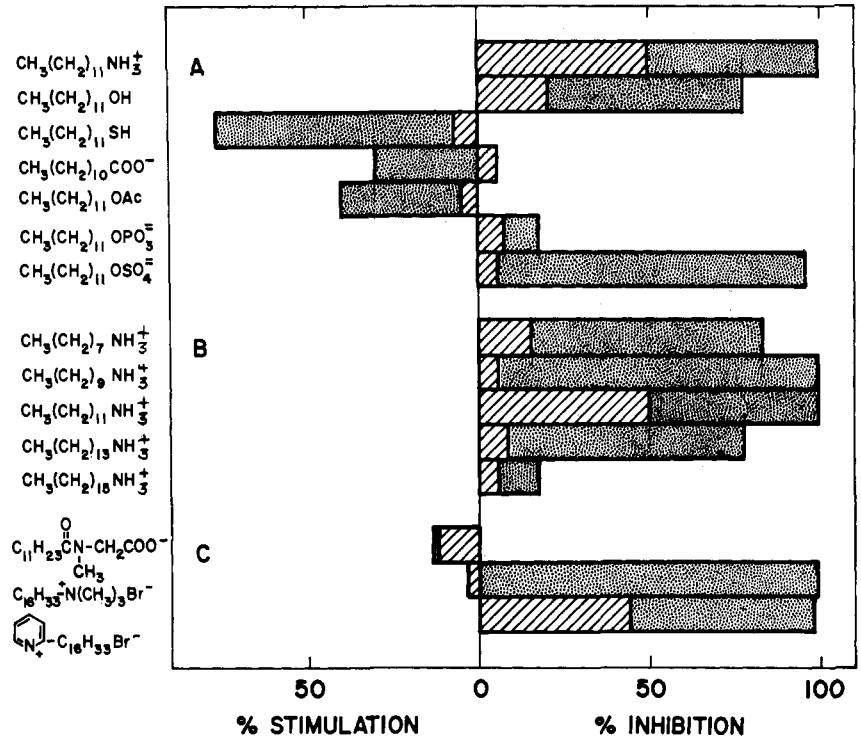

FIGURE 4: Specificity profile of surfactant effects. The exchange assay was used with either 6×10^{-4} M (/////) or 3×10^{-3} M (⋮⋮⋮⋮) surfactant and 0.6 mg of membranes. Dioxane (20%, v/v) was incorporated into the reaction mixture for series A and B to ensure the solubility of the surfactants. Series C does not contain dioxane. The amount of UMP exchanged in the presence of 20% dioxane was 1.09 times that exchanged in the absence of dioxane. The surfactants were preincubated with the membrane preparation for 10 min before initiating the reaction.

tions is an effective inhibitor. The results with sodium lauroyl sarcosinate are identical in both assays. From Figure 3A,B it is evident that two of the surfactants have different effects on the assays.

Inhibition by Dodecylamine. During the course of testing various surfactants, it was observed that dodecylamine was an effective inhibitor of the exchange reaction. The specificity of inhibition is illustrated in Figure 4. Dodecylamine, dodecyl sulfate, and 1-dodecanol are effective inhibitors of the exchange assay (Figure 4A). Dodecylmercaptan, dodecanoic acid, and dodecyl acetate stimulate the exchange assay. The optimum chain length for inhibition by primary alkylamines is twelve carbons (Figure 4B). Two additional surfactants, *N,N,N*-trimethylhexadecylammonium bromide and 1-hexadecylpyridinium chloride, were also found to be effective inhibitors of the exchange reaction (Figure 4C). At low concentrations, sodium lauroyl sarcosinate stimulates to a small extent (see also Figure 3B). With these surfactants no precipitation of the enzyme preparation was detected.

In Figure 5, radioactivity profiles of chromatographic separations from reaction mixtures performed in the presence (Figure 5A) and absence (Figure 5B) of 3×10^{-3} M dodecylamine are shown. The addition of dodecylamine to a reaction mixture results in the formation of phospho-*N*-acetylmuramyl-pentapeptide in an amount comparable to the amount of lipid product formed in the absence of dodecylamine.

