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Biosynthesis of Spin-Labeled Peptidoglycan: Spin–Spin Interactions†

Laurance S. Johnston and Francis C. Neuhaus*

ABSTRACT: Membrane preparations from *Gaffkya homari* catalyzed the in vitro biosynthesis of soluble uncross-linked spin-labeled peptidoglycan, a uniformly labeled polynitroxide, from the spin-labeled nucleotide UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-2,2,5,5-tetramethyl-1-pyrrolin-1-oxyl-3-carbonyl)-DAla-DAla (I) and UDP-GlcNAc. Soluble spin-labeled peptidoglycan was separated from membrane fragments and its spin-labeled precursor by centrifugation and gel filtration. The molecular weight distribution of the polymer was examined by agarose gel filtration. Spin-labeled [^{14}C]peptidoglycan was polydisperse with a peak of radioactivity corresponding to a molecular weight of 5.0×10^5. The electron spin resonance spectrum of spin-labeled peptidoglycan was extensively broadened by spin–spin exchange interactions. These interactions were modified by changes in temperature, reduction by ascorbate, hydrolysis by lysozyme, and complexation with the antibiotic, vancomycin. Spin–spin exchange was reduced or eliminated in spin-labeled peptidoglycan by the random reduction of free radicals by ascorbate. A rotational correlation time of 0.37 ns was calculated for the probe in partially reduced spin-labeled peptidoglycan. This compares to a correlation time of 0.13 ns for the substrate (I). Raising the temperature increases spin–spin exchange line broadening. No transition points were observed for spin-labeled peptidoglycan as measured by this method. Degradation of spin-labeled peptidoglycan by lysozyme eliminated the observed spin–spin exchange and yielded products with a mobility similar to I. Complexation of spin-labeled peptidoglycan with vancomycin resulted in both pronounced free-radical immobilization and a decrease in spin–spin exchange. The exchange effects are consistent with distance measurements in molecular models for peptidoglycan.

The biosynthesis of peptidoglycan occurs in three major phases (Ghuysen and Shockman, 1973). The first phase involves the synthesis in the cytoplasm of the nucleotide precursors, UDP-GlcNAc[1] and UDP-MurNAc-pentapeptide. In the second phase, phospho-MurNAc-pentapeptide and GlcNAc, as well as other intermediates, are translocated to the membrane where associated enzymes catalyze the synthesis of nascent peptidoglycan using the carrier, undecaprenyl phosphate. In the extracellular phase, nascent peptidoglycan is transferred to preexisting peptidoglycan as a result of two enzyme-catalyzed reactions, transglycosylation and transpeptidation. Probes that reflect the microenvironment in these phases will be useful in unravelling this complex biosynthetic system.

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 environment surrounding the probe. In a preceeding paper, Johnston and Neuhaus (1975) reported the synthesis of spin-labeled UDP-MurNAc-pentapeptide and spin-labeled undecaprenyl diphosphate-MurNAc-pentapeptide.

It is the purpose of this paper to report the synthesis and partial characterization of spin-labeled, uncross-linked peptidoglycan. The spectrum of spin-labeled peptidoglycan is discussed in terms of spin–spin exchange interactions, spin-label mobility, and parameters that affect them. Preliminary accounts of some aspects of this work have been presented (Johnston et al., 1974, 1975).

Experimental Procedure

Materials. UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-2,2,5,5-tetramethyl-1-pyrrolin-1-oxyl-3-carbonyl)-DAla-DAla was prepared by the procedure described by Johnston and Neuhaus (1975). Vancomycin was a gift of Dr. Otto K. Behrens, Lilly Research Laboratories. Fraction IV of this antibiotic was isolated from the preparation by the method described by Best

† From the Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60201. *Received October 12, 1976.* 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*-oxylpyrrolin-3-carbonyl; MurNAc, N-acetylmuramyl; GlcNAc, *N*-acetylglucosamine; *m*Dap, *meso*-α,ϵ-diaminopimelic acid; UDP, uridine diphosphate.

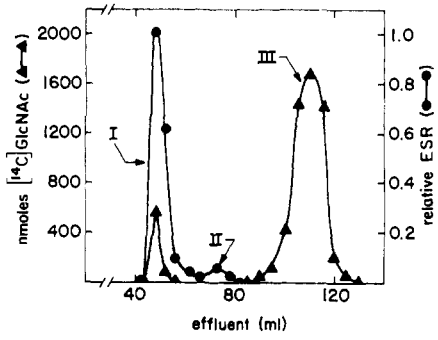


FIGURE 1: Separation of spin-labeled [^{14}C]peptidoglycan from nucleotide precursors. The supernatant fraction from the incubation mixture was applied to column of Bio-Gel P-6 (1.5 × 90 cm) equilibrated with 0.25 M NH_4HCO_3. The column was eluted with the same buffer. The column effluent was monitored for radioactivity (▲) and for ESR (●). The ESR was measured on a 300-μL aliquot of each fraction that had been incubated with 0.15 mg of lysozyme for 60 min at 25 °C. Peaks I, II, and III are spin-labeled [^{14}C]peptidoglycan, spin-labeled UDP-MurNAc-pentapeptide, and UDP-[^{14}C]GlcAc, respectively.

et al. (1968) and desalted by the procedure by Nieto and Perkins (1971). UDP-[^{14}C]GlcNAc (300 mCi/mmol) was purchased from Amersham/Searle. Penicillin G and lysozyme were products of Sigma Chemical Co. Dextrans of molecular weight 70 000 and 500 000 were purchased from Pharmacia Fine Chemicals. Dextrans of molecular weight 250 000, 150 000, and 20 000 were a gift from David A. Weber of Pharmacia Fine Chemicals. Membrane fragments from *Gaffkya homari* were prepared according to the procedure described by Hammes and Neuhaus (1974a).

