Characterization of Octapeptin–Membrane Interactions Using Spin-Labeled Octapeptin†

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ABSTRACT: Octapeptin is a membrane-active peptide antibiotic that contains a C_{10} fatty acid covalently attached to the peptide through an amide bond. Interactions of octapeptin with bacterial membranes and phospholipids were characterized by using spin-labeling techniques and octapeptin derivatives containing fatty acids of varying chain length. Acyl modification of octapeptin demonstrated that the fatty acid of the antibiotic contributed to the antimicrobial activity of octapeptin and its affinity for membranes. The influence of octapeptin and C_2 acyloctapeptin on the rates of ascorbate reduction of several membrane-bound doxyl stearates was also examined. These studies demonstrated that octapeptin increased the rate of diffusion of ascorbate into the lipid bilayer and suggested that the acyl chain contributed to this activity. In addition, an acyl spin-labeled analogue of octapeptin was prepared and shown to retain biological activity. Spectral analysis showed that octapeptin does not aggregate in solution over a wide concentration range. However, the isotropic splitting constant indicated that the acyl chain of octapeptin is not completely exposed to water. It is proposed that the acyl chain of octapeptin in solution interacts with hydrophobic amino acids in the peptide, which partially shields the acyl chain from water. Spectral features of the spin-labeled antibiotic bound to phospholipid dispersions were consistent with directional binding of octapeptin to lipid bilayers with insertion of the fatty acid into the hydrocarbon domain.

The octapeptins are a closely related class of broad-spectrum peptide antibiotics isolated from *Bacillus circulans* (Meyers et al., 1973a,b, 1976; Parker & Rathnum, 1973). These antibiotics contain a C_{10} or C_{11} β-hydroxy fatty acid covalently attached to a cyclic peptide through a peptide bond (Figure 1). The peptide contains four residues of diaminobutyric acid (DAB) which make the antibiotic tetracationic at physiological pH.

The primary site of action of the octapeptins and polymyxins is the plasma membrane of microorganisms (Storm et al., 1977). A variety of biochemical and biophysical studies have established that octapeptin binds to bacterial membranes and enhances the permeability of the membrane to protons and other cations (Rosenthal et al., 1976, 1977; LaPorte et al., 1977; Swanson & Storm, 1979). There is evidence that the polymyxins and octapeptins interact preferentially with anionic phospholipids in the membrane (Few, 1955; Newton, 1956; Pache et al., 1972; Imai et al., 1973; Rosenthal et al., 1976; Teuber & Miller, 1977; Hartman et al., 1978). However, the mechanism for the perturbation of the membrane structure by octapeptin has not been elucidated and the contribution of the fatty acid moiety to the biological properties of the peptide and binding to membranes is undefined.

In the present report, interactions between octapeptin and membranes were studied by using several structural analogues of the antibiotic and by spin-labeling techniques. This study indicates that the peptide antibiotic has measurable effects on the membrane structure and that the fatty acid of the antibiotic inserts itself into the hydrophobic core of membranes.

Experimental Procedures

Materials. Octapeptin tetrahydrochloride and 4-Cbz-deacyloctapeptin hydrochloride[1] were provided by the Squibb Institute for Medical Research. 5-, 12-, and 16-DS spin-labels were purchased form Syva. Synthetic chemicals were the highest purity available from Aldrich, and Pt_2O catalyst was from Matheson Coleman and Bell. 1-^{14}C-labeled fatty acids were purchased from NEN, Amersham, or ICN, and all phospholipids were from Sigma.

Instrumentation. Electron spin resonance (ESR) spectra were recorded on a Varian E-3 spectrophotometer equipped with a variable temperature controller. Samples were contained in sealed disposable pipets or 5-μL capillary tubes unless indicated otherwise. A Varian E-9 instrument equipped for time averaging was used when higher resolution was required.

Synthesis of [^{14}C]Acyloctapeptins. 4-Cbz-deacyloctapeptin (1 mmol), 1-^{14}C-labeled fatty acid (or Na^+ salt) (1.2 mmol), and *N*-hydroxybenzotriazole (1.2 mmol) were dissolved in 100 mL of dry DMF, and the pH was adjusted to 7.5 with TEA. Dicyclohexylcarbodiimide (DCCD) (1.5 mmol) was added slowly and the pH maintained with TEA. After 2 h the solvent was removed in vacuo. The residue was taken up in AcOEt, filtered, and then washed successively with H_2O, 5% $NaHCO_3$, H_2O, 10% $KHSO_4$, and H_2O. The solution was dried (Na_2SO_4) and the AcOEt removed in vacuo. The residue was taken up in MeOH and passed through a column of Dowex AG50W-X2(H^+) equilibrated in MeOH to remove unreacted 4-Cbz-deacyloctapeptin. MeOH was removed in vacuo to yield a glassy solid.

The peptide was deblocked in 10–12 mL of 6 M HBr in AcOH for 1 h at room temperature and precipitated with Et_2O. The product was washed 1 time each with Et_2O and

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[1] Abbreviations used: 5-DS, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy; 12-DS, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy; 16-DS, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy; MIC, minimal inhibitory concentration; 9-DU-octapeptin, (9-doxylundecanoyl)octapeptin; PC, phosphatidylcholine; PG, phosphatidylglycerol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Cbz, carbobenzyloxy; TEA, triethylamine.

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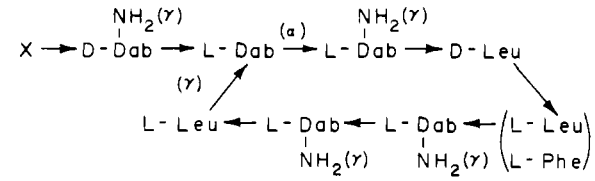

FIGURE 1: Structure of octapeptin illustrating the position of the fatty acid (X).

AcOEt. The final workup included taking the residue into a small amount of MeOH, decolorizing it with activated charcoal, precipitating it with Et_2O, and removing the solvent under high vacuum.

Each derivative gave one ninhydrin-positive spot of radioactivity on silica gel TLC developed with 1-PrOH–pyridine–AcOH–H_2O–AcOEt (5:1:2:2:2 v/v): [^{14}C]acetyloctapeptin (R_f 0.47); [^{14}C]stearyloctapeptin (R_f 0.65). Anal. [$C_{40.5}H_{75}O_9N_{13}Cl_4$ + $(CH_2)_nCH_3$] C, H, N, Cl.

