(1971), *Biochemistry 10,* 3825.
Goodman, D. B. P., Haussler, M. R., and Rasmussen, H. (1972), *Biochem. Biophys. Res. Commun. 46,* 80.
Goodman, D. B. P., Wong, M., and Rasmussen, H. (1975), *Biochemistry* (in press).
Grisham, C. M., and Barnett, R. E. (1973), *Biochemistry 12,* 2635.
Handler, J. S., Preston, A. S., and Orloff, J. (1969), *J. Clin. Invest. 48,* 823.
Hutchinson, J. H., and Porter, G. A. (1970), *Biochem. Med. 3,* 498.
Kimelberg, H. K., and Papahadjopoulos, D. (1974), *J. Biol. Chem. 249,* 1071.
Kobayashi, Y., and Maudsley, D. V. (1970), in The Current Status of Liquid Scintillation Counting, Bransome, E. D., Ed., New York, N.Y., Grune & Stratton, Inc., pp 76–81.
Leaf, A., Anderson, J., and Page, C. B. (1958), *J. Gen. Physiol. 41,* 657.
Lien, E. L., Goodman, D. B. P., and Rasmussen, H. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 658.
Ling, G. A. (1962), A Physical Theory of the Living State, New York, N.Y., Blaisdel, Appendix H.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Maragoudakis, M. E. (1969), *J. Biol. Chem. 244,* 5005.
Maragoudakis, M. E. (1970), *Biochemistry 9,* 413.
Maragoudakis, M. E. (1971), *J. Biol. Chem. 246,* 4046.
Maragoudakis, M. E., and Hankin, H. (1971), *J. Biol. Chem. 246,* 348.
Reddy, J., Svaboda, D., and Azarnoff, D. (1973), *Biochem. Biophys. Res. Commun. 52,* 537.
Schacht, U., and Granzer, E. (1970), *Biochem. Pharmacol. 19,* 2963.
Ussing, H. H., and Zerahn, K. (1951), *Acta Physiol. Scand. 23,* 110.

Initial Membrane Reaction in the Biosynthesis of Peptidoglycan. Spin-Labeled Intermediates as Receptors for Vancomycin and Ristocetin†

Laurance S. Johnston and Francis C. Neuhaus*

ABSTRACT: Phospho-*N*-acetylmuramyl-pentapeptide translocase (UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla:undecaprenyl phosphate, phospho-MurNAc-pentapeptide transferase) catalyzes the initial membrane reaction in the biosynthesis of peptidoglycan. The spin-labeled nucleotide, UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-2,2,5,5-tetramethyl-*N*-oxylpyrroline-3-carbonyl)-DAla-DAla, was used as a substrate by this enzyme for the synthesis of membrane-associated undecaprenyl-diphosphate-MurNAc-Ala-DGlu-Lys(N^{ϵ}-Tempyo)-DAla-DAla. The spin-labeled substrate and product complex with the antibiotics vancomycin and ristocetin. The association constants for the spin-labeled nucleotide are 6.2×10^5 and 6.2×10^4 M^{-1} for vancomycin and ristocetin, respectively. The association constants for the spin-labeled lipid intermediate are 3.0×10^4 and 2.1×10^4 M^{-1} for vancomycin and ristocetin, respectively. These results indicate that the acyl-DAla-DAla termini of membrane-associated spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide are accessible to vancomycin and ristocetin and that the association constants are smaller than those determined for the corresponding antibiotic spin-labeled UDP-MurNAc-pentapeptide complexes.

The biosynthesis of peptidoglycan is catalyzed by a series of membrane-associated enzymes that utilize two nucleotide-activated precursors, UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-pentapeptide (Ghuysen and Shockman, 1973). The initial enzyme of this series, phospho-MurNAc-pentapeptide[1] translocase, catalyzes the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenyl phosphate with the formation of undecaprenyl-diphosphate-MurNAc-pentapeptide (Neuhaus, 1971). This reaction results in the transfer of a precursor from the cytoplasm to the membrane. A reporter group that reflects the microenvironment in each of these phases would be useful in probing this reaction.

Spin-labels provide sensitive probes to study intermediates in enzyme-catalyzed reactions. The electron spin resonance (ESR) spectrum of the spin-label is a function of the motion that the probe experiences and the polarity of the solvent surrounding the probe. Thus, information about the mobility of the spin-label and its microenvironment can be deduced from its spectrum. It is the purpose of this paper to report the synthesis of spin-labeled UDP-MurNAc-pentapeptide and membrane-associated spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide and to utilize these compounds as receptors for the antibiotics, vancomycin and ristocetin. These experiments indicate that the acyl-

† From the Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60201. *Received January 27, 1975.* This work was supported in part by a grant (AI-04615) from the National Institute of Allergy and Infectious Diseases.

[1] Unless stated, all abbreviations of residues denote the L configuration. The omission of the hyphen, i.e., -DAla- for -D-Ala, conforms with the suggestion cited in *Biochemistry 5,* 2485 (1966). Although not stated, all D-glutamic acid residues are linked through the γ-carboxyl group to the α-amino group of the diamino acid. In UDP-MurNAc-pentapeptide the residues are numbered as follows: UDP-MurNAc-Ala^1-DGlu^2-Lys^3-DAla^4-DAla^5. Abbreviations used are: Tempyo, 2,2,5,5-tetramethyl-*N*-oxylpyrroline-3-carbonyl-; MurNAc, *N*-acetylmuramyl; GlcNAc, *N*-acetylglucosamine; *m*Dap, *meso*-α,ε-diaminopimelic acid; UDP, uridine diphosphate.

