Sugimoto, K., Okamoto, T., Sugisaki, H., and Takanami, M. (1975), *Nature (London) 253,* 410.
Takanami, M., Sugimoto, K., Sugisaki, H., and Okamoto, T. (1976), *Nature (London) 260,* 297.
Walz, A., and Pirrotta, V. (1975), *Nature (London) 254,* 118.
Weiss, B., Live, T. R., and Richardson, C. C. (1968), *J. Biol. Chem. 243,* 4530.
Weiss, B., and Richardson, C. C. (1967), *J. Mol. Biol. 23,* 405.
Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L., and Treiber, G. (1970), *Virology 40,* 734.

Disruption of *Escherichia coli* Outer Membranes by EM 49. A New Membrane Active Peptide Antibiotic†

Ken S. Rosenthal,‡ Paul E. Swanson,‡ and Dan R. Storm*,§

ABSTRACT: A new peptide antibiotic, EM 49, is shown to disrupt the structure of *Escherichia coli* outer membranes and release outer membrane fragments into the surrounding media. Evidence supporting this conclusion includes EM 49 stimulated release of outer membrane phospholipids, lipopolysaccharide, and membrane fragments having a phospholipid and polypeptide composition similar to outer membranes. The density of the membrane fragments released by EM 49 was 1.22 g/cm^3, which was identical to isolated outer membranes. Approximately 10 to 15% of the *E. coli* lipopolysaccharide was released upon treatment with EM 49. Both scanning and transmission electron microscopy revealed that the antibiotic caused the formation of numerous protrusions or blebs on the surface of *E. coli*, with apparent release of membrane vesicles from the cells. Direct interaction between EM 49 and outer membranes was demonstrated using outer membranes labeled with the fluorescent dye diphenylhexatriene. Treatment of the fluorescent-labeled outer membranes with EM 49 increased fluorescence intensity and decreased polarization, indicating that the peptide perturbed outer-membrane structure. In addition, strong interactions between EM 49 and purified *E. coli* phospholipids were detected using the Hummel and Dreyer technique. Association constants between the peptide and phospholipids were approximately 10^5 M^{-1}. A model for the disruptive effect of EM 49 on outer-membrane structure is proposed in which the fatty acid chain of the antibiotic is inserted into the hydrophobic core of the membrane. This orientation would allow the polycationic, peptide portion of the antibiotic to disrupt the normal electrostatic interactions between divalent cations and components of the outer membrane. Evidence supporting this conclusion includes specific protection of *E. coli* from EM 49 by Mg^{2+} and Ca^{2+} and inhibition of EM 49 stimulated phospholipid release by these cations. Disruption of the outer membrane would allow the antibiotic to penetrate to the inner membrane, which is probably the primary killing site of EM 49.

EM 49 is a new broad spectrum antibiotic active against gram-positive and negative bacteria, as well as yeasts, fungi, and protozoa (Meyers et al., 1973a,b, 1974; Parker and Rathnum, 1973, 1975; Rosenthal and Storm, 1975). The antibiotic is a mixture of closely related peptides bearing structural resemblance to the polymyxins (Figure 1). Both families of peptides are cyclic peptide antibiotics containing a high percentage of 2,4-diaminobutyric acid with a fatty acid attached to the peptide through an amide bond. However, there are important structural differences between the polymyxins and EM 49. EM 49 is an octapeptide containing a C:10 or C:11 β-hydroxy fatty acid with no threonine residues, whereas the polymyxins are decapeptides containing a C:8 or C:9 fatty acid with 2 threonine residues/molecule. In addition, the biological properties of the polymyxins and EM 49 are significantly different. The antimicrobial spectrum of EM 49 is much broader than the polymyxins, and the antibiotics are not cross-resistant with respect to several polymyxin resistant *E. coli* strains (Meyers et al., 1974).