Synthesis of (9-Doxylundecanoyl)octapeptin. 9-Doxylundecanoic acid was prepared according to the synthetic scheme described by Keana et al. (1967). Changes in the experimental procedures of Hubbel & McConnell (1971) for preparation of doxyl stearates from chloroformyl esters have been made only as required in the workup of synthetic intermediates. The method for coupling of 9-doxylundecanoic acid to 4-Cbz-deacyloctapeptin is identical with that described for the [^{14}C]acyloctapeptins. Hydrogenation as opposed to HBr–AcOH was used for deblocking.

(*A*) *Monomethyl Monochloroazelate.* Monomethylazealic acid was refluxed with a 1.5 M excess of thionyl chloride for 3 h. Excess thionyl chloride was distilled off, and the monomethyl monochloroazealate was vacuum distilled (102 °C at 2 mmHg): IR max (cm^{-1}) 1725 and 1775. Anal. ($C_{10}H_{17}O_3Cl$) C, H, Cl.

(*B*) *9-Ketoundecanoic Acid Methyl Ester.* Monomethyl monochloroazealate in dry benzene was added slowly to a suspension of diethylcadmium in benzene at 0 °C, previously prepared from the Grignard reagent. The crude product was chromatographed over silica gel by eluting with hexane–AcOEt (9:1). Dinitrophenylhydrazone (DNPH)-positive fractions were pooled, and the oily keto ester was recrystallized 3 times from pentane at −20 °C: IR max (cm^{-1}) 1710 and 1730. Anal. ($C_{12}H_{22}O_3$) C, H.

(*C*) *9-Doxylundecanoic Acid.* 9-Ketoundecanoic acid methyl ester was converted to the oxazolidine by refluxing in toluene containing 2-amino-2-methyl-1-propanol and *p*-toluenesulfonic acid monohydrate and was oxidized directly with *m*-chloroperbenzoic acid in Et_2O at 0 °C. The crude mixture was first applied to a silica gel column and eluted with hexane–Et_2O (7:3). The yellow doxyl ester (fast moving) was finally purified by using preparative TLC on silica gel eluted with toluene–AcOEt (99:1), R_f 0.35.

The purified doxyl ester was deesterified directly to yield a yellow waxy product which melted at ~25 °C and showed one spot on silica gel TLC eluted with ether–hexane–AcOEt (75:25:1): R_f 0.50; IR max (cm^{-1}) 1695–1705. Anal. ($C_{15}H_{28}O_4N$) C, H, N.

(*D*) *4-Cbz-(9-doxylundecanoyl)octapeptin.* Following coupling and workup, the orange product showed one spot on silica gel TLC developed with $CHCl_3$–MeOH–HAc (90:10:1), R_f 0.65. Comparative ESR spectra before and after coupling confirmed transfer and recovery of the spin-label was >95%. Anal. ($C_{86.5}H_{122}O_{19}N_{14}$) C, H, N.

(*E*) *(9-Doxylundecanoyl)octapeptin Hydrochloride.* Only marginal recovery of the spin-label could be achieved after hydrogenation over various Pd/C and Pt/C catalysts. However, successful removal of the blocking groups was achieved by using PtO_2 in AcOH–H_2O with quantitative recovery of the spin-label under mildly alkaline conditions and exposure to air.

Typically, 100 mg of 4-Cbz-(9-doxylundecanoyl)octapeptin was dissolved in 10 mL of AcOH–H_2O (9:1) and hydrogenated over 50 mg of PtO_2 for 20–24 h until uptake of H_2 ceased. The catalyst was filtered and the solvent stripped in vacuo. The residue was taken up in 5 mL of BuOH and partitioned over 5 mL of saturated Na_2HCO_3. The mixture was stirred vigorously with exposure to air for 4 h, sufficient to maximally recover the label (70–80%). The mixture was centrifuged, the Na_2HCO_3 phase removed, and the BuOH phase washed carefully with H_2O (2 times). Avoiding the use of strong anion-exchange resins to prepare the HCl salt, we partitioned the BuOH solution over 0.1 N HCl. After the removal of the BuOH phase, the aqueous phase was back-extracted 2 times with BuOH and all washes were combined. The antibiotic was assayed with a Lowry procedure (Lowry et al., 1951) using octapeptin tetrahydrochloride as a standard. TLC on silica gel developed with the solvent system previously described for the [^{14}C]acyloctapeptins showed one spot (R_f 0.53), as compared to R_f 0.52 for authentic octapeptin. Anal. ($C_{54.5}H_{102}O_{11}N_{14}Cl_4$) C, H, N, Cl.

Antimicrobial Characterization of Modified Octapeptins. Stationary phase cultures of *Escherichia coli* or *Bacillus subtilis* were inoculated (1:100) into enriched media containing antibiotics. MIC values are defined as the lowest concentration required to prevent growth once controls have reached the stationary phase.

Membrane Vesicles. E. coli ML-308-225 was grown on minimal medium A containing 1% sodium succinate with a trace of $FeSO_4 \cdot 7H_2O$. Vesicles were prepared according to Kaback (1971) and stored under liquid N_2 in 100 mM potassium phosphate (pH 6.6) at 3–5 mg of protein per mL.

Binding of [^{14}C]Acyloctapeptins to Membrane Vesicles. Binding was determined by rapid filtration on cellulose acetate filters (0.45 μm). Potassium phosphate (100 mM, pH 6.6) was used throughout to dilute and wash samples. Rapidly thawed vesicles were first diluted to 1 mg of protein per mL. Samples of 100 μL were treated with [^{14}C]acyloctapeptins for 30 s, diluted with 2 mL of the buffer, filtered immediately, and washed with an additional 2 mL. Filters were dried and counted in 5 mL of Aquasol. All values were corrected for nonspecific binding to the filters.

Binding of [^{14}C]Acyloctapeptins to Whole Bacteria. Bacteria were grown on minimal medium A to the late log phase, sedimented at 7000*g* for 10 min, and suspended in fresh medium for binding studies. The remaining protocol was identical with that just described for membrane vesicles except that minimal medium was used to dilute and wash the samples.

Labeling of Membrane Vesicles with 5-, 12-, and 16-DS. Aliquots of 1% spin-label in EtOH were transferred to small round-bottom flasks and taken to dryness under N_2. Vesicles were thawed rapidly and labeled (10 μg of label per mg of membrane protein) for 4 h at 4 °C with stirring. The membranes were sedimented at 25000*g* for 15 min, washed once and resuspended to the desired concentration in cold 100 mM potassium phosphate (pH 6.6), and stored at 4 °C.