The K_i for dodecylamine has been established to be 6.5×10^{-4} M from Dixon plots (Dixon, 1953). Since these plots have a common intercept on the abscissa, it is concluded that dodecylamine is a noncompetitive inhibitor with respect to both UDP-*N*-acetylmuramyl-pentapeptide and UMP in the exchange assay.

The action of dodecylamine has been further examined in the reverse reaction (Table II). At a concentration of 3×10^{-3} M, dodecylamine is not an effective inhibitor of the reverse reaction. In addition, phospho-*N*-acetylmuramyl-pentapeptide is not released from lipid diphosphate-*N*-acetylmuramyl-pentapeptide when incubated in the presence of dodecylamine.

Discussion

The results of Struve *et al.* (1966) and Anderson *et al.* (1965, 1966) have established the presence of an enzyme that catalyzes the transfer of phospho-*N*-acetylmuramyl-pentapeptide from UMP to a lipid acceptor phosphate associated with the membrane. The lipid acceptor is a C_{55}-isoprenoid alcohol (Higashi *et al.*, 1967). The present work provides three observations that must be correlated with the available data on the translocase. These observations are: (1) apparent pyrophosphatase activity for an intermediate stage of the reaction, (2) different surfactant effects in the exchange and transfer assays, and (3) uncoupling by dodecylamine.

We propose that an initial transphosphorylation to a nucleophilic center on the enzyme occurs according to the following reaction sequence shown in eq 9–11

E + UMP-phospho-*N*-acetylmuramyl-pentapeptide ⇌
E···UMP-phospho-*N*-acetylmuramyl-pentapeptide (9)

E···UMP-phospho-*N*-acetylmuramyl-pentapeptide ⇌
UMP···E-phospho-*N*-acetylmuramyl-pentapeptide (10)

UMP···E-phospho-*N*-acetylmuramyl-pentapeptide ⇌
E-phospho-*N*-acetylmuramyl-pentapeptide + UMP (11)

where E···UMP-phospho-*N*-acetylmuramyl-pentapeptide is the Michaelis complex of enzyme (E) and UDP-*N*-acetylmuramyl-pentapeptide, E-phospho-*N*-acetylmuramyl-pentapeptide is the covalent intermediate between phospho-*N*-acetylmuramyl-pentapeptide and enzyme, and UMP···E-phospho-*N*-acetylmuramyl-pentapeptide is the Michaelis complex of the covalent intermediate and UMP.

In the presence of lipid acceptor phosphate a second transphosphorylation occurs with the formation of lipid acceptor diphosphate-*N*-acetylmuramyl-pentapeptide according to the following reactions.

E-phospho-*N*-acetylmuramyl-pentapeptide + acceptor P ⇌
acceptor P···E-phospho-*N*-acetylmuramyl-pentapeptide (12)

acceptor P···E-phospho-*N*-acetylmuramyl-pentapeptide ⇌
E···acceptor P-phospho-*N*-acetylmuramyl-pentapeptide (13)

E···acceptor P-phospho-*N*-acetylmuramyl-pentapeptide ⇌
E + acceptor P-phospho-*N*-acetyl muramyl-pentapeptide (14)

The exchange assay involves reactions 9–11, whereas the transfer assay involves reactions 9–14.

The ratio of the exchange rate to the transfer rate has been established under a variety of conditions and found to vary from 6 to 24. If all components are present under saturating conditions, this ratio would indicate that the pseudo-composite rate constant, k_e, for reactions 9–11 is larger than the pseudo-composite rate constant, k_t, for reactions 9–14. Thus, partial inhibition of reactions 9–11 will not result in an apparent inhibition of the transfer (reactions 9–14).

The differences in surfactant behavior in the two assays are consistent with this model. For example, deoxycholate and cutscum inhibit at a site(s) in reactions 9–11 as shown by the effective inhibition of the exchange reaction. In contrast, these surfactants appear to enhance the transfer of phospho-*N*-acetylmuramyl-pentapeptide to the lipid acceptor. The biphasic stimulation–inhibition is the result of the enhancement in reactions 12–14 coupled with inhibition of reactions 9–11. Since partial inhibition of reactions 9–11 would not affect the over-all transfer, a biphasic curve showing stimulation followed by inhibition can occur.