The mechanism for the antimicrobial activity of EM 49 has not been elucidated; however, preliminary electron microscopy studies have indicated that EM 49 caused the accumulation of numerous blebs on the outer surface of *E. coli* with release of membrane vesicles from the bacteria (Meyers et al., 1974). Data presented in this report establish that the antibiotic disrupts outer-membrane structure and releases outer-membrane fragments from *E. coli*. Furthermore, the antibiotic is shown to interact strongly with isolated *E. coli* phospholipids and outer-membrane preparations. A model for the disruptive effect of EM 49 on outer-membrane structure is proposed and discussed in terms of the antibiotic activity of these peptides against gram-negative bacteria.

Experimental Procedure

Materials

EM 49 (lot no. SQ 21, 286) as well as *E. coli* strains SC 9251, SC 9252, and SC 9253 were kindly supplied by the Squibb Institute for Medical Research. Ammonium 2-keto-3-deoxyoctanoate used for standards was supplied by Dr. H. E. Conrad. Polymyxin B sulfate was purchased from Sigma Chemical Co. Lysozyme, DNase, and RNase were obtained from Worthington. Enriched media consisted of 1% beef peptone, 0.5% NaCl, and 0.1% yeast extract at pH 7.0. Syn-

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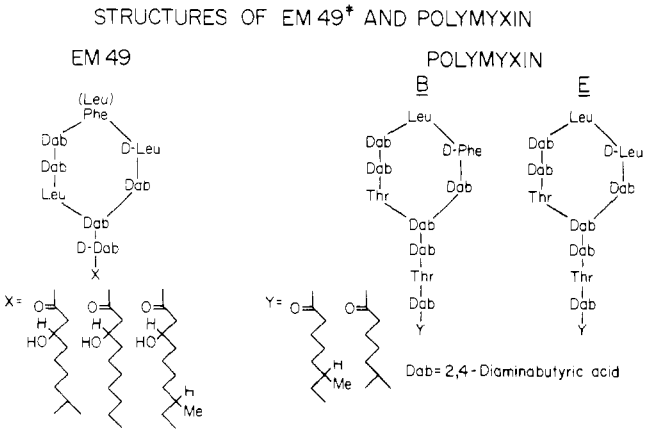

FIGURE 1: Structure of EM 49 (Parker and Rathnum, 1975) and polymyxins B and E (Nakajima, 1967). The abbreviation Dab stands for 2,4-diaminobutyric acid.

thetic media consisted of 50 mM potassium phosphate, 1 mM ammonium sulfate, 0.12 mM $MgCl_2$ (optional), 20 mM sorbitol, and casein hydrolysate (1 g/l.) neutralized to pH 7.0 with NaOH. Diphenylhexatriene was purchased from Aldrich Chemical Co.

Methods

Preparation of ^{32}P-Labeled Phospholipids for Lipid-Binding Studies. E. coli B was grown for 10 h in 1 l. of enriched media containing 24 mCi of $H_3{}^{32}PO_4$. The cells were harvested by centrifugation at 400*g* for 15 min and then washed four times with 0.1 M Tris-HCl pH 7.5. Phospholipids were extracted using the method of Bligh and Dyer (Bligh and Dyer, 1959). Organic solvents contained 50 μg/ml of 2,6-di-*tert*-butylcresol as an antioxidant. Phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin were obtained in pure form by preparative silica gel thin-layer chromatography using either chloroform–methanol–acetic acid (65:24:8), or Skipski and Barclay's solvent I or II for the separation of acidic phospholipids (Skipski and Barclay, 1969). Phospholipid fractions were located on thin-layer plates by autoradiography, eluted from silica gel with chloroform–methanol (2:1) and stored at −20 °C under nitrogen. Phospholipid standards were run to aid in the identification of bacterial phospholipids.