Kinetics of Reduction of Membrane-Bound Spin-Labels by Ascorbate. An aqueous quartz ESR cell fitted with a Hamilton syringe allowed for the addition and rapid mixing of sodium ascorbate without readjustment of instrument settings. The rate of decay in the height of the low-field line was used to follow the kinetics of reduction since the ascorbate free

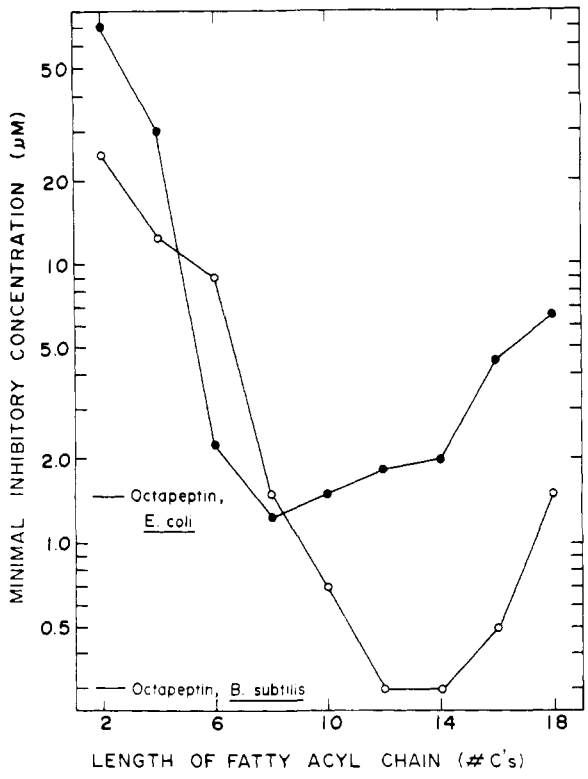

FIGURE 2: MIC as a function of chain length for acyl-modified octapeptins from C_2 (acetyl) to C_{18} (stearyl). *E. coli* SC 9251 (●); *B. subtilis* GSY 201 (○).

radical spectrum generated during the reaction is upfield from this line. Typically, 250 μL of vesicles suspended at 9 mg of protein per mL was brought to room temperature, treated with antibiotic, and placed in the cell. The instrument was adjusted for maximal low-field peak height and sodium ascorbate added to 10^{-2} M. Eight to ten spectra were then recorded at set time intervals (3–5 min) by offsetting the magnetic field. As described by Schreier-Muccillo et al. (1976), the rate of decay of the ESR signals was first order; linear plots of log line height vs. time yield a pseudo-first-order rate constant (k') and a half-time for reduction ($t_{1/2}$).

Liposomes. Stock solutions of phospholipids were evaporated to dryness under N_2, and the residual solvent was removed under high vacuum. Sonicated dispersions were prepared as described by Horwitz & Klein (1972) using 50 mM KCl–150 mM NaCl–10 mM Tris–10 μM EDTA, pH 7.0, as a buffer. Spin-labels were coevaporated with phospholipid stock solutions at a molar ratio of 1:100 unless indicated otherwise.

General Methods. Membrane protein was determined according to Lowry et al. (1951) with BSA as a standard. Lipids were extracted by the method fo Bligh & Dyer (1959) as described by Ames (1968). Total phosphate analysis was according to Ames (1966).

Results

Antibiotic Activity of Acyl-Modified Octapeptin. A number of octapeptin derivatives were prepared which contained a series of straight-chained fatty acids (C_2–C_{18}) in place of the natural β-hydroxy fatty acid. The activities of these derivatives against *E. coli* SC 9251 and *B. subtilis* GSY 201 are reported in Figure 2. Antimicrobial activity against both bacterial strains was optimal at intermediate fatty acid chain lengths: C_8 for *E. coli* and C_{12} for *B. subtilis*. Also illustrated are the MIC values of the native antibiotic. It is clear that the β-hydroxy fatty acid is not an obligatory requirement for antibiotic activity. These data illustrate that the potency of octapeptin systematically increased with the length of the fatty acid. For example, the MIC against *B. subtilis* decreased from 25 to 0.2 μM when the fatty acid increased from C_2 to C_{12}. On the other hand, the C_{16} and C_{18} derivatives were less active than the C_{12} or C_{14} analogues.

Binding of Acyl-Modified Octapeptins to E. coli Membrane Vesicles. Binding of various [^{14}C]acyloctapeptins to membrane vesicles is reported in Figure 3A as Scatchard plots and binding isotherms (Figure 3B). Individual Scatchard plots were nonlinear, indicating two or more membrane binding sites for octapeptin. Qualitatively, it is evident that a reduction in acyl chain length from C_{10} to C_2 decreased binding, approaching a lower limit at C_4. Comparison of the various binding isotherms reveals differences in both the slopes and overall shapes. Therefore, the relationship between acyl chain length and affinity for the membranes is complex and there may be several classes of binding sites. However, when compared at several constant antibiotic concentrations, the differences between binding of the C_2, C_4, C_6, C_8, and C_{10} analogues remained relatively constant. When binding was treated as a partitioning between the aqueous and membrane phases, plots of $\Delta G_{memb} - \Delta G_{H_2O}$ vs. the number of carbon atoms in the fatty acid were linear. The contribution of one methylene group to binding was ~100–200 cal/mol over antibiotic concentrations ranging from 20 to 500 μM. It has been determined that a methylene group contributes 825–850 cal/mol for partitioning of amphiphiles between H_2O and hydrocarbon phases (Tanford, 1973). Although the fatty acid of octapeptin contributes to membrane binding, its contribution is substantially less than that expected for transfer of a fatty acid acyl chain from an aqueous to a hydrophobic environment.

Correlation between Antimicrobial Activity and Binding of Acyl-Modified Octapeptins. Binding between the acyl-modified octapeptins and whole *B. subtilis* cells was also examined (data not shown). The binding isotherms for the various analogues were similar to those reported in Figure 3 for *E. coli* membranes. The data discussed thus far indicate that increases in the length of the octapeptin fatty acid chain increase binding to membranes, but only by a factor of approximately 10-fold. The corresponding MIC values varied over 2 orders of magnitude. Therefore, the number of moles of each analogue bound per cell at its MIC was determined (Figure 4). These data illustrate that the C_2 analogue must be absorbed to the membrane at a significantly higher density to elicit the same biological response as the C_{10} or C_{12} derivatives. Therefore, the fatty acid chain of octapeptin not only increases the affinity of the peptide for membranes but also actively contributes to the biological activity of the peptide.

Effect of Octapeptin and C_2 Octapeptin on the Rates of Reduction of Membrane-Bound Doxyl Stearates by Ascorbate. Nitroxide spin-labels are rapidly reduced by ascorbate with loss of paramagnetism. However, membrane-bound spin-labels such as the doxyl stearates are reduced at much slower rates due to limited penetration of ascorbate into the hydrophobic domain of the membrane (Schreier-Mucillo et al., 1976). The half-times ($t_{1/2}$) for reduction of three membrane-bound doxyl stearates (5-, 12-, and 16-DS) in the presence of 10^{-2} M sodium ascorbate at 19 °C were examined. There was a linear relationship between $t_{1/2}$ for reduction and the depth of the doxyl group in the bilayer, which is consistent with slow diffusion of ascorbate into the bilayer as the rate-limiting step.