DAla-DAla termini of the membrane-associated undecaprenyl-diphosphate-MurNAc-pentapeptide are accessible to vancomycin and ristocetin, and that the association constants are smaller than those determined for the corresponding antibiotic spin-labeled UDP-MurNAc-pentapeptide complexes. Preliminary accounts of this work have been presented (Johnston et al., 1974, 1975).

Experimental Procedure

Materials. UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla from *Staphylococcus aureus* Copenhagen was prepared by procedures described by Hammes and Neuhaus (1974a). 2,2,5,5-Tetramethyl-1-pyrrolin-1-oxyl-3-carboxylic acid *N*-hydroxysuccinimide ester, prepared by the method of Hoffman et al. (1969), was a gift from Dr. Brian Hoffman. Hexamethylphosphoramide was purchased from Aldrich Chemical Co. Vancomycin was kindly given by Dr. Otto K. Behrens, Lilly Laboratories. Fraction IV of this antibiotic was isolated from this preparation by the procedure described by Best et al. (1968) and desalted by the procedure of Nieto and Perkins (1971a). Ristocetin (Lot 757-2493) was kindly given by Dr. Geiszler, Abbott Laboratories, and is a mixture of 90% ristocetin A and 10% ristocetin B. The sources of other chemicals have been previously described (Hammes and Neuhaus, 1974a–c).

UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-2,2,5,5-tetramethyl-1-pyrrolin-1-oxyl-3-carbonyl)-DAla-DAla was prepared in a reaction mixture that contained: 3 μmol of UDP-MurNAc-pentapeptide (salt free) in 0.5 ml of water, 15 μmol of 2,2,5,5-tetramethyl-1-pyrrolin-1-oxyl-3-carboxylic acid *N*-hydroxysuccinimide ester, 2 ml of hexamethylphosphoramide, and 10 μl of triethylamine. The reaction mixture was maintained at 37° for 20 hr. Water (20 ml) was added to the reaction mixture, and the mixture was extracted with 30 ml of diethyl ether. The aqueous fraction was then extracted with six portions (30 ml) of chloroform. The spin-labeled nucleotide was isolated by ion exchange chromatography on DEAE-cellulose (Figure 1). The spin-label is only associated with the high affinity peak. Both nucleotides are active in the exchange reaction (see below) catalyzed by phospho-MurNAc-pentapeptide translocase. The yield of spin-labeled nucleotide averaged 80%. Since the ϵ-amino group of the lysine residue is unreactive to 1-fluoro-2,4-dinitrobenzene, it is concluded that the spin-label is attached to this functional group. At pH 8 the spin-labeled nucleotide has a net negative charge of −3 in contrast to −2 for UDP-MurNAc-pentapeptide. The spin-labeled nucleotide has a 280/260 ratio of 0.497 and a 250/260 ratio of 0.872.

Membrane Fragments and Membrane-Associated Spin-Labeled Undecaprenyl-diphosphate-MurNAc-pentapeptide. Membrane fragments from *S. aureus* Copenhagen were prepared as described by Struve et al. (1966). For the preparation of spin-labeled membrane fragments, phospho-MurNAc-pentapeptide covalently linked to undecaprenyl phosphate was first removed from the membranes in order to generate the highest concentration of undecaprenyl phosphate. The incubation mixture contained: 0.21 *M* KCl; 50 m*M* Tris-HCl (pH 7.8); 42 m*M* $MgCl_2$; 0.21 m*M* UMP; and membranes (120 mg of protein) in a total volume of 10 ml. After 10 min at 25°, the membranes were sedimented by centrifugation for 45 min at 105,000*g*, washed four times in 20 m*M* Tris-HCl (pH 7.8) containing 1 *M* KCl, and resuspended in the same buffer. In the second stage for the introduction of the spin-label the reaction mixture contained: 0.61 *M* KCl; 150 m*M* Tris-HCl (pH 7.8); 120 m*M* $MgCl_2$; 42 μ*M* UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide; 53 units of bacterial alkaline phosphatase; and membranes (80 mg of protein) in a total volume of 10.0 ml. After 24 hr at 22°, the membranes were sedimented by centrifugation for 90 min at 230,000*g*, washed twice in 20 m*M* Tris-HCl (pH 7.8) containing 1 *M* KCl, and resuspended in the same buffer. The spin-labeled membranes were stored at −178°.

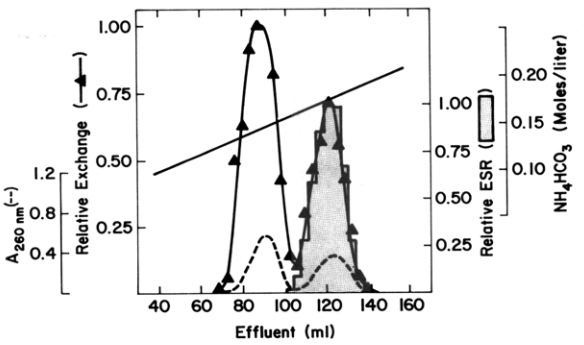


FIGURE 1: Separation of UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide and UDP-MurNAc-pentapeptide on DEAE-cellulose. For isolating UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide the aqueous fraction was applied to a column of DEAE-cellulose (0.9 × 55 cm) and developed with a linear gradient between 0.1 *M* NH_4HCO_3 (125 ml) and 0.3 *M* NH_4HCO_3 (125 ml). To illustrate the separation a mixture containing 0.6 μmol of UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide and 0.8 μmol of UDP-MurNAc-pentapeptide was applied to the column. The gradient is indicated by (—). The nucleotides were located by their absorbance at 260 nm (- -) and by their activity in the exchange reaction catalyzed by phospho-MurNAc-pentapeptide translocase (▲). The amount of spin-label in each fraction (dotted bar graph) was quantitated as described in the Experimental Procedure and is presented relative to the maximum amount in the peak tube.