Peptidoglycan Synthesis. The synthesis of peptidoglycan was routinely assayed by determining the incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc. The amount of this glycan was determined from the radioactivity that was immobilized on descending paper chromatography using an isobutyric solvent system (isobutyric acid-concentrated NH_4OH-H_2O, 66:2:33). The labeled fraction was concluded to be peptidoglycan on the basis of its sensitivity to lysozyme and its dependence on both UDP-activated precursors for synthesis. The procedure is identical with that described by Hammes and Neuhaus (1974a). Values for K_m and V_{max} are calculated from Lineweaver-Burk plots. At low substrate concentrations, the ratio V_{max}/K_m is used as an index for comparing substrates (Hammes and Neuhaus, 1974a). At high substrate concentration, V_{max} is used for comparison.

A typical incubation for the synthesis of spin-labeled peptidoglycan contained: 17 mM ATP; 1.4 mM UDP-[^{14}C]-GlcNAc (1300 dpm/nmol); 8.2 mM NH_4Cl; 50 mM Tris-HCl (pH 7.8); 0.21 M KCl; 50 mM $MgCl_2$; 260 μg/mL benzylpenicillin; 0.37 mM UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-2,2,5,5-tetramethyl-1-pyrrolin-1-oxyl-3-carbonyl)-DAla-DAla, and membrane fragments (21 mg) in a total volume of 3 mL. After 12 h at 22 °C with gentle stirring, the membranes were sedimented by centrifugation at 230 000*g*. The supernatant fraction was applied to a column (1.5 × 90 cm) of Bio-Gel P-6 equilibrated with 0.2 M NH_4HCO_3. The spin-labeled peptidoglycan, which is excluded from the gel (Figure 1), was collected, desalted, and concentrated. The final yield of the spin-labeled polymer averaged 65% of the starting spin-labeled substrate.

Molecular-Weight Estimations. A calibrated Bio-Gel A-5m column (1.5 × 60 cm) equilibrated with 1% NaCl was used for molecular-weight determinations. The column was calibrated with dextrans of molecular weight 20 000, 70 000, 150 000, 250 000, and 500 000. The dextrans were assayed by the anthrone reaction (Scott and Melvin, 1953).

TABLE I: Requirements for [^{14}C]GlcNAc Incorporation into Spin-Labeled Peptidoglycan.[a]

Additions	Incorporation (pmoles/15 min) UDP-MurNAc-pentapeptide	UDP-MurNAc-(N^{ϵ}-Tempyo)-pentapeptide
Complete	170	128
−UDP-MurNAc-peptide	6	4
$-NH_4^+$	162	85
$-Mg^{2+}$	3	2
−ATP	5	2
+Lysozyme	2	4

[a] The reaction mixture contained: 3.0×10^{-5} M UDP-MurNAc-peptide, 3.8×10^{-4} M UDP-[^{14}C]GlcNAc (14.8 dpm/pmol); 0.05 M $MgCl_2$; 8.3×10^{-3} M NH_4Cl; 0.21 M KCl; 6.7×10^{-3} M ATP; 0.05 M Tris-HCl, pH 8.0; membrane fragments (94 μg of membrane protein); 250 μg of benzylpenicillin per mL in a total volume of 60 μL. Lysozyme (300 μg) was added to a complete reaction mixture after terminating synthesis at 15 min and incubated for 1 h at 37 °C. The amount of peptidoglycan was determined by the method described under Experimental Procedure.

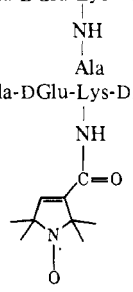
Analytical Procedures. Unless otherwise stated, all ESR measurements were made in a Varian E-4 spectrometer (X-band) at 22 ± 1 °C in a standard aqueous flat cell purchased from Wilmad Glass Co. The spectra were recorded at a modulation amplitude of 0.63 G, a microwave power of 50 mW, a time constant of 1.0 s, and a scan time of 8 min. For variable temperature studies, a low temperature, aqueous solution, sample cell (Wilmad Glass Co.) was used with a liquid nitrogen boil-off temperature unit. The rotational correlation times (τ) were calculated with the expression formulated by Stone et al. (1965) using the quadratic term. The rotational correlation times calculated from the spectra recorded at 50 mW are similar (within 10%) to the values calculated for spectra recorded at lower power settings (2–5 mW). Spin–spin exchange was characterized by the empirical parameter α (Devaux and McConnell, 1972), which depends only on the shape of the spectrum and does not require normalization. The spin–spin exchange parameter, α, is defined by the ratio of peak amplitudes. It equals the peak to peak height of the low field line divided by two times the distance between the midpoint of the vertical line determining the peak to peak height and the baseline.