Phospholipid and Fatty Acid Composition of E. coli Strains. One liter of *E. coli* was grown to late log phase in either enriched or minimal media containing 1 mCi of $H_3{}^{32}PO_4$. Phospholipids were extracted and analyzed by silica gel thin-layer chromatography as described above. Fatty acid compositions were determined by transesterification of the phospholipid extracts using methanol and concentrated HCl (20:1, v/v) followed by gas-liquid chromatography of the fatty acid methyl esters (Ferguson et al., 1975). The column used was 10% SP-2340 on 100–200 mesh Chromasorb P-AW-PMCS. Methyl ester standards of myristic, palmitic, palmitoleic, steric, oleic, and vaccenic acids were used to aid in the identification of fatty acid methyl esters.

Release of Phospholipids from E. coli by EM 49. E. coli was grown to late log phase in 1 l. of enriched media containing 10 mCi of $H_3{}^{32}PO_4$. After the cells were harvested, they were washed three times in 0.01 M Tris-HCl, pH 7.5. The washed cells were gently suspended in 100 ml of the same buffer and divided into 10-ml fractions containing equivalent numbers of cells. EM 49 or polymyxin B was then added at concentrations of 4, 40, and 400 μg/ml and incubations were carried out for 30 min at 30 °C. Controls were treated with buffer in the absence of antibiotics. The cells were then centrifuged at 10 000*g* for 15 min and the supernatants were removed and analyzed for ^{32}P-labeled material. An aliquot of the supernatant was counted for ^{32}P-labeled compounds released from the bacteria into the media. The remaining supernatant was extracted with chloroform–methanol (2:1), concentrated, and submitted to thin-layer silica gel chromatography developed with chloroform–methanol–acetic acid (65:24:8).

Lipopolysaccharide Release by EM 49. E. coli was grown to late log phase in enriched media and then harvested by centrifugation at 3000*g* for 10 min at 4 °C. The cells were resuspended in 10 mM $NaHCO_3$, pH 7.5, divided into equivalent fractions of 0.5 g of wet cell/fraction, and recentrifuged at 3000*g*. The pellets were weighed and resuspended in 10 ml of 10 mM $NaHCO_3$ containing varying concentrations of EM 49. These samples were incubated at 25 °C for 1 h and then centrifuged at 3000*g* for 20 min. When the kinetics of lipopolysaccharide release were determined, the cells were spun down at 10 000*g* for 2 min following various incubation periods with EM 49. The supernatants obtained from these samples were carefully removed and evaporated down to dryness. The residue was dissolved in 0.1 ml of 0.02 N H_2SO_4, hydrolyzed at 100 °C for 30 min, and then assayed for 2-keto-3-deoxyoctanoic acid (KDO[1]) using the thiobarbituric acid assay (Weissbach and Hurwitz, 1959). The chromogen was extracted into cyclohexanone and the absorbance at 548 nm was read immediately due to the instability of the chromophore. KDO determined in this manner was used as a quantitative marker for lipopolysaccharide. DNA will interfere with the thiobarbituric acid assay for KDO, since deoxyribose is detected by this assay. In order to correct for DNA contamination, samples were treated with DNase and dialyzed extensively to remove nucleotides. Samples of pure KDO were used for quantitative standardization of this assay.

Isolation of Outer-Membrane Fragments Released by EM 49. Four liters of *E. coli* SC 9251 was grown to late log phase in enriched media containing 5 mCi of $H_3{}^{32}PO_4$. The harvested cells were treated with EM 49 as described earlier for those experiments measuring the release of KDO from *E. coli*. The supernatants were concentrated either by lyophilization or by ultrafiltration using an Amicon XM 50 membrane. These samples were suspended in 1 mM $NaHCO_3$ and applied to a discontinuous sucrose gradient ranging from 30 to 55% sucrose in 10% steps. This gradient was then centrifuged at 25 000 rpm in a SW 25.2 rotor for up to 22 h. The sucrose gradient profile was identical following 2.5-, 12-, or 22-h spins. The gradients were fractionated into 2-ml aliquots and 100-μl fractions were counted for ^{32}P in Aquosol. Peak fractions were pooled and dialyzed against distilled water or dilute buffers prior to subsequent analyses.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis of Outer Membranes and EM 49 Released Membrane Fragments. Outer membranes were isolated by the method of Mizushima and Yamada (Mizushima and Yamada, 1975). Fraction II (43–49% sucrose), from the sucrose density gradient (Figure 5), was isolated and dialyzed first against 100 mM phosphate buffer at pH 7.2 and then 10 mM NH_4HCO_3. Both samples (70 μg of membrane protein) were lyophilized prior to electrophoresis and dissolved in 100 μl of sample buffer containing 10 mM phosphate, pH 7.2, 1% sodium dodecyl