Analytical Procedures. The ESR measurements were made in a Varian E-4 spectrometer (X-band) at 22 ± 1°. Spectra were recorded at a modulation amplitude of 0.63 G, microwave power of 50 mW, time constant of 1.0 sec, and a scan time of 8 min. A standard aqueous flat cell purchased from Wilmad Glass Co. was used. The rotational correlation times (τ) were calculated with the expression formulated by Stone et al. (1965) using the quadratic term. Although the value may not be absolute (Mukai et al., 1972), τ provides a useful measure of relative mobility.

For spectra from two component mixtures, the peak to peak height of the center line (h_0) was used in analyzing all data. The fraction bound is

$$f_b = (h_f - h_{exp})/(h_f - h_{exp})_\infty \quad (1)$$

where the numerator is the difference between the peak height of uncomplexed spin-label (h_f) and the observed peak height of the experimental mixture (h_{exp}) and the denominator is the maximum difference in the two states established from a double reciprocal plot. The presence of non-base line isoclinic points indicates two components (bound and unbound) in the experimental mixture (Marriott and Griffith, 1974).

Calculation of Association Constants. To establish the association constant of the spin-labeled UDP-MurNAc-pentapeptide–antibiotic complex, increasing concentrations of antibiotic are added to a fixed concentration of spin-labeled UDP-MurNAc-pentapeptide. The fraction of bound spin-label (f_b) was calculated from (1). The concentration of nonbound antibiotic [A] is equivalent to the total antibiotic concentration minus the concentration of bound spin-label. This is based on the 1:1 binding stoichiometry for

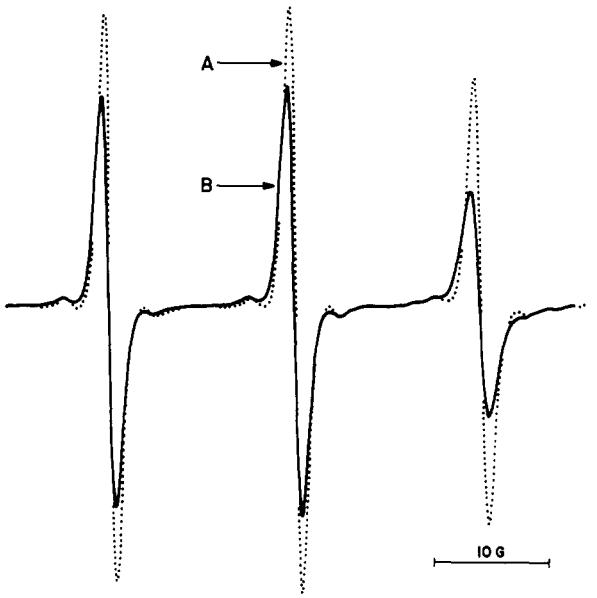


FIGURE 2: Superposition of three-line ESR spectra of UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide (A) and UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide with vancomycin (B). The test solutions contained 2.1×10^{-5} M UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide and 67 mM phosphate buffer (pH 7.0) and B contained in addition 1.1×10^{-4} M vancomycin.

both vancomycin and ristocetin established by Nieto and Perkins (1971a,b) for a molecular weight of 1800. For calculating the association constant (K), the following expression is used:

$$f_b = n - (f_b/K[A]) \quad (2)$$

A graph of f_b vs. $f_b/[A]$ yields a plot whose intercept on the abscissa is the association constant (Thompson and Klotz, 1971). On the basis of the 1:1 binding stoichiometry cited above the number of binding sites (n) is one.

Exchange Assay. For determining the exchange rate catalyzed by phospho-MurNAc-pentapeptide translocase, the exchange of [^{3}H]UMP with the unlabeled UMP moiety of UDP-MurNAc-peptide was measured according to the following reaction:

$$\text{UDP-MurNAc-peptide} + [^3\text{H}]\text{UMP} \overset{K^+Mg^{2+}}{\rightleftharpoons} \text{UMP} + [^3\text{H}]\text{UDP-MurNAc-peptide} \quad (3)$$

In the second stage of the assay, the [^{3}H]UMP was separated from [^{3}H]UDP-MurNAc-peptide by first hydrolyzing the [^{3}H]UMP with alkaline phosphatase and then separating the [^{3}H]uridine from the [^{3}H]UDP-MurNAc-peptide on DEAE-cellulose. The procedure is identical with that described by Hammes and Neuhaus (1974a). Values for K_m and R_{max} are calculated from Lineweaver–Burk plots. At low substrate concentrations, the ratio R_{max}/K_m is used as an index for comparing substrates (Hammes and Neuhaus, 1974a). At high substrate concentrations R_{max} is used for comparison.