Results

Synthesis of Spin-Labeled Peptidoglycan. The requirements for peptidoglycan synthesis by membrane fragments from *G. homari* are illustrated in Table I. The incorporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc into peptidoglycan requires UDP-MurNAc-pentapeptide and is stimulated by the addition of NH_4^+, Mg^{2+}, and ATP. When UDP-MurNAc-pentapeptide is replaced by spin-labeled UDP-MurNAc-pentapeptide, 128 pmol of lysozyme-sensitive polymer is synthesized in 15 min, compared with 170 pmol for UDP-MurNAc-pentapeptide. The difference in stimulation between nucleotides by NH_4^+ is caused by variable amounts of NH_4^+ in the nucleotide preparation.

Frequently, the introduction of a spin-label as a probe in a

TABLE II: Specificity of Nascent Peptidoglycan Synthesis in *Gaffkya homari*.

Substrate	V_{max}[c]	K_m (μM)	V_{max}/K_m (L min^{-1} × 10^6)
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla[a]	270	57	4.7
UDP-MurNAc-Ala-DGlu-Lys-DAla[a]	200	77	2.6
UDP-MurNAc-Ala-DGlu-Lys[a]	22	74	0.3
UDP-MurNAc-Ala-DGlu-*m*Dap-DAla-DAla[a]	180	67	2.7
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla[b]	200	55	3.6
			
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla	117	54	2.1

[a] Hammes and Neuhaus, 1974a. [b] Swenson, 1974. [c] V_{max} = pmol of [^{14}C]GlcNAc incorporated (94 μg)$^{-1}$ min^{-1}.

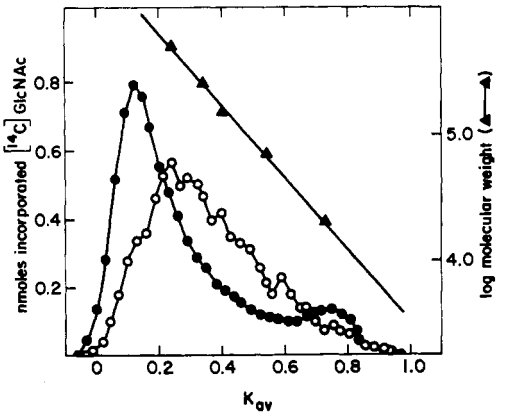


FIGURE 2: Molecular-weight distribution of spin-labeled [^{14}C]peptidoglycan (○) and non-spin-labeled [^{14}C]peptidoglycan (●). Samples of peptidoglycan from the Bio-Gel P-6 column were applied individually to a column of Bio-Gel A-5m (1.5 × 60 cm) equilibrated with 1% NaCl. The column was eluted with 1% NaCl. For purposes of comparison, the total radioactivity applied has been normalized. The column was calibrated (▲) with 1-mg samples of dextrans of 20 000, 70 000, 150 000, 250 000, and 500 000 molecular weight.

biological system perturbs the system it is meant to study. The effect of the spin-label attached to residue 3 on peptidoglycan synthesis is illustrated in Table II. At low substrate concentration, the apparent first-order rate constant, V_{max}/K_m, of the spin-labeled nucleotide is 0.45 of that for UDP-MurNAc-pentapeptide. At high substrate concentrations, the maximum velocity is 0.43 of that observed for the reference nucleotide. Other modifications of the peptide side chain are presented for comparison. Thus, the introduction of this bulky substituent inhibits the synthetic activity of the system by 55%.

Initial experiments indicated that 80–90% of the in vitro synthesized uncross-linked spin-labeled peptidoglycan was released into the supernatant fraction from the membrane fragments. It is this released, soluble spin-labeled peptidoglycan that is described in this paper. The spin-labeled peptidoglycan was separated from its spin-labeled precursor by filtration on Bio-Gel P-6 (Figure 1). This is important because small quantities of the more mobile UDP-MurNAc-(N^{ϵ}-Tempyo)pentapeptide will have a significant effect on the overall appearance of the ESR spectrum of the spin-labeled peptidoglycan.

Spin-labeled peptidoglycan is a polymer consisting of multiple nitroxide radicals in close proximity. Since interactions between nitroxides can affect the spectral shape, an examination of the degree of polymerization will be important in the interpretation of the spectra of spin-labeled peptidoglycan. The molecular-weight distribution was examined by agarose gel filtration on a column calibrated with a series of dextran standards of known molecular weight. As shown in Figure 2, spin-labeled [^{14}C]peptidoglycan is polydisperse with a peak of radioactivity corresponding to a molecular weight of 500 000. This compares with a molecular weight of 10^6 for non-spin-labeled peptidoglycan prepared by procedures identical to that of the spin-labeled polymer. Approximately 34% of the radioactivity is in spin-labeled peptidoglycan that is eluted before dextran at 500 000 compared with 58% for non-spin-labeled peptidoglycan. Forty nine percent of the radioactivity is incorporated in spin-labeled peptidoglycan which is eluted between 70 000 and 500 000 compared with only 27% for non-spin-labeled peptidoglycan. These data, however, do not indicate that a molecule of spin-labeled peptidoglycan has an average molecular weight of 500 000. On the contrary, much of the radioactivity is in a small fraction of the spin-labeled peptidoglycan molecules. For example, approximately 4% of the spin-labeled peptidoglycan molecules contain 34% of the radioactivity which elutes before the 500 000 molecular weight marker. Spin-labeled peptidoglycan of molecular weight 500 000 corresponds to 440 disaccharide-pentapeptide units. Non-spin-labeled peptidoglycan of 10^6 molecular weight corresponds to 1000 disaccharide-pentapeptide units. Thus, the membrane preparation synthesizes a spin-labeled polymer at maximum incorporation of [^{14}C]GlcNAc that is 44% the size of the non-spin-labeled polymer.

Spin–Spin Exchange. The spectrum of spin-labeled peptidoglycan has elements characteristic of spin–spin exchange interactions. Spin–spin exchange is frequently observed when there are high or high local concentrations of spin label (Jost and Griffith, 1972). Since each of the lysine residues in spin-labeled peptidoglycan is acylated with a nitroxide, it was expected that spin–spin exchange would contribute to the spectral shape. The presence of spin–spin exchange renders the calculation of rotational correlation times (τ) difficult.

Spin–spin exchange is brought about by direct electronic overlap of free radicals due to conformational collisions and requires that the free radicals be virtually in van der Waals contact (Ferruti et al., 1970). Electronic overlap leads to an exchange of the spin states of two interacting radicals and thus induces flip-flops between oppositely oriented spins (Sackmann and Trauble, 1972). Any process that increases contact between spins will increase the rate of transitions between electron spin states and therefore decrease the lifetime of the spin states. Decreasing the lifetime will increase the line width. Increasing the temperature will increase the kinetic energy that will result in more rapid, unhindered tumbling and bending which is favorable to spin–spin exchange. Thus, temperature affects spin–spin exchange by promoting thermally activated conformational collisions of the nitroxide units.

Spin–spin exchange can be characterized by the empirical parameter, α, (Devaux and McConnell, 1972), which is described under Experimental Procedure. This parameter depends only on the shape of the spectrum and does not require

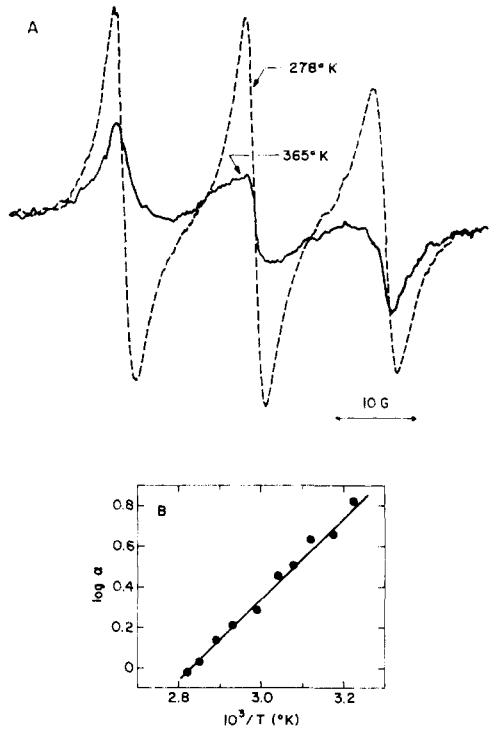


FIGURE 3: The effect of temperature on spectral shape (A) and the spin-spin exchange parameter (α) (B). The superposition of ESR spectra of spin-labeled peptidoglycan at 278 and 365 K is shown in A. In B, log α is plotted against reciprocal temperature. The test solutions contained 5.8×10^{-5} M [^{14}C]GlcNAc incorporated in spin-labeled peptidoglycan.

normalization. As spin-spin exchange decreases, α approaches infinity; as interactions increase, α approaches zero. The spectral shape of spin-labeled peptidoglycan is greatly modified by temperature-dependent spin-spin exchange, as shown in Figure 3A. As the temperature increases, spin-spin exchange increases and α decreases. Log α is linearly related to the reciprocal temperature, as shown in Figure 3B. There is no transition temperature for spin-labeled peptidoglycan, as measured by spin-spin exchange. In addition to line broadening, spin-spin exchange leads to a simultaneous movement of the high- and low-field peaks to the center of the spectrum (Sackmann and Trauble, 1972). This is also observed for spin-labeled peptidoglycan, as shown in Figure 3A. As the temperature increases from 278 to 365 K, the separation between the outer peaks decreases from 31.7 to 28.0 G. When the polynitroxide is degraded, the spectra are essentially temperature independent as expected from a large population of noninteracting free radicals.

Spin-spin exchange interactions can be reduced or eliminated in spin-labeled peptidoglycan by the random reduction of the nitroxide labels by ascorbate. Ascorbate reduces nitroxides to their diamagnetic, hydroxylamine analogues (Kornberg and McConnell, 1971). The partial random reduction of spin-labeled peptidoglycan by ascorbate will result in a polymer in which the majority of the remaining free radicals are of sufficient distance apart so that they are not interacting. The spectra of partially reduced and nonreduced spin-labeled peptidoglycan are shown in Figure 4A. The spectrum of partially reduced spin-labeled peptidoglycan is characteristic of a population of noninteracting spin-labels. Line broadening by spin-spin exchange interactions has been eliminated. Spectral shape is now predominately determined by free-radical mobility. From the spectrum in Figure 4A, a rotational correlation time is calculated to be approximately

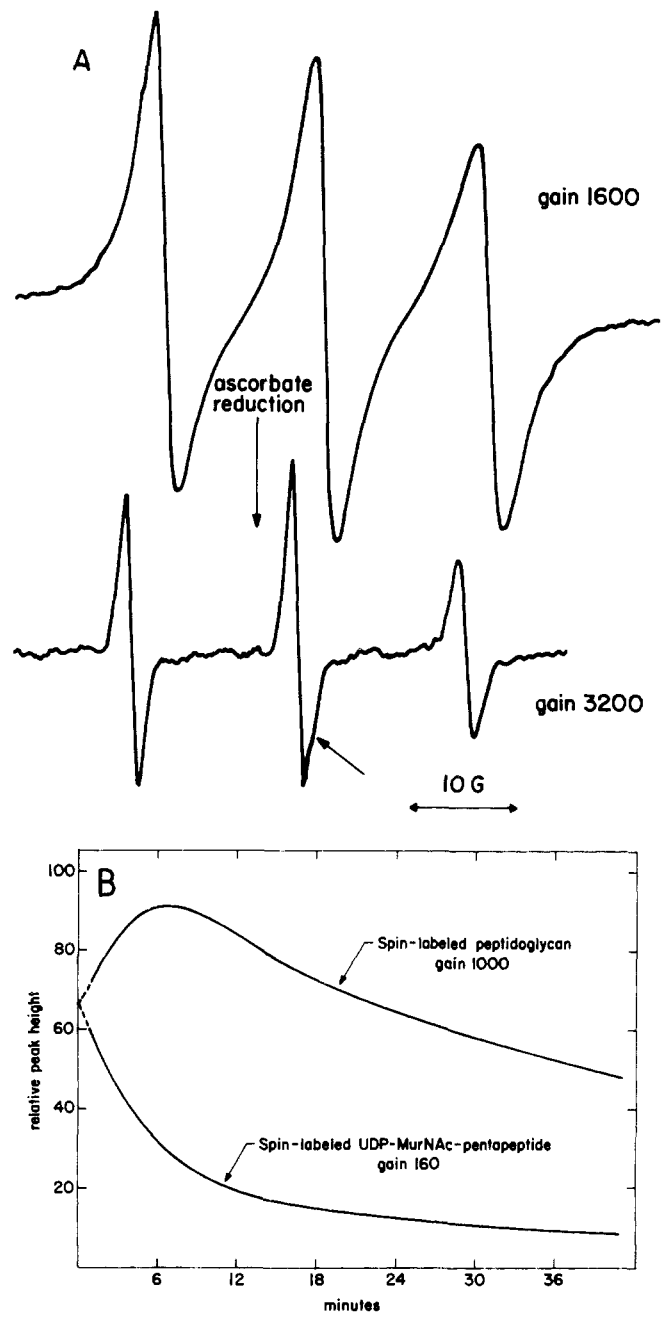


FIGURE 4: Reduction of spin-labeled peptidoglycan by ascorbate. The spectra of spin-labeled peptidoglycan before and after partial reduction by ascorbate is shown in A. In A, the test solution contained 5.6×10^{-5} M [^{14}C]GlcNAc incorporated in spin-labeled peptidoglycan. The reduction was for 20 min with 40 mM ascorbate (pH 7.0) at 22 °C. The centerfield peak (h_0) has contributions from a transient ascorbate free radical (see arrow). A time course of reduction by ascorbate for spin-labeled peptidoglycan and spin-labeled UDP-MurNAc-pentapeptide was recorded (B) by setting the spectrometer to 0-G sweep and measuring peak height of the low-field peak (h_{+1}) as a function of time. Both test solutions contained 10 mM ascorbate (pH 7.0). The concentrations of spin-labeled UDP-MurNAc-pentapeptide and [^{14}C]GlcNAc incorporated in spin-labeled peptidoglycan were 6.2×10^{-5} and 4.9×10^{-5} M, respectively. The time courses of reduction have been normalized to give the same initial peak height. Only the shape of the two curves should be compared.

0.37 ns. This compares to 0.13 ns for spin-labeled UDP-MurNAc-pentapeptide and 0.54 ns for spin-labeled undecaprenyl diphosphate-MurNAc-pentapeptide (Johnston and Neuhaus, 1975).

Spin-spin exchange in spin-labeled peptidoglycan can be further characterized by observing the random reduction of

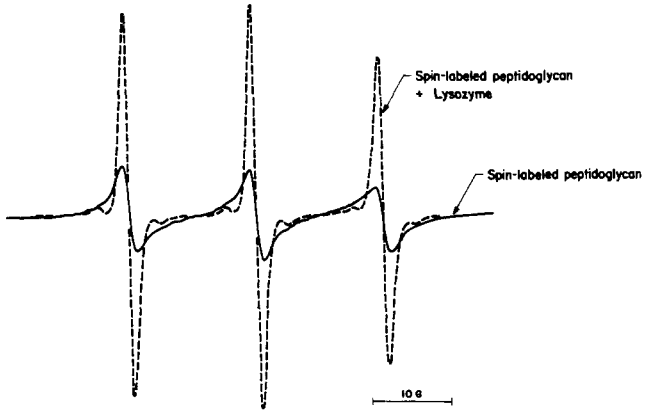


FIGURE 5: Superposition of ESR spectra of spin-labeled peptidoglycan before and after incubation with lysozyme. The test solutions contained 3.8×10^{-5} M [^{14}C]GlcNAc incorporated in spin-labeled peptidoglycan. The incubation was for 1 h at 22 °C with 3.9 μg/mL lysozyme.

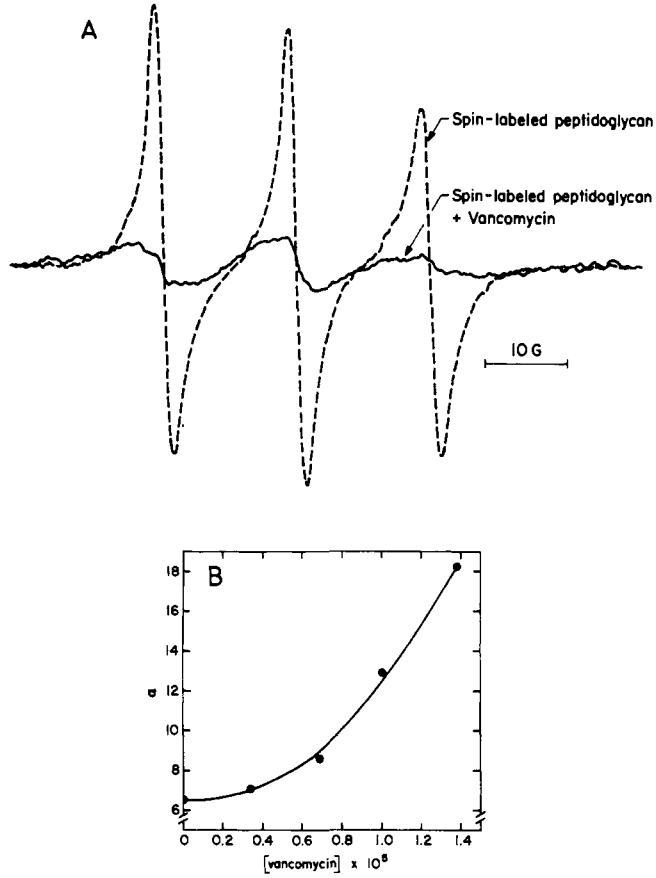


FIGURE 6: Complexation of spin-labeled peptidoglycan with vancomycin. Spectra of spin-labeled peptidoglycan before and after complexation with vancomycin is shown in A. The test solutions contained 4.9×10^{-5} M [^{14}C]GlcNAc incorporated in spin-labeled peptidoglycan and in the indicated spectrum 3.2×10^{-4} M vancomycin. Part B shows the increase in the spin-spin exchange parameter, α, as a function of vancomycin concentration. The test system contained 1.9×10^{-5} M [^{14}C]GlcNAc incorporated in spin-labeled peptidoglycan and increasing concentrations of vancomycin.

the polymer by ascorbate as a function of time. As illustrated in Figure 4B, the addition of ascorbate to spin-labeled UDP-MurNAc-pentapeptide will decrease peak height characterized by first-order kinetics. First-order kinetics, however, are not observed in the case of spin-labeled peptidoglycan. After addition of ascorbate, there is an initial increase in peak height that is followed by a gradual decrease. The initial increase in peak height results from a decrease in spin-spin exchange. Random reduction by ascorbate will increase the average distance between free radicals on the polymer and, therefore, decrease spin-spin exchange. Any diminution of spin-spin exchange will decrease line broadening and increase spectral amplitude. The continued reduction of spin-labels will eventually decrease peak height until all spin-labels are reduced and there is no signal. Thus, spin-spin exchange, which significantly modifies spectral shape, is diminished by reduction with ascorbate.

Hydrolysis of Spin-Labeled Peptidoglycan by Lysozyme. Spin-labeled peptidoglycan is a substrate for lysozyme. Lysozyme is a muramidase that hydrolyzes β (1 → 4) linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues of peptidoglycan. Lysozyme hydrolysis results in the formation of the disaccharide GlcNAc-MurNAc-pentapeptide and smaller amounts of oligosaccharides. As shown in Figure 5, incubation of the spin-labeled peptidoglycan with lysozyme results in marked sharpening of the spectrum. The resulting degradation products have a spectrum similar to that of spin-labeled UDP-MurNAc-pentapeptide. This is expected, since one of the major degradation products of lysozyme hydrolysis, GlcNAc-MurNAc-pentapeptide, has a molecular weight similar to that of UDP-MurNAc-pentapeptide. The rotational correlation time (τ) calculated from the spectrum of the degradation products is 0.15 ns. This value is considerably smaller than the 0.37 ns for partially reduced, spin-labeled peptidoglycan; it is similar to the value of 0.13 ns for spin-labeled UDP-MurNAc-pentapeptide. In addition, spin-spin exchange interactions can no longer be observed. As in the case of reduction by ascorbate, incubation with lysozyme decreases spin-spin exchange line broadening, resulting in an increase in spectral height. Thus, the sharpening of the spectrum of spin-labeled peptidoglycan on the addition of lysozyme results from both an increase in mobility and a decrease in spin-spin exchange of the products.

Interaction of Spin-Labeled Peptidoglycan with Vancomycin. The mechanism of action of vancomycin is related to its ability to complex with acyl-DAla-DAla termini that are present at various phases of peptidoglycan synthesis (Perkins and Nieto, 1973, 1974). UDP-MurNAc-tetrapeptide and UDP-MurNAc-tripeptide do not complex with vancomycin (Perkins, 1969; Hammes and Neuhaus, 1974b). Johnston and Neuhaus (1975) showed that spin-labeled UDP-MurNAc-pentapeptide and spin-labeled undecaprenyl diphosphate-MurNAc-pentapeptide are both receptors for vancomycin. Addition of vancomycin to spin-labeled peptidoglycan results in pronounced broadening of the spectra (Figure 6A). Thus, spin-labeled peptidoglycan is also able to complex with vancomycin.

The reduction in peak height upon complexation with the antibiotic originates from the immobilization of nitroxides which are in close proximity to the acyl-DAla-DAla binding sites. The proximity of the spin-label and the bound antibiotic sterically decreases collision dependent spin-spin exchange interactions. As the concentration of vancomycin increases, there is a diminution of spin-spin exchange as reflected by an increase in α (Figure 6B). The reduction in peak height of spin-labeled peptidoglycan as function of vancomycin concentration is illustrated in Figure 7. Vancomycin has a high affinity for spin-labeled peptidoglycan with little of the polymer remaining unbound in the presence of an equimolar quantity of antibiotic. Since spectral changes result from both mobility and spin-spin exchange factors, the calculation of an associ-

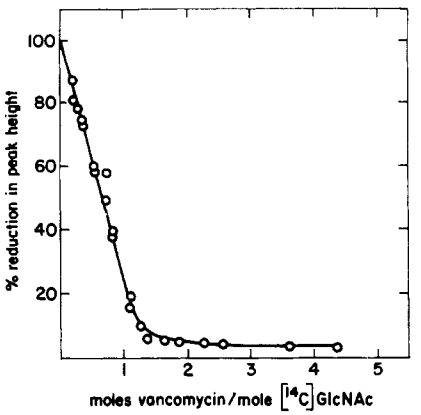


FIGURE 7: Percent reduction in peak height of the centerfield peak (h_0) as a function of vancomycin concentration. The test system contained 4.9 $\times 10^{-5}$ M [^{14}C]GlcNAc incorporated in spin-labeled peptidoglycan and increasing concentrations of vancomycin. This is based on a molecular weight of 1800 for vancomycin (Nieto and Perkins, 1971).

ation constant for the vancomycin–spin-labeled peptidoglycan complex is rendered difficult.

Discussion

This paper describes the synthesis and partial characterization of uncross-linked, spin-labeled peptidoglycan. The ESR spectrum of the spin-labeled polymer is evaluated in terms of spin–spin exchange interactions and mobility parameters. Four factors that affect these terms are discussed.

Only a few syntheses of polynitroxides have been reported (Griffith et al., 1967; Drefahl et al., 1968; Ferruti et al., 1970). The preparation of most of these polynitroxides involved the random attachment of spin-labels to previously synthesized polymers and, thus, were not uniformly labeled. In contrast, the enzymatic synthesis of spin-labeled peptidoglycan from the spin-labeled precursor results in a uniformly labeled, polydisperse polymer of high molecular weight. The ESR spectrum of this polynitroxide in aqueous solution is extensively broadened by spin–spin exchange interactions. Any factor that will modify exchange interactions will also modify spectral shape. In this paper, temperature, reduction by ascorbate, hydrolysis by lysozyme, and complexation with vancomycin are used to modify spin–spin exchange in spin-labeled peptidoglycan. These agents act either by reducing collisions between free radicals or decreasing the population of interacting spin-labels.

The magnitude of spin–spin exchange in spin-labeled peptidoglycan is dependent on temperature. Increases in temperature enhance spin–spin exchange by promoting conformational collisions between nitroxides. In addition, increased thermal energy may affect the degree and nature of hydrogen bonding in spin-labeled peptidoglycan (cf. Kelemen and Rogers, 1971; Formanek et al., 1974). A reduction in hydrogen bonding will increase rotational freedom which would favor spin–spin exchange. Spin-labels have been used to detect thermally induced transitions in many biological macromolecules. However, in the case of spin-labeled peptidoglycan no transition was observed in the temperature range investigated, as measured by spin–spin exchange.

Random reduction by ascorbate is another way to affect spin–spin exchange. Even though reduction will lower the concentration of free radicals, it will produce an initial increase in spectral amplitude by reducing line broadening from spin–spin exchange. The spectrum of partially reduced spin-labeled peptidoglycan will depend primarily on nitroxide mobility and not on spin–spin exchange. Free-radical mobility (τ) in this labeled glycan (0.37 ns) is greater than that in spin-labeled, membrane-bound undecaprenyl diphosphate-MurNAc-pentapeptide (0.54 ns) and less than that in spin-labeled UDP-MurNAc-pentapeptide (0.13 ns). The immobilization of the spin-label alone cannot account for the magnitude of line broadening for spin-labeled peptidoglycan. Thus, spectral shape in this spin-labeled polymer is primarily determined by spin–spin exchange contributions.

Spectral shape is also modified by incubation of spin-labeled peptidoglycan with lysozyme. The spectra of the lysozyme degradation products do not show spin–spin exchange. The rotational correlation time (τ) calculated from the spectrum of the degradation products is similar to that of spin-labeled UDP-MurNAc-pentapeptide. As spin-labeled peptidoglycan is hydrolyzed, there is a reduction of spin–spin exchange. As this exchange is reduced, mobility parameters will be the primary determinant of spectral shape. The observed spectral sharpening upon degradation provides the basis for a sensitive lysozyme assay, which will be presented in a future paper.

Complexation of vancomycin with spin-labeled peptidoglycan is the fourth way to affect spin–spin exchange. Complexation of the antibiotic with either spin-labeled UDP-MurNAc-pentapeptide or spin-labeled undecaprenyl diphosphate-MurNAc-pentapeptide results in immobilization of the spin-label (Johnston and Neuhaus, 1975). This is also observed for spin-labeled peptidoglycan. However, in addition to nitroxide immobilization, vancomycin causes spectral changes that result from reducing spin–spin exchange. Any phenomenon which reduces the collision frequency between nitroxides will decrease spin–spin exchange. This is the case for vancomycin, whose close proximity to the spin-label sterically decreases collisions.

Spin–spin exchange interactions require that the free radicals be within approximately a 6-Å collision distance (Ferruti et al., 1970). Exchange will fall off rapidly with increasing distance. Thus, the spin-labeled peptidoglycan must have sufficient rotational freedom for free-radical interaction. The nitroxide by itself has a moderate degree of mobility based on its rotational correlation time. The distance between peptide attachment points is 10.3 Å (Kelemen and Rogers, 1971; Formanek et al., 1974), which indicates that the greatest average distance between nitroxides is 20.6 Å. This would occur when the peptide side chains are directed in opposite directions along the glycan backbone. However, various models propose a relatively linear glycan backbone in which the peptide side chains are aligned parallel (Tipper, 1970; Kelemen and Rogers, 1971; Formanek et al., 1974). The parallel arrangement of the side chains in these models dictates a 10.3-Å average distance between nitroxides. This is a distance in which nitroxide collisions will occur at a moderate frequency if they possess sufficient freedom of motion. If the peptide side chains are aligned in other conformations, the collision frequency will be lower. If molecular motion is arrested, the average distance between nitroxides would be greater than the 6-Å interaction distance proposed by Ferruti et al. (1971). Thus, any process that reduces this freedom of motion should reduce or eliminate spin–spin exchange. This is the case for frozen spin-labeled peptidoglycan where spin–spin exchange has been reduced or eliminated and the primary nitroxide–nitroxide interaction is the magnetic, anisotropic, dipole–dipole interaction (Johnston and Neuhaus, unpublished observations). The dipole–dipole interaction, which is inversely proportional to the third power of the distance between free radicals (Jost and Griffith, 1972),

has a longer range than collision dependent spin–spin exchange. The free radicals of frozen spin-labeled peptidoglycan will be within a range of moderate dipole–dipole interactions regardless of which arrangement the peptide side chains may take.

Spin-labeled peptidoglycan is one in a series of spin-labeled intermediates that reflect various phases of peptidoglycan biosynthesis. These intermediates are the basis of a useful technique for investigating the membrane translocation of intermediates involved in the assembly of peptidoglycan. In addition, they can be used to study the mode of action of various antimicrobial agents that interact with these intermediates. The experiments with spin-labels complement those using other commonly employed techniques.

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References

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

Devaux, P., and McConnell, H. M. (1972), *J. Am. Chem. Soc. 94,* 4475.

Drefahl, G., Hoerhold, H. H., and Hofmann, K. D. (1968), *J. Prakt. Chem. 37,* 137.

Ferruti, P., Gill, D., Klein, M. P., Wang, H. H., Entine, G., and Calvin, M. (1970), *J. Am. Chem. Soc. 92,* 3704.

Formanek, H., Formanek, S., and Wawra, H. (1974), *Eur. J. Biochem. 46,* 279.

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

Griffith, O. H., Keana, J. F. W., Rottschaefer, S., and Warlick, T. A. (1967), *J. Am. Chem. Soc. 89,* 5072.

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

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

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 Topics in Infectious Diseases, Drews, J., and Hahn, F. E., Ed., Vienna, Springer-Verlag, p 269.

Johnston, L. S., and Neuhaus, F. C. (1975), *Biochemistry 14,* 2754.

Jost, P., and Griffith, O. H. (1972), *Methods Pharmacol. 2,* 223.

Kelemen, M. V., and Rogers, H. J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 992.

Kornberg, R. D., and McConnell, H. M. (1971), *Biochemistry 10,* 1111.

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

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.

Sackmann, E., and Trauble, H. (1972), *J. Am. Chem. Soc. 94,* 4492.

Scott, T. A., and Melvin, E. H. (1953), *Anal. Chem. 25,* 1656.

Stone, T. J., Buckman, T., Nordio, P. L., and McConnell, H. M. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 1010.

Swenson, J. C. (1974), Thesis, Northwestern University, Evanston, Ill.

Tipper, D. J. (1970), *Int. J. Syst. Bacteriol. 20,* 361.