[1] Abbreviations used are: KDO, 2-keto-3-deoxyoxtanoic acid; DPH, diphenylhexatriene; LPS, lipopolysaccharide; CTAB, cetyltrimethylammonium bromide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

sulfate, 0.14 M β-mercaptoethanol, 10% (v/v) glycerol, and 0.002% bromphenol blue by boiling for 5 min. These samples were electrophoresed on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Gels were stained with 0.25% Coomassie blue in 25% 2-propanol–10% acetic acid and destained in 25% 2-propanol–10% acetic acid for 2 h, followed by 5% methanol–5% acetic acid for 36 h. Destained gels were scanned with a Gilford Linear transport system at 550 nm. Proteins of known molecular weight were run as standards for molecular weight determinations.

Effect of Divalent Cations on EM 49 Antibiotic Activity. Ten milliliters of enriched media containing varying concentrations of divalent cations was inoculated with 0.1 ml of fully grown *E. coli* culture and then incubated for 1 h at 37 °C with shaking. EM 49 was then added at varying concentrations and the cultures were grown for another 9 h. Final growth was quantitated by measuring light scattering in Klett units.

Binding between EM 49 and E. coli Phospholipids. Interaction between EM 49 and ^{32}P-labeled phospholipid was quantitated by the general method of Hummel and Dreyer (Hummel and Dreyer, 1962). Solutions of purified phospholipids in chloroform–methanol were brought down to dryness in silanized pear-shaped flasks under vacuum. The phospholipids were then sonicated in 15 ml of 0.1 M Tris, pH 7.5, for 30 min using 10-s bursts followed by 20-s pauses for cooling. The flask was immersed in an ice bath during sonication. After the sonication procedure, a sample of the phospholipid was chromatographed on silica gel in several solvent systems to test for decomposition of the lipid during sonication. No breakdown of the phospholipids occurred during sonication as long as the phospholipid dispersion was kept cool during sonication. The sonicated phospholipid dispersions were filtered through Whatman no. 1 filter paper immediately before use. The phospholipid dispersion was then passed through a 4-ml Sephadex G-25 column and 200-μl fractions were collected and counted (Figure 2). After a baseline of radioactivity was established, a weighed amount of the antibiotic was added to the top of the column in 200 μl of the buffer containing [^{32}P]-phospholipid. Under these conditions, the phospholipid in an aggregated form was excluded from Sephadex G-25. EM 49 and polymyxin B both disrupted phospholipid aggregates such that some fraction of the phospholipid was included in the gel. This was manifested in the elution profile as a trough of radioactivity followed by a peak of equivalent area. EM 49 and polymyxin B applied to the column were found to elute in the peak of radioactivity. It was assumed that these peptides formed complexes with the phospholipids which were included in the gel. The ratio of peptide bound lipid to unbound lipid can be approximated from the average counts per minute in the peak, trough, and baseline. The peptide was in large molar excess relative to the phospholipid such that the amount of bound peptide was negligible relative to total peptide. Knowing the ratio of bound to unbound lipid and the amount of peptide present in the binding assay, it is possible to derive approximate binding constants for the following equilibrium (Storm and Strominger, 1973, 1974):

$$(\text{lipid})_n + \text{peptide} \rightleftarrows (\text{peptide}\cdot\text{lipid}_x) + (\text{lipid})_{n-x}$$

Fluorescence Polarization of EM 49 Treated Outer Membranes. E. coli SC 9251 was grown to late log phase in enriched media and outer membranes were isolated by the method of Mizushima and Yamada (Mizushima and Yamada, 1975). Membranes were suspended in 0.15 M KCl and were used immediately after preparation for fluorescence studies. Membrane suspensions were treated with an equal volume of

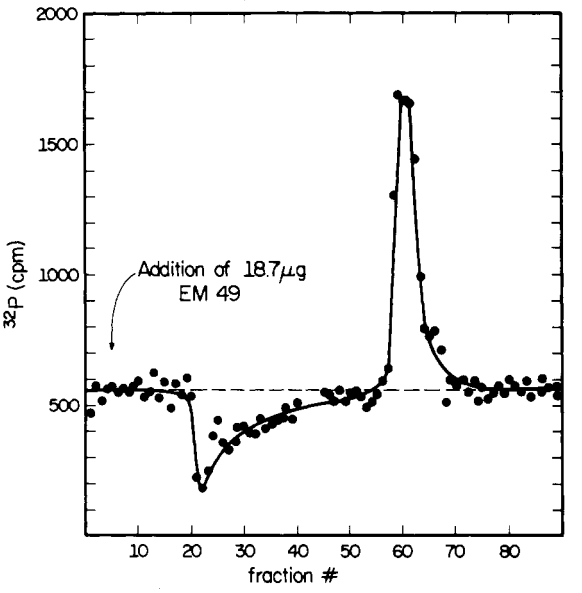

FIGURE 2: Interaction between EM 49 and *E. coli* phosphatidylethanolamine. ^{32}P-labeled phosphatidylethanolamine, prepared as described under Methods, was dispersed by sonication in 0.1 M Tris, pH 7.5, and passed through a 4-ml Sephadex G-25 column. EM 49 (18 μg) was added at fraction 5 in buffer containing the labeled phosphatidylethanolamine and 0.2-ml fractions were collected and counted.

2 μM 1,6-diphenyl-1,3,5-hexatriene (DPH) in 0.15 M KCl as described by Shinitsky (Shinitsky and Inbar, 1974). Membrane samples prepared identically in the absence of DPH were used as scatter controls. Following a 60-min incubation with DPH at room temperature, the membranes were pelleted at 25 000*g* for 20 min and then resuspended in 0.15 M KCl. These suspensions were then used immediately for fluorescence measurements. Fluorescence polarization and fluorescence intensity measurements were carried out at an exciting wavelength of 366 nm from a mercury arc and detected in two cross-polarized channels following passage through 2 M aqueous $NaNO_2$ to cut off scattered light below 390 nm. Temperature was maintained at 25 °C throughout. Values for fluorescence polarization and intensity were obtained by simultaneously measuring fluorescence intensity parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarized exciting beam. Fluorescence intensity (F) and polarization (P) are given by eq 1 and 2:

$$F = I_{\perp}[(I_{\parallel}/I_{\perp}) + 2] \quad (1)$$

$$P = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 1} \quad (2)$$

In all cases, DPH-labeled membranes were treated with EM 49, polymyxin B, or buffer for 10 min at 25 °C before fluorescence measurements were taken. Polarization and fluorescence were corrected for scattering using eq 3 and 4:

$$P_{\text{corrected}} = \frac{[(I_{\parallel}/I_{\perp}) - 1] - [I^s_{\perp}/I_{\perp}\,(I^s_{\parallel}/I^s_{\perp} - 1)]}{[(I_{\parallel}/I_{\perp}) + 1] - [I^s_{\perp}/I_{\perp}\,(I^s_{\parallel}/I^s_{\perp} + 1)]} \quad (3)$$

$$F_{\text{corrected}} = [I_{\perp}\,(I_{\parallel}/I_{\perp} + 2)] - [I^s_{\perp}\,(I^s_{\parallel}/I^s_{\perp} + 2)] \quad (4)$$

Values for $I^s_{\perp}$ and $I^s_{\parallel}$ were obtained from scatter controls treated with EM 49, polymyxin B, or buffer.

Scanning Electron Microscopy. Ten milliliters of *E. coli* SC 9252 were routinely grown to midlog phase in shaker culture at 37 °C. EM 49 or polymyxin B was added to a concentration of 50 μg/ml and incubated for 30 min. Controls were treated identically in the absence of added antibiotic. The cells were

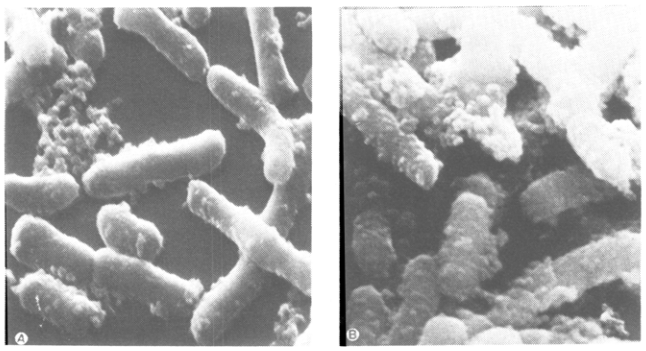

FIGURE 3: Scanning electron microscopy of EM 49 treated and untreated *E. coli* cells. *E. coli* SC 9252 grown to midlog phase were treated with 50 μg/ml of EM 49 and allowed to grow for another 30 min (B). Controls (A) were treated identically in the absence of added antibiotic. The bacteria were fixed in 2% glutaraldehyde, added directly to the media at 4 °C for 4 h, and then postfixed in 0.6% $KMnO_4$ for 6 h. The fixed cells were dehydrated in ethanol and critical-point dried onto glass cover slips, coated with Au/Pd (60%/40%), and viewed with a JEOL U-3 scanning electron microscope.

TABLE I: ^{32}P-Labeled Material Released from *E. coli* Y-10 by EM 49 or Polymyxin B.

Treatment[a]	^{32}P Released[b] (%)	^{32}P Released Extracted into $CHCl_3$–MeOH[c] (%)
Buffer only	1.6	<0.1
Polymyxin, 400 μg/ml	10.4	<0.1
Polymyxin, 40 μg/ml	3.9	<0.1
Polymyxin, 4 μg/ml	2.0	<0.1
EM 49, 400 μg/ml	9.6	5.0
EM 49, 40 μg/ml	3.1	2.0
EM 49, 4 μg/ml	1.9	1.0
EM 49, 40 μg/ml + 1 mM $MgCl_2$ + 1 mM $CaCl_2$	<0.1	<0.1

[a] *E. coli* Y-10 treated with 10 ml of 0.01 M Tris-HCl, pH 7.5, with or without the indicated amounts of EM 49 or polymyxin B as described under Methods. [b] Each sample contained 2.6×10^7 cpm ^{32}P. Data represent percentages of total cellular ^{32}P released upon treatment. [c] Percentages represent the fraction of released ^{32}P that partitioned into the organic phase when treated with $CHCl_3$–MeOH (2:1).

double fixed first with glutaraldehyde and then with $KMnO_4$. Glutaraldehyde was added directly to the cultures to a concentration of 2% and the samples were stored at 4 °C for 4 h. They were then washed twice in phosphate-buffered saline and resuspended in the phosphate buffer containing 0.6% $KMnO_4$. After 6 h, the cells were washed with cold 25% ethanol, resuspended in the ethanol, and allowed to warm to room temperature. Dehydration with ethanol was followed by critical-point drying using carbon dioxide. Samples were coated with gold–palladium and viewed with a JEOL U3 scanning electron microscope. Postfixation of these samples in this manner aids in the retention of lipid in the dehydration process and maintenance of fine structures of the bacterium (Hayat, 1970).

Protein was determined by the Lowry method using Bovine serum albumin as a standard (Lowry et al., 1951