Results

Interaction of Spin-Labeled UDP-MurNAc-pentapeptide with Vancomycin and Ristocetin. As a model for studying immobilization of the spin-label, we have examined the complex formed between vancomycin and spin-labeled UDP-MurNAc-pentapeptide. The mechanism of action of this antibiotic is related to its ability to complex with

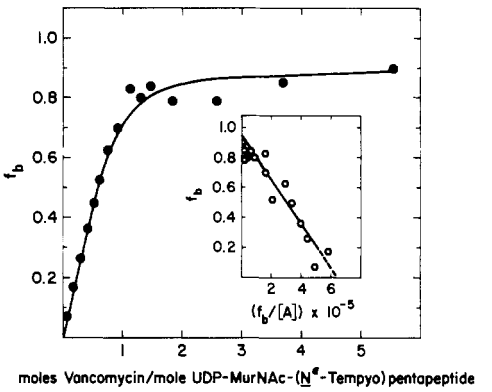


FIGURE 3: Fraction of spin-labeled UDP-MurNAc-pentapeptide bound (f_b) as a function of the total vancomycin concentrations. In the inset f_b is plotted vs. $f_b/[A]$ where [A] is the concentration of nonbound vancomycin. The test system contained: 1.9×10^{-5} M UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide, 67 mM phosphate buffer (pH 7.0), and increasing concentrations of vancomycin.

acyl-D-Ala-D-Ala termini that are present at various phases of polymer synthesis (Perkins and Nieto, 1973, 1974). The antibiotic complexes with the terminal D-Ala-D-Ala moiety of UDP-MurNAc-pentapeptide. UDP-MurNAc-tetrapeptide and UDP-MurNAc-tripeptide do not complex with vancomycin (Perkins, 1969). The composition and structure studies on vancomycin and ristocetin are reviewed by Perkins and Nieto (1974).

Complex formation with vancomycin results in isotropic broadening of the nitroxide spectrum (Figure 2). As a result the rotational correlation time of the spin-label increases from 0.13 to 0.35 nsec. The fraction of the spin-labeled nucleotide bound as a function of the vancomycin concentration is shown in Figure 3. In the inset the association constant (6.2×10^5 M^{-1}) is determined from the abscissa intercept. The calculation of the association constant assumes that the molecular weight of vancomycin is 1800 and is based on the 1:1 binding stoichiometry established by Nieto and Perkins (1971a). For comparison, Nieto and Perkins (1971a) established the association constant of the vancomycin–UDP-MurNAc-Ala-D-Glu-mDap-D-Ala-D-Ala complex to be 7.2×10^5 M^{-1}. In the present work residue 3 is lysine.

The reversibility of vancomycin binding to spin-labeled UDP-MurNAc-pentapeptide is illustrated in Figure 4. In this experiment varying amounts of UDP-MurNAc-pentapeptide were added to a solution containing vancomycin–UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide complex. Addition of unlabeled nucleotide–pentapeptide causes the fractional amount of the spin-labeled nucleotide bound to antibiotic to decrease. Thus, the unlabeled UDP-MurNAc-pentapeptide competes with spin-labeled UDP-MurNAc-pentapeptide for the site on vancomycin. Addition of UDP-MurNAc-tripeptide to the spin-labeled complex results in no change. This observation is consistent with the specificity of interaction between nucleotide and vancomycin (Perkins, 1969). The association constant, 6.3×10^5 M^{-1}, for the vancomycin–UDP-MurNAc-pentapeptide complex was calculated by the competitive displacement technique (Klotz et al., 1948).

Ristocetin has a mechanism of action similar to that described for vancomycin (Wallas and Strominger, 1963). There are, however, certain specificity differences that have been noted (Nieto and Perkins, 1971b). For example, replacement of D-alanine (residue 5) by D-leucine does not result in a change in the association constant of the diacetyl-

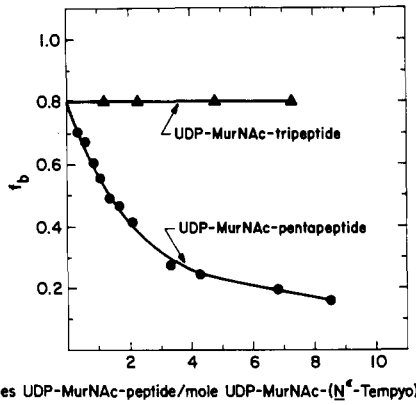


FIGURE 4: Reversibility of vancomycin binding to UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide. Increasing amounts of UDP-MurNAc-Ala-DGlu-Lys (▲) and UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla (●) were added to test solutions containing 1.5×10^{-5} *M* UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide, 2.1×10^{-5} *M* vancomycin, and 67 m*M* phosphate buffer (pH 7.0).

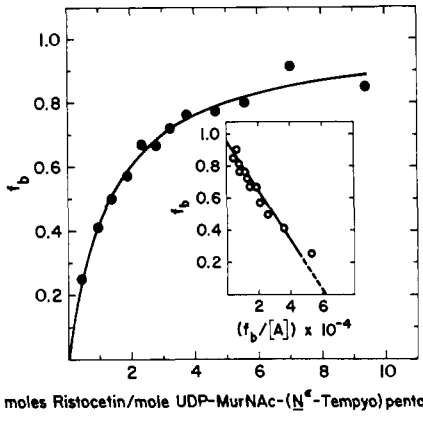


FIGURE 5: Fraction of spin-labeled UDP-MurNAc-pentapeptide bound (f_b) as a function of ristocetin concentration. In the inset f_b is plotted vs. $f_b/[A]$ where [A] is the concentration of nonbound ristocetin. The test solution contained: 2.1×10^{-5} *M* UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide, 67 m*M* phosphate buffer (pH 7.0), and increasing concentrations of ristocetin.