The influence of octapeptin on the $t_{1/2}$ for ascorbate reduction of three membrane-bound doxyl stearates is reported

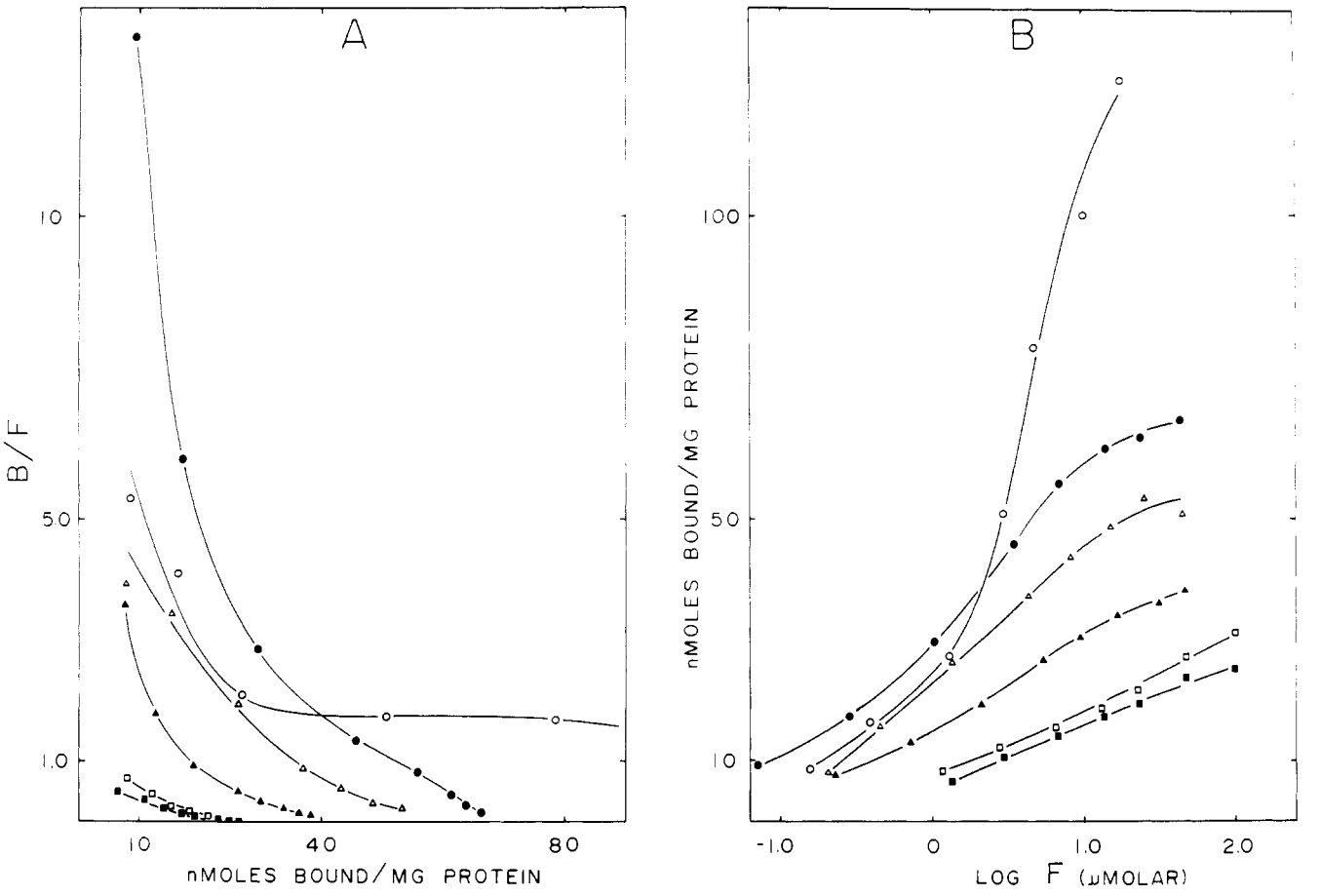

FIGURE 3: Binding of C_2 through C_{12} octapeptins to *E. coli* ML-308-225 membrane vesicles (1 mg of protein per mL): C_{12} (○); C_{10} (●); C_8 (△); C_6 (▲); C_4 (□); C_2 (■). (A) Scatchard plot; (B) binding isotherm.

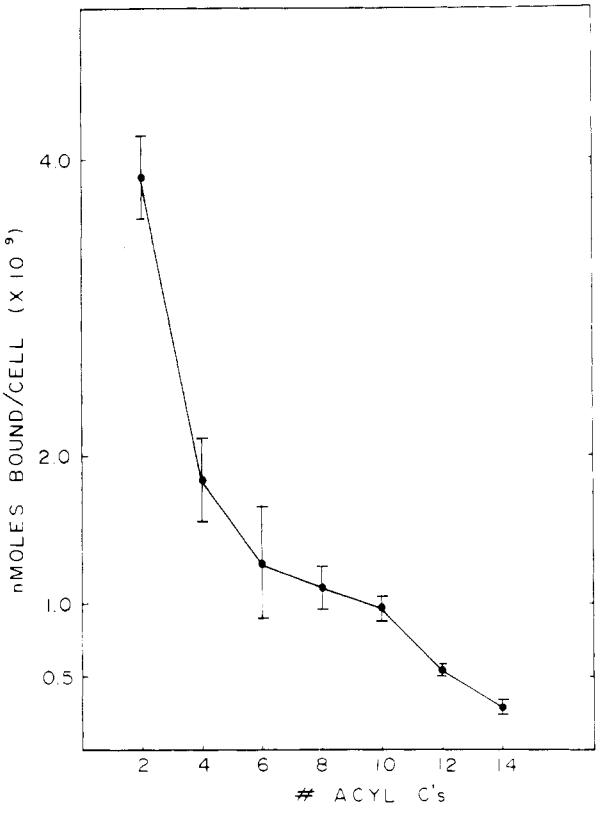

FIGURE 4: Binding of C_2 through C_{14} octapeptins to whole cells of *B. subtilis* determined at the respective MIC values for each analogue: C_2, 25 μM; C_4, 11 μM; C_6, 9 μM; C_8, 1.6 μM; C_{10}, 0.8 μM; C_{12}, 0.3 μM; C_{14}, 0.3 μM.