The apparent pyrophosphatase activity is consistent with the proposed enzyme intermediate. With the depletion of free lipid acceptor in extended periods of reaction, hydrolysis could occur according to the following reaction.

H_2O + E-phospho-*N*-acetylmuramyl-pentapeptide → E + phospho-*N*-acetylmuramyl-pentapeptide (15)

The lag in phospho-*N*-acetylmuramyl-pentapeptide formation is consistent with the hydrolysis of either the lipid product or the covalent intermediate. The zero-order kinetics of phospho-*N*-acetylmuramyl-pentapeptide release are also consistent with the turnover of an enzyme intermediate.

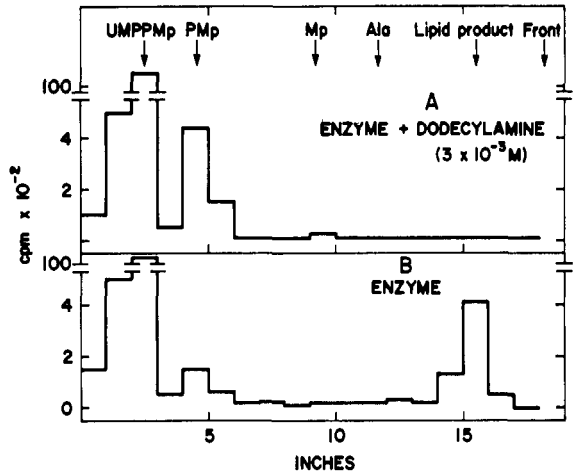

FIGURE 5: Analysis of reaction products in the presence of dodecylamine. The reaction mixture of transfer assay A was used with 0.3 mg of membranes. The mixture was incubated for 60 min at 25° and the reaction was terminated by air drying the mixture on the chromatographic paper. The products were analyzed by descending chromatography in solvent A. The positions of authentic standards are shown in profile A (top). In part A, 3×10^{-3} M dodecylamine was included in the reaction mixture while in part B the complete system is shown.

The "uncoupling" by dodecylamine may be caused by either the absence of the lipid acceptor from its specific site or the nucleophilic attack of dodecylamine on the enzyme intermediate. Dodecylamine does not compete with either UMP or UDP-*N*-acetylmuramyl-pentapeptide. Moreover, it does not cause the hydrolysis of the lipid product. The experiments suggest that dodecylamine prevents the lipid acceptor from participating in the transfer reaction. As a result, the system behaves as if it were depleted of lipid acceptor and thus hydrolysis proceeds as described above in the kinetic experiments. The lack of inhibition by dodecylamine in the reverse reaction (*cf.* Table II) is consistent with the proposed sequence of steps (reactions 9–14). This proposal is based on the following two conditions of the reaction sequence: (1) the rate constant for reaction 15 (the hydrolysis of E-phospho-*N*-acetylmuramyl-pentapeptide) is smaller than the composite rate constant for the reverse of reactions 9–11; (2) the composite rate constant for the reverse of reactions 9–11 is larger than the composite rate constant for the reverse of reactions 12–14. The second condition is based on a comparison of exchange rates to transfer rates. Under these conditions dodecylamine would not be expected to have an effect in the reverse reaction.

Gilby and Few (1957) observed that dodecylamine (1.8×10^{-4} M) is a more effective lytic agent for protoplasts from *M. lysodeikticus* than dodecyl sulfate. Moreover, dodecylamine (2.1×10^{-4} M) is also a more effective bactericidal agent than dodecyl sulfate. The

"uncoupling" of the translocase by dodecylamine may be the mechanism of action of this bactericidal agent.

The identification of the proposed intermediate, enzyme–phospho-*N*-acetylmuramyl-pentapeptide, will contribute to our understanding of the mechanism of this enzyme. Attempts (Heydanek and Neuhaus, 1968) to isolate this intermediate are being made with solubilized preparations of the translocase.

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