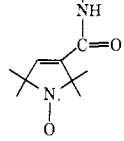
Lys-DAla-DAla-ristocetin complex whereas it has a significant effect on the association constant of the vancomycin complex. In Figure 5, the effect of increasing concentrations of ristocetin on the fraction of spin-label complexed with antibiotic is illustrated. On the basis of a molecular weight of 1800 and a 1:1 binding stoichiometry (Nieto and Perkins, 1971b), K was established to be $6.2 \times 10^4\ M^{-1}$ for the ristocetin-UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide complex (Figure 5, inset). The rotational correlation time for the probe in this complex is 0.48 nsec. As in the case of vancomycin, complex formation is reversible. From these competition studies the association constant for the formation of the ristocetin-UDP-MurNAc-pentapeptide complex is $2.3 \times 10^5\ M^{-1}$. Antibiotics such as bacitracin (Siewert and Strominger, 1967) and moenomycin (Huber and Nesemann, 1968, Lugtenberg et al., 1971, Hammes and Neuhaus, 1974c) that inhibit peptidoglycan synthesis by other mechanisms do not complex with the nucleotide and, therefore, do not result in the partial immobilization of the spin-label. Thus, complex formation of spin-labeled MurNAc-pentapeptide with either vancomycin or ristocetin may provide a useful approach to detect acyl-DAla-DAla termini at the lipid-intermediate stage of peptidoglycan synthesis.

Synthesis of Spin-Labeled Undecaprenyl-diphosphate-MurNAc-(N^{ϵ}-Tempyo)pentapeptide. Phospho-MurNAc-pentapeptide translocase catalyzes the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenyl phosphate according to eq 4.

$$\text{UDP-MurNAc-pentapeptide} + \text{undecaprenyl phosphate} \overset{Mg^{2+}K^{+}}{\rightleftharpoons} \text{undecaprenyl-diphosphate-MurNAc-pentapeptide} + \text{UMP} \quad (4)$$

In order to assess the effect of substituting a spin-label on residue 3, we have compared the activity of the spin-labeled nucleotide with five UDP-MurNAc-peptides (Table I). The forward transfer assay (eq 4) requires UDP-MurNAc-[^{14}C]peptide. Since spin-labeled UDP-MurNAc-pentapeptide with a ^{14}C label was not available, the exchange assay (eq 3) was used. At low substrate concentrations, the apparent first-order rate constant (R_{max}/K_m) for the spin-labeled

Table I: Comparison of UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide with Nucleotide-Activated Precursors in the Exchange Reaction Catalyzed by Phospho-MurNAc-pentapeptide Translocase.[a]

Substrate	R_{max} (M min^{-1} × 10^7)	K_m (μM)	R_{max}/K_m (min^{-1} × 10^3)
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla[b]	4.9	20	25
UDP-MurNAc-Ala-DGlu-Lys-DAla[b]	6.0	58	10
UDP-MurNAc-Ala-DGlu-Lys[b]	0.3	180	0.17
UDP-MurNAc-Ala-DGlu-*m*Dap-DAla-DAla[b]	3.6	22	16
UDP-MurNAc-Ala-DGlu-Lys(NH-Ala)-DAla-DAla[c]	4.5	18	25
UDP-MurNAc-Ala-DGlu-Lys(NH-C(=O)- )-DAla-DAla	4.6	52	8.8

[a] The parameters, R_{max} and K_m, were established with the exchange assay described in the Experimental Procedure. [b] Hammes and Neuhaus, 1974a. [c] Swenson, 1974.

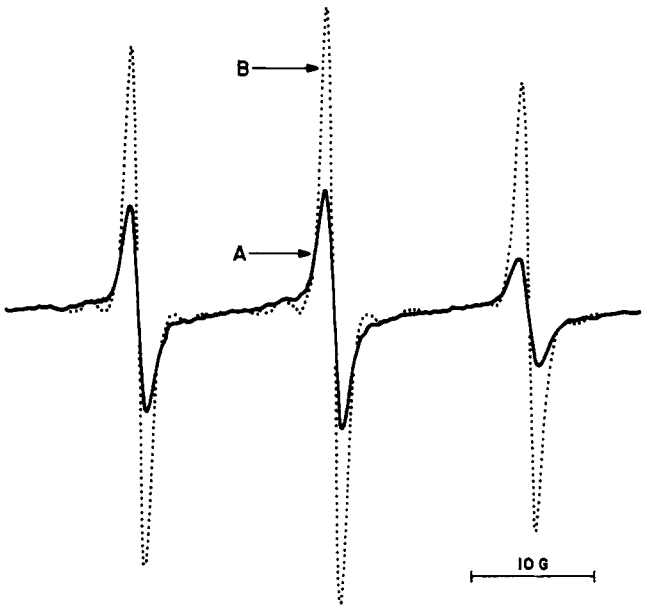


FIGURE 6: Superposition of three-line ESR spectra of membrane-associated undecaprenyl-diphosphate-MurNAc-(N^{ϵ}-Tempyo)pentapeptide (A) and after incubation with UMP (B). For A the test mixture contained: membrane fragments (6.6 mg of protein) containing 3.7 nmol of undecaprenyl-diphosphate-MurNAc-(N^{ϵ}-Tempyo)pentapeptide, 5 μmol of Tris-HCl (pH 7.8), and 250 μmol of KCl in a total volume of 250 μl. For B 190 nmol of UMP was added to the reaction mixture used in A and incubated for 5 min at 22°. Assuming that the product of this reaction is spin-labeled UDP-MurNAc-pentapeptide, the concentration of spin-labeled lipid intermediate equals the concentration of the spin-labeled UDP-MurNAc-pentapeptide formed. This determination is the assay for the amount of spin-labeled lipid intermediate in the membrane fragments.

nucleotide is 0.35 of that for UDP-MurNAc-pentapeptide. At high substrate concentrations, the rate of exchange is virtually identical for the spin-labeled UDP-MurNAc-pentapeptide and the reference nucleotide. Substitutions involving amino acid residue 3 do not markedly affect activity. For example, the replacement of lysine by *meso*-diaminopimelic acid results in a 1.5-fold decrease in R_{max}/K_m and with ornithine in no change (Hammes and Neuhaus, 1974a). As shown by Swenson (1974), acylation of the ϵ-amino group with L-alanine does not affect the activity. From the results in Table I we conclude that the introduction of a spin-label on residue 3 does not affect the activity to a large extent. Thus, it is feasible to synthesize spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide associated with the membrane and use it as a possible receptor for ristocetin and vancomycin.