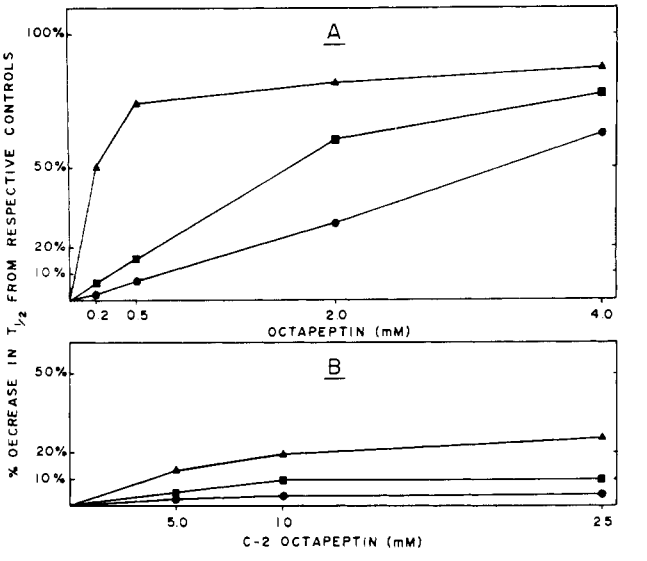

FIGURE 5: Percentage decrease in the $t_{1/2}$ from control values for membrane bound 5-DS, (▲), 12-DS (■), and 16-DS (●) in the presence of 10^{-2} M sodium ascorbate as a function of antibiotic concentration. (A) Octapeptin; (B) C_2 octapeptin. $t_{1/2}$ controls for 5-, 12-, and 16-DS are 29.5, 37.1, and 42.5 min, respectively.

in Figure 5A. An overall increase in the rate of ascorbate reduction in the presence of the antibiotic is not unexpected because binding of a tetracationic peptide to the membrane surface should facilitate localization of ascorbate at the membrane surface. Therefore, similar experiments were carried out with the C_2 acyl derivative which carries an equivalent charge (Figure 5B). Although both octapeptin and

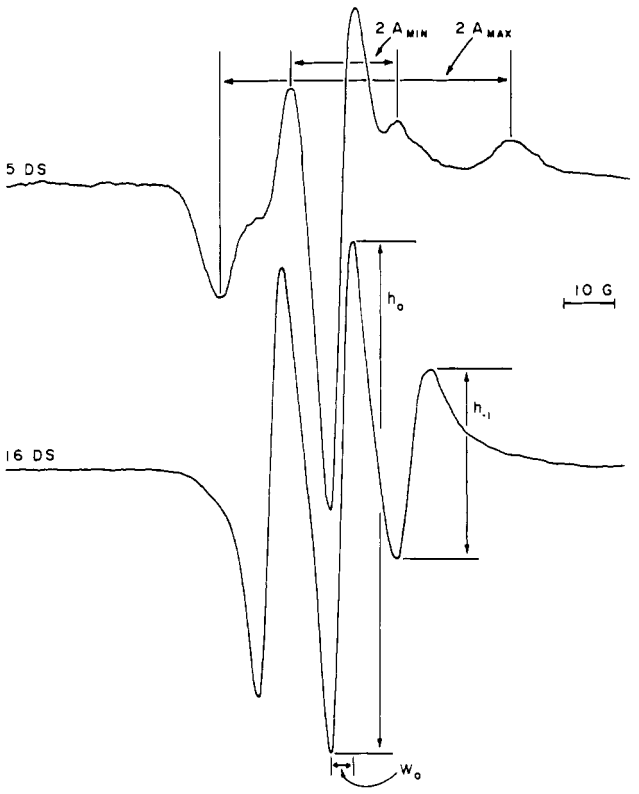

FIGURE 6: ESR spectra of 5-DS and 16-DS bound to *E. coli* ML-308-225 membrane vesicles (9 mg/mL protein) in the presence of 500 μM octapeptin at 19 °C to illustrate the determination of spectral parameters A_{max}, A_{min}, h_0, h_{-1}, and W_0.

the C_2 analogue enhanced the rate of ascorbate reduction, the native antibiotic was much more effective in this respect than the C_2 derivative. Equivalent amounts of the C_2 octapeptin and native octapeptin were bound to the membranes at 25 and 500 μM, respectively. Under these conditions, the C_2 analogue caused a 25% increase in the rate of ascorbate reduction of 5-DS whereas octapeptin enhanced the rate 75%. A similar trend was observed with the 12- and 16-DS labels, although it is clear from Figure 5 that the reduction rates of the deeper probes were less sensitive to the antibiotic. These data demonstrate that octapeptin increases the rate of diffusion of polar molecules into the hydrophobic domain of a membrane system and suggest that the fatty acid chain contributes to this effect.

Effect of Octapeptin on Lipid Motion in E. coli Membrane Vesicles. The data discussed thus far suggest that the acyl chain of octapeptin may insert itself into the hydrophobic domain of membranes. The effect of the antibiotic on the motional parameters of 5-, 12-, and 16-DS was examined in order to assess the influence of octapeptin at different levels of the bilayer. Stearate spin-labels have been widely used to monitor changes in order and motion at varying depths in phospholipid bilayers (Griffith & Jost, 1976).

Representative spectra for the 5- and 16-DS probes in *E. coli* membrane vesicles at 19 °C are reported in Figure 6. The motion of 5-DS was sufficiently anisotropic to determine both A_{max} and A_{min} from the spectra. An order parameter (S) introduced by Seelig (1970) was calculated as described by Esser & Lanyi (1973):

$$S = 0.568(A_{max} - A_{min})/a'$$

$$a' = (1/3)(A_{max} + 2A_{min})$$

A_{max} was not determinable from the spectra of the more isotropic 12-DS and 16-DS probes. The motion of these spin-labels can then be characterized by rotational correlation times introduced by McConnell (1956) and Kivelson (1960). As described by Keith et al. (1970), an approximate motion parameter (τ_n) has been calculated according to

$$\tau_n = (6.5 \times 10^{-10})W_0[(h_0/h_{-1})^{1/2} - 1]$$

where W_0, h_0, and h_{-1} are defined in Figure 6.

Figures 7, 8, and 9 illustrate the effect of temperature and octapeptin on the various spectral parameters (S, τ_n, and A_{max}) which reflect the order and motion of these three probes in the membrane. Examination of the control curve for 5-DS illustrates that S varied from 0.6 to 0.8 from 37 to 4 °C (Figure 7B). Similar results for the temperature dependence of the 5-DS order parameter have been obtained with phospholipid extracts and biological membranes (Esser & Lanyi, 1973). Addition of octapeptin resulted in significant restriction of 5-DS motion over the entire temperature range (Figure 7). Both S and A_{max} have been reported to illustrate this effect because of the loss of the second spectral maximum when $S > 0.85$. The temperature dependence of S between 25 and 37 °C was qualitatively similar in the presence or absence of octapeptin. Below 22 °C, the ordering effect of the antibiotic was even more pronounced. Values of A_{max} for both control and octapeptin-treated membranes approached 31 G at 4 °C which is indicative of a highly ordered state.