The transfer of spin-labeled phospho-MurNAc-pentapeptide to undecaprenyl phosphate results in the formation of membrane-associated spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide. For the preparation of membranes with the maximum concentration of undecaprenyl phosphate, membranes were first incubated with UMP to discharge endogenous undecaprenyl-diphosphate-MurNAc-pentapeptide. After incubation with spin-labeled UDP-MurNAc-pentapeptide, the spin-labeled membranes were washed to remove spin-labeled UDP-MurNAc-pentapeptide. The resulting nitroxide spectrum of the lipid intermediate has been isotropically broadened relative to that of spin-labeled UDP-MurNAc-pentapeptide (Figure 6A). The rotational correlation time of the probe is 0.54 nsec. For comparison, τ for UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide is 0.13 nsec. The probe of the spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide has a spectrum similar to that for spin-labeled UDP-MurNAc-pentapeptide in a 50% glycerol solution. The spectrum and the correlation time are derived from membrane-associated spin-labeled lipid intermediate and not from free undecaprenyl-diphosphate-MurNAc-(N^{ϵ}-Tempyo)pentapeptide. Incubation of undecaprenyl-diphosphate-MurNAc-pentapeptide associated with membrane fragments with UMP results in the formation of UDP-MurNAc-pentapeptide (eq 4) (Struve et al., 1966). Incubation of the spin-labeled lipid intermediate associated with membrane fragments with UMP results in isotropic sharpening of the spectrum (Figure 6B). The value of 0.15 nsec for τ is similar to that observed for spin-labeled UDP-MurNAc-pentapeptide. This observation is consistent with the participation of the spin-labeled lipid intermediate in the reverse transfer reaction.

Interaction of Vancomycin and Ristocetin with Spin-Labeled Undecaprenyl-diphosphate-MurNAc-pentapeptide. With the available methodology (Nieto and Perkins, 1971a,b), it is difficult to study the interaction of the membrane-associated undecaprenyl-diphosphate-MurNAc-pentapeptide with either vancomycin or ristocetin. An examination of these interactions and a knowledge of the association constants are of primary importance to our understanding of the mechanism of action of vancomycin and ristocetin. The fraction of the spin-labeled lipid intermediate bound as a function of the vancomycin concentration is shown in Figure 7A. From the plot (Figure 7A, inset) the association constant is established to be $3.0 \times 10^4\ M^{-1}$. In a similar experiment with ristocetin (Figure 7B) the association constant is calculated to be $2.1 \times 10^4\ M^{-1}$.

Discussion

The spin-labeled substrate described in this paper provides the basis for a sensitive method to study the interaction of acyl-DAla-DAla termini with vancomycin and ristocetin. Acyl-DAla-DAla termini of the nucleotide-activated precursor, the lipid intermediates, and the uncross-linked (nascent) peptidoglycan are all potential sites for complex formation. A quantitative assessment of the interaction of these termini in each of these intermediates with antibiotic will contribute to a more precise definition of the primary site of antibiotic action.

The interaction of spin-labeled UDP-MurNAc-pentapeptide with vancomycin and ristocetin provides a model for studying the partial immobilization of the spin-label. The association constant, $6.2 \times 10^5\ M^{-1}$, for vancomycin–UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-Tempyo)-DAla-DAla is similar to that described for vancomycin–UDP-MurNAc-Ala-DGlu-*m*Dap-DAla-DAla ($7.2 \times 10^5\ M^{-1}$) (Nieto and Perkins, 1971a). Although the association constant for the ristocetin–UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide is smaller, $6.2 \times 10^4\ M^{-1}$, the degree of immobilization of the probe is larger (τ = 0.48 nsec compared with 0.35 nsec for the vancomycin complex). Antibiotics such as bacitracin and moenomycin that inhibit peptidoglycan synthesis by other mechanisms do not complex with the nucleotide and, thus, do not result in the immobilization of the spin-label.

Although vancomycin has a significant effect on the synthesis of undecaprenyl-diphosphate-MurNAc-pentapeptide, its primary effect appears to be concerned with the utilization of lipid-linked intermediates for peptidoglycan synthesis (Izaki et al., 1968, Perkins and Nieto, 1974). Recently it was concluded that the formation of a complex between vancomycin and a postulated cell-wall acceptor or between

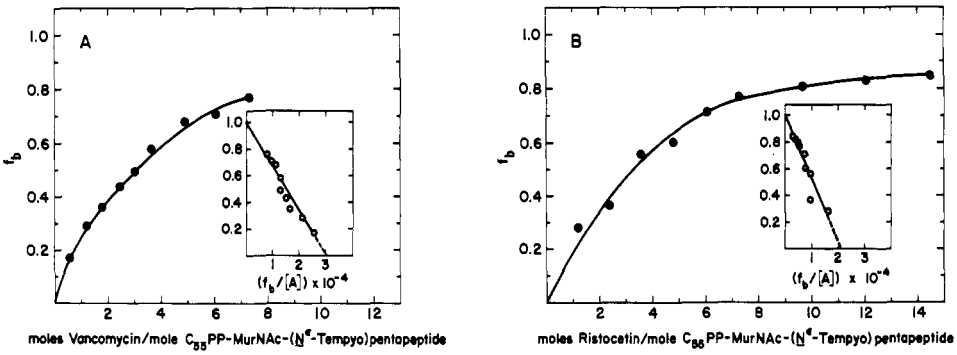


FIGURE 7: Fraction of undecaprenyl-diphosphate-MurNAc-(N^{ϵ}-Tempyo)pentapeptide bound (f_b) as a function of the concentration of vancomycin (A) and ristocetin (B). In the insets f_b is plotted vs. $f_b/[A]$ where [A] is the concentration of nonbound antibiotic. The test solutions contained: membrane fragments (6.6 mg of protein), 3.7 and 4.7 nmol of undecaprenyl-diphosphate-MurNAc-(N^{ϵ}-Tempyo)pentapeptide for A and B, respectively, 5 μmol of Tris-HCl (pH 7.8), 250 μmol of KCl, and increasing amounts of either vancomycin or ristocetin in a total volume of 250 μl.

vancomycin and the enzymes involved in peptidoglycan synthesis does not contribute to the inhibitory action of the antibiotic (Hammes and Neuhaus, 1974c). Ward (1974) proposed that the affinity of vancomycin for some intermediate in peptidoglycan synthesis is greater than the affinity for the precursor, UDP-MurNAc-pentapeptide. This intermediate may be indecaprenyl-diphosphate-MurNAc-pentapeptide, undecaprenyl-diphosphate-MurNAc(pentapeptide)-GlcNAc, or the linear glycan attached to undecaprenyl diphosphate. The synthesis of the lipid intermediate, membrane-associated undecaprenyl-diphosphate-MurNAc-Ala-DGlu-Lys(N^{ϵ}-Tempyo)-DAla-DAla, provides a test system for establishing the association constant for the antibiotic–lipid intermediate complex for comparison with the corresponding constants for the formation of the antibiotic–nucleotide complex. On the basis of the data we conclude that both vancomycin and ristocetin have a higher affinity for UDP-MurNAc-pentapeptide than for membrane-associated undecaprenyl-diphosphate-MurNAc-pentapeptide. These experiments do not provide an assessment of the affinity of undecaprenyl-diphosphate-disaccharide or the postulated undecaprenyl-diphosphate-glycan for either vancomycin or ristocetin.

Complex formation between lipid intermediate and vancomycin reflects the accessibility of the acyl-DAla-DAla termini of the intermediate to external reagents. Thus, we have a sensitive method for establishing the accessibility of the termini at various stages of synthesis. If the termini were imbedded in the lipid bilayer or the peptidoglycan synthesizing complex, they might be inaccessible to the antibiotic.

The synthesis of spin-labeled undecaprenyl-diphosphate-MurNAc-pentapeptide provides methodology to study the intramembranal events of peptidoglycan synthesis. In preliminary reports Johnston et al. (1974, 1975) demonstrated that the peptidoglycan synthesizing system utilizes spin-labeled UDP-MurNAc-pentapeptide for the synthesis of spin-labeled peptidoglycan. Thus, with this probe it may be possible to detect the intramembranal translocation process involved in the biosynthesis of peptidoglycan.

Acknowledgments

We thank Dr. Harvey Lazar, Gillette Research Institute, and Roger Franz for collaboration on some of the initial experiments of this project. We thank Professor Brian M. Hoffman for the spin-labeling reagent and discussions of the data. In addition, we thank Professors I. M. Klotz and P. Loach and Drs. Walter Hammes and John Swenson for discussions. For excellent technical assistance we thank Sue Goyer.

References

Best, G. K., Best, N. H., and Durham, N. N. (1968), *Antimicrob. Agents Chemother. 8,* 115.

Ghuysen, J.-M., and Shockman, G. D. (1973), *in* Bacterial Membranes and Walls, Leive, L., Ed., New York, N.Y., Marcel Dekker, p 37.

Hammes, W. P., and Neuhaus, F. C. (1974a), *J. Biol. Chem. 249,* 3140.

Hammes, W. P., and Neuhaus, F. C. (1974b), *J. Bacteriol. 120,* 210.

Hammes, W. P., and Neuhaus, F. C. (1974c), *Antimicrob. Agents Chemother. 6,* 722.

Hoffman, B. M., Schofield, P., and Rich, A. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 1195.

Huber, G., and Nesemann, G. (1968), *Biochem. Biophys. Res. Commun. 30,* 7.

Izaki, K., Matsuhashi, M., and Strominger, J. L. (1968), *J. Biol. Chem. 243,* 3180.

Johnston, L. S., Hammes, W. P., Lazar, H. A., and Neuhaus, F. C. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1274.

Johnston, L. S., Hammes, W. P., Lazar, H. A., and Neuhaus, F. C. (1975), *in* Proceedings of Symposium on Drug Receptor Interactions in Antimicrobial Chemotherapy, Drews, J., and Hahn, F. E., Ed., Vienna, Springer-Verlag (in press).

Klotz, I. M., Triwush, H., and Walker, F. M. (1948), *J. Am. Chem. Soc. 70,* 2935.

Lugtenberg, E. J. J., van Schijndel-Van Dam, A., and van Bellegem, T. H. M. (1971), *J. Bacteriol. 108,* 20.

Marriott, T. B., and Griffith, O. H. (1974), *J. Magn. Reson. 13,* 45.