The effect of octapeptin on the motion of 12-DS is reported in Figure 8. Variation in A_{max} and τ_n with temperature was qualitatively similar to that of the 5-DS probe. Again, the most pronounced effect of octapeptin on the motion of the spin-labeled fatty acid occurred at temperatures below 22 °C. The τ_n temperature dependence for 16-DS exhibited a clear break in the range of 16–19 °C (Figure 9). Thermal transition points are most readily detected when the spin-label is located toward the end of the fatty acid (Esser & Lanyi, 1973). Octapeptin also restricted the motion of 16-DS, but the thermal transition point and the slopes of the lines were unaffected.

The data obtained with 5-, 12-, and 16-DS indicate that octapeptin causes restricted motion of the fatty acid at varying levels in the lipid bilayer. Based on the assumption that these spin-labeled stearates monitor the most fluid regions of the membrane (Oldfield et al., 1972), this is the consequence of tighter, more ordered packing of the lipid phase or direct interactions between the antibiotic and phospholipids.

Antimicrobial Activity of Spin-Labeled Octapeptin. (9-Doxylundecanoyl)octapeptin (9-DU-octapeptin) was assayed for antimicrobial activity against *E. coli* ML-308-225. The MIC for octapeptin, C_{12} octapeptin, and 9-DU-octapeptin were 3.0, 4.0, and 5.5 μM, respectively. The presence of the spin-label decreased the potency of the antibiotic somewhat, but the spin-labeled derivative was still quite active.

Spectral Characteristic of 9-DU-Octapeptin in Solution. The ESR spectrum of 1 mM 9-DU-octapeptin in aqueous solutions was highly isotropic, indicative of rapid reorientation of the label (Figure 10A). In addition, exchange broadening was not observed. No changes in the spectral shape were detected as the concentration of 9-DU-octapeptin was varied from 100 μM to 50 mM or when the spin-labeled antibiotic was diluted with unlabeled antibiotic. These results indicate that 9-DU-octapeptin and presumably the native octapeptin do not aggregate in solution.

The isotropic splitting constant, A, of 9-DU-octapeptin in solution was 15.4 ± 0.05 G. This indicates that the fatty acid chain of octapeptin in solution is not completely exposed to water and that the polarity of its microenvironment is comparable to that of EtOH–H_2O (1:1) (Griffith et al., 1974).

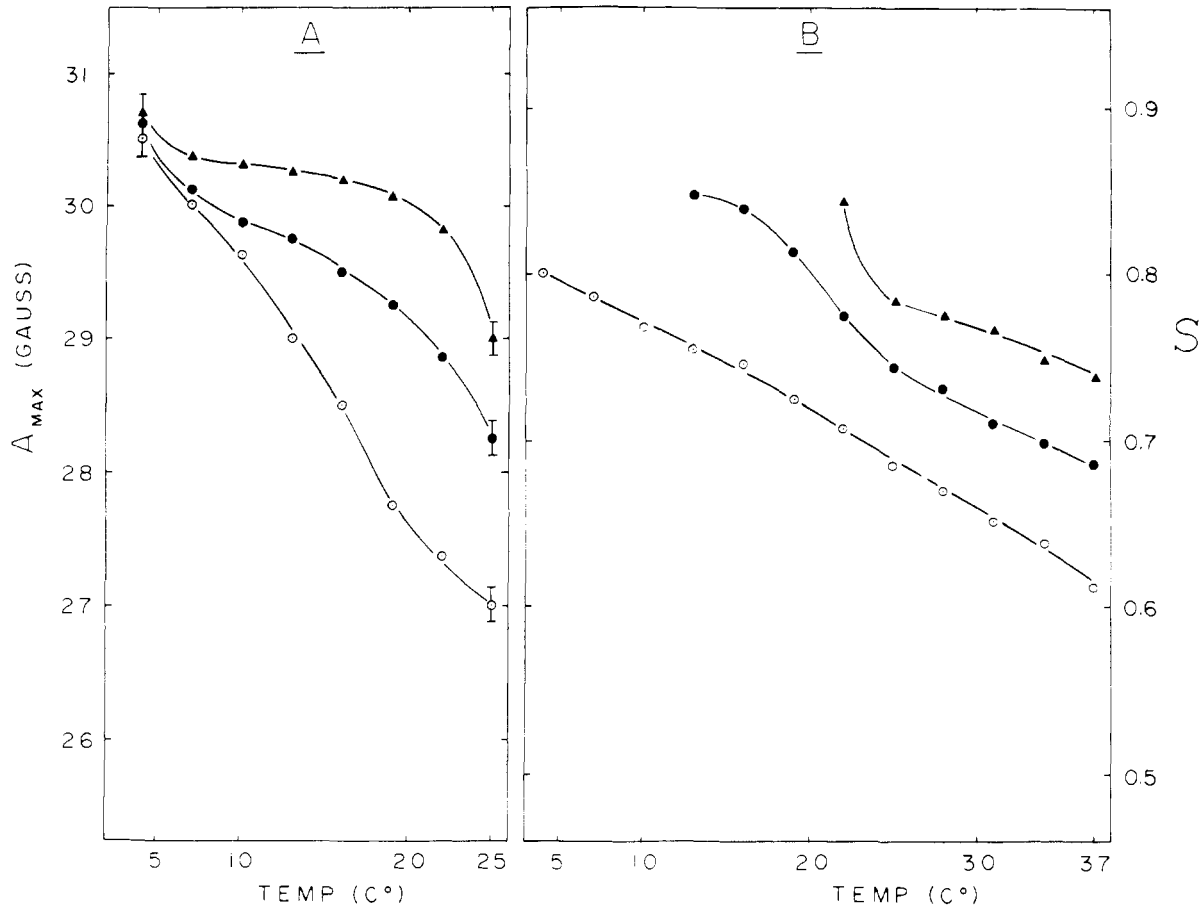

FIGURE 7: A_{max} (A) and S (B) as a function of temperature for 5-DS-labeled *E. coli* ML-308-225 membrane vesicles (9 mg/mL protein) in the presence of octapeptin. Untreated (⊙); 500 μM octapeptin (●); 1 mM octapeptin (▲).

It is likely that the fatty acid chain of the antibiotic interacts with hydrophobic amino acids of the peptide. These data partially explain why binding to membranes was only enhanced 100–200 cal/mol for each methylene group in the fatty acid. In the presence of excess ascorbate (10^{-2} M), 1 mM 9-DU-octapeptin was rapidly reduced ($t_{1/2}$ = 20 s), indicating that the spin-labeled fatty acid was readily accessible to water. These data also indicate that the antibiotic does not form micelles in solution with the fatty acid unexposed to solvent. When 9-DU-octapeptin was bound to *E. coli* membrane vesicles, the rate of ascorbate reduction was much slower ($t_{1/2}$ = 12.0 min). This observation is consistent with the proposal that the fatty acid of the antibiotic is inserted into the membrane.

Spectral Characteristics of 9-DU-Octapeptin Bound to Lipid Dispersions. Lipid dispersions were formed in the presence of 9-DU-octapeptin at a high lipid/antibiotic ratio (200:1) to maximize the amount of 9-DU-octapeptin bound to phospholipids for spectral analysis with minimal exchange broadening. Under these conditions, there was not contribution to the spectra from free 9-DU-octapeptin. The spectra of spin-labeled octapeptin bound to phosphatidylcholine and phosphatidylglycerol are given in Figure 10B. Under these conditions, 9-DU-octapeptin was bound to both the zwitterionic phosphatidylcholine and the anionic phosphatidylglycerol. The two spectra were nearly identical and were quite similar to a deeper membrane-bound stearate probe. The approximate motion parameters (τ_n) were 2.8×10^{-9} and 2.5×10^{-9} s for the phosphatidylcholine and phosphatidylglycerol systems, respectively. The motion of 12-DS under identical conditions was slightly more rapid, yielding τ_n values of 1.4×10^{-9} and 1.6×10^{-9} s in the phosphatidylcholine and phosphatidylglycerol systems. This indicates that the motion of the 9-DU-octapeptin doxyl group was slightly more ordered than that of the 12-DS probe, consistent with its location on the ninth carbon of the fatty acid. The characteristics of these spectra strongly suggest directional binding of the antibiotic to the phospholipid phase with anisotropic motion about the fatty acyl axis.

When a total *E. coli* phospholipid extract was dispersed with 9-DU-octapeptin, two bound spectral components were discernible (Figure 10C). The innermost component of the low-field line is similar in shape and location to those observed with the pure phosphatidylglycerol and phosphatidylcholine systems, whereas the outer peak is indicative of more restricted motion. The relative proportions of these two spectral components were altered by changes in the phospholipid/antibiotic ratio. The relative amount of the more restricted component increased with increases in the phospholipid/antibiotic ratio. The antibiotic apparently existed in two distinct domains which resulted in different orientations and/or immobilization of the peptide. This may reflect bound antibiotic in both a phase-separated and fluid domain and also suggests that its interaction with anionic phospholipids (phosphatidylglycerol and cardiolipin) may be distinct from the interaction with zwitterionic phosphatidylethanolamine in this mixed system.

Discussion

The data presented in this study illustrate the importance of the octapeptin fatty acid chain for the biological activity of the antibiotic and its affinity for membrane systems. The contribution of the fatty acid to membrane binding was considerably less than would be expected for partitioning of a fatty acid from an aqueous environment to a hydrophobic domain.

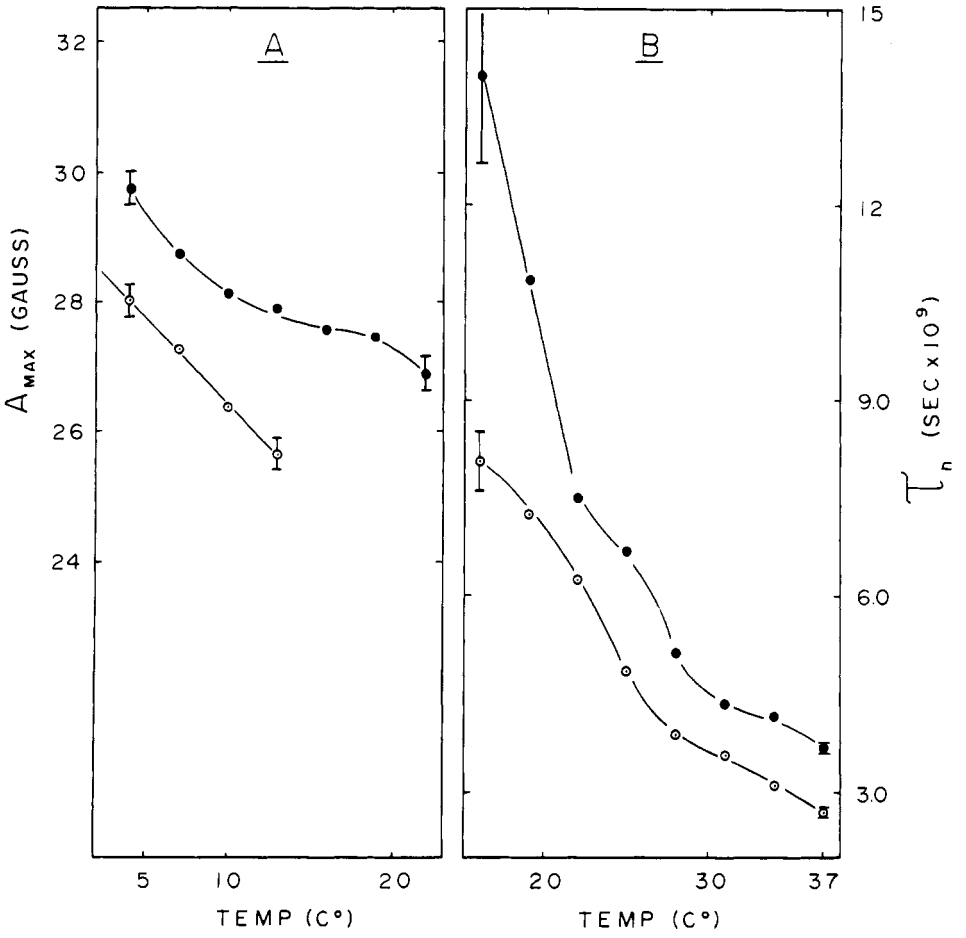

FIGURE 8: A_{max} (A) and τ_n (B) as a function of temperature for 12-DS-labeled *E. coli* ML-308-225 membrane vesicles (9 mg/mL protein) in the presence of octapeptin. Untreated (⊙); 1 mM octapeptin (●).

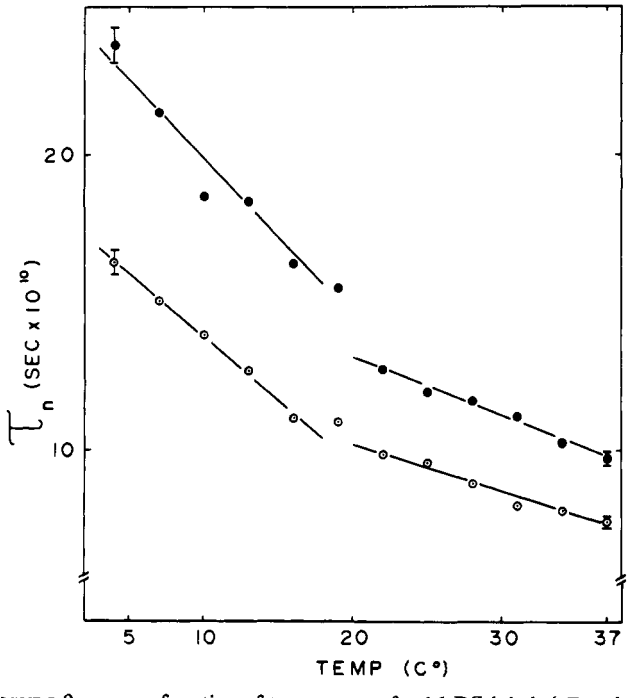

FIGURE 9: τ_n as a function of temperature for 16-DS-labeled *E. coli* ML-308-225 membrane vesicles (9 mg/mL protein) in the presence of octapeptin. Untreated (⊙); 1 mM octapeptin (●).

The ESR spectral properties of 9-DU-octapeptin in solution suggested that the fatty acid chain is partially removed from the solvent, perhaps through hydrophobic interactions with apolar amino acid residues of the peptide. These data were also inconsistent with the formation of large aggregates or micelles of the antibiotic which would tend to diminish the contribution of the fatty acid to membrane binding.

A number of experiments strongly suggest that the fatty acid chain of octapeptin inserts itself into the hydrophobic domain of phospholipid bilayers. For example, the ESR spectra of 9-DU-octapeptin bound to either phosphatidylglycerol or phosphatidylcholine indicated directional binding of the antibiotic to the bilayer with anisotropic motion about the fatty acyl axis. Ascorbate reduction of 9-DU-octapeptin free in solution was quite rapid whereas binding of this derivative to membranes greatly retarded the rate of ascorbate reduction. Both octapeptin and its C_2 acyl derivative enhanced the rate of ascorbate reduction of membrane-bound doxyl stearates (5-, 12-, and 16-DS); however, octapeptin was much more effective than its C_2 acyl derivative. Although this effect was much more pronounced with the shallower probe, octapeptin did increase the rate of ascorbate reduction of the deeper probe (16-DS). In addition, ESR studies with 5-, 12-, and 16-DS indicated that all levels of the membrane experienced motional restriction in the presence of octapeptin.

We have previously proposed that binding of octapeptins and polymyxins to membranes involves electrostatic interactions between the antibiotic amino groups and lipid phosphates with insertion of the fatty acid into the center of the membrane. The primary focus of this study has been to define the contribution of the fatty acid for membrane interactions and to determine whether or not the fatty acid chain is indeed inserted into the membrane. Taken collectively, the evidence supports

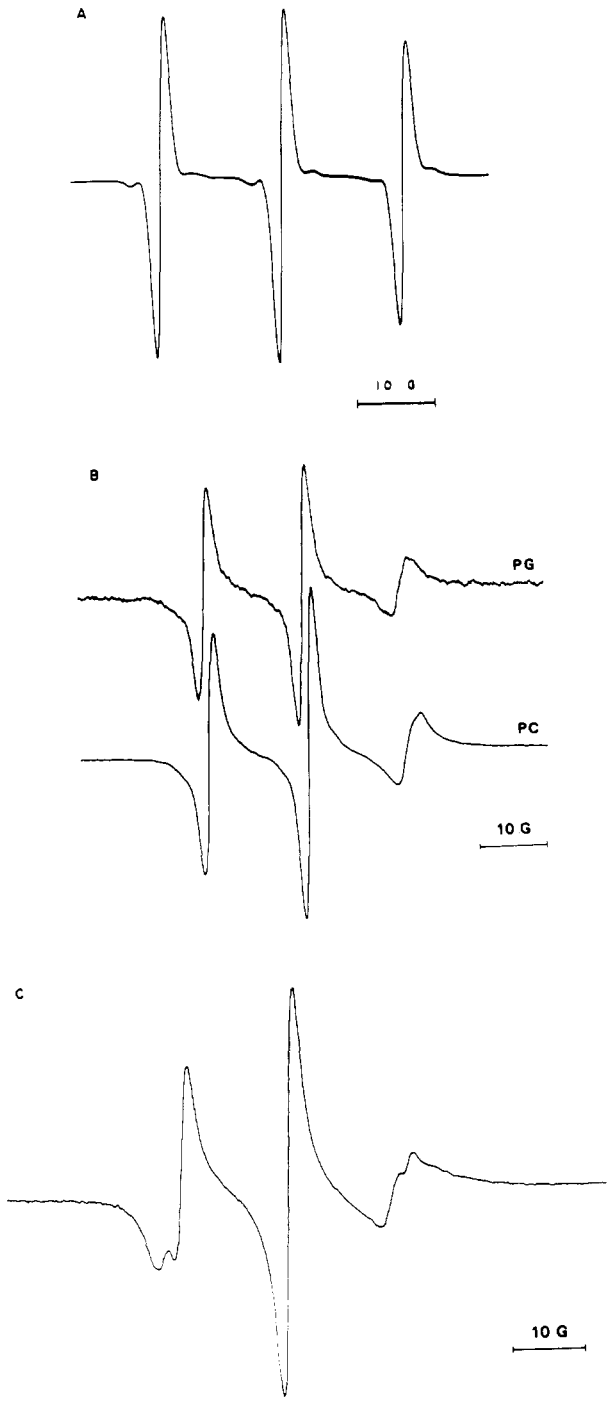

FIGURE 10: ESR spectra of 9-DU-octapeptin free and bound to membranes. (A) 1 mM 9-DU-octapeptin in 65 mM KCl–65 mM NaCl–20 mM Tris-HCl (pH 7.0). (B) 9-DU-octapeptin codispersed with PC or PG at a 200:1 molar ratio of lipid to antibiotic in 50 mM KCl–50 mM NaCl–10 mM Tris–10 M EDTA (pH 7.0). (C) 9-DU-octapeptin codispersed with *E. coli* total lipid as described in (B). All spectra were run at 25 °C.

this general model. The incorporation of a spin-labeled fatty acid into the antibiotic represents a structural modification of the native antibiotic, and therefore extrapolation of these observation requires some caution. However, the spin-labeled antibiotic possessed antimicrobial activity comparable to the native antibiotic and results obtained with this derivative probably approximate interactions of the native antibiotic with phospholipids.

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