Mukai, K., Lang, C. M., and Chesnut, D. B. (1972), *Chem. Phys. Lipids 9,* 196.

Neuhaus, F. C. (1971), *Acc. Chem. Res. 4,* 297.

Nieto, M., and Perkins, H. R. (1971a), *Biochem. J. 123,* 789.

Nieto, M., and Perkins, H. R. (1971b), *Biochem. J. 124,* 845.

Perkins, H. R. (1969), *Biochem. J. 111,* 195.

Perkins, H. R., and Nieto, M. (1973), *Pure Appl. Chem. 35,* 371.

Perkins, H. R., and Nieto, M. (1974), *Ann. N.Y. Acad. Sci.*

235, 348.
Siewert, G., and Strominger, J. L. (1967), *Proc. Natl. Acad. Sci. U.S.A. 57*, 767.
Stone, T. J., Buckman, T., Nordio, P. L., and McConnell, H. M. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010.
Struve, W. G., Sinha, R. K., and Neuhaus, F. C. (1966), *Biochemistry 5*, 82.
Swenson, J. C. (1974), Ph.D. Thesis, Northwestern University.
Thompson, C. J., and Klotz, I. M. (1971), *Arch. Biochem. Biophys. 147*, 178.
Wallas, C. H., and Strominger, J. L. (1963), *J. Biol. Chem. 238*, 2264.
Ward, J. B. (1974), *Biochem. J. 141*, 227.

A Link between Rhodopsin and Disc Membrane Cyclic Nucleotide Phosphodiesterase. Action Spectrum and Sensitivity to Illumination†

James J. Keirns, Naomasa Miki, Mark W. Bitensky,* and Mary Keirns

ABSTRACT: Frog (*Rana pipiens*) rod outer segment disc membranes contain guanosine 3′,5′-cyclic monophosphate phosphodiesterase (EC 3.1.4.1.c) which, in the presence of ATP, is stimulated 5- to 20-fold by illumination. The effectiveness of monochromatic light of different wavelengths in activating phosphodiesterase was examined. The action spectrum has a maximum of 500 nm, and the entire spectrum from 350 to 800 nm closely matches the absorption spectrum of rhodopsin, which is apparently the pigment which mediates the effects of light on phosphodiesterase activity. *trans*-Retinal alone does not mimic light. Half-maximal activation of the phosphodiesterase occurs with a light exposure which bleaches $1/2000$ of the rhodopsins. Half-maximal activation can also be achieved by mixing 1 part of illuminated disc membranes in which the rhodopsin is bleached with 99 parts of unilluminated membranes. Regeneration of bleached rhodopsin by addition of 11-*cis*-retinal to illuminated disc membranes reverses the ability of these membranes to activate phosphodiesterase in unilluminated membranes. If the rhodopsin regenerated by 11-*cis*-retinal is illuminated again, it regains the ability to activate phosphodiesterase. These studies show that the levels of cyclic nucleotides in vertebrate rod outer segments are regulated by minute amounts of light and clearly indicate that rhodopsin is the photopigment whose state of illumination is closely linked to the enzymatic activity of disc membrane phosphodiesterase.

In 1971 Bitensky et al. reported an effect of illumination on the concentrations of adenosine 3′,5′-cyclic monophosphate (cAMP)[1] in suspensions of frog rod outer segment disc membranes. At first this effect was attributed to inhibition of adenylate cyclase by illumination, since in the absence of ATP illumination had no effect on phosphodiesterase activity. Later, however, Miki et al. (1973, 1974) found that the light-sensitive enzyme is phosphodiesterase. In the presence of ATP, or other nucleoside triphosphates, the activity of phosphodiesterase is stimulated 5- to 20-fold by visible light. The disc phosphodiesterase has a lower K_m for guanosine 3′,5′-cyclic monophosphate (cGMP) than for cAMP, so the enzyme is better designated as a cGMP phosphodiesterase (at 10^{-6} *M* or below, cGMP is hydrolyzed 23 times more rapidly than cAMP). The percentage activation of phosphodiesterase by light in the presence of ATP is comparable for the two substrates (Miki et al., 1973).

At the time when the locus of light regulation was thought to be adenylate cyclase, Bitensky et al. (1972) examined the apparent change in cyclase activity as a function of the fraction of rhodopsins bleached, and also examined the action spectrum for the apparent change in cyclase activity. These early experiments suggested that the change in cyclase activity was proportional to the fraction of rhodopsins bleached, and that the action spectrum had a maximum around 500 nm.

With the knowledge that the light-sensitive enzyme is phosphodiesterase rather than cyclase and more information about the technical factors which affect the stability of the light-regulated enzyme, we decided to reexamine the questions of action spectrum and sensitivity to illumination with the following changes in procedure: the more active phosphodiesterase was monitored, permitting the use of more dilute suspensions of disc membranes which allowed more uniform illumination of the sample; we used fresh, fully dark adapted frog retinae; and, finally, we took precautions (rapid assay and incorporation of dithiothreitol) to prevent the gradual decline in the activity of phosphodiesterase which follows activation. These changes in technique have permitted us to generate a very precise action spectrum for the activation of phosphodiesterase, and to precisely determine the relationship between the fraction of rhodopsin bleached and the activity of phosphodiesterase.

We had found (Miki et al., 1973) that complete activa-

† From the Departments of Pathology and Chemistry, Yale University, New Haven, Connecticut. *Received January 13, 1975.* This work was supported by U.S. Public Health Service Grants AM 15016 and CA 13444 and by American Cancer Society Grant BC-106. When this work was performed J.J.K. was a fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

[1] Abbreviations used are: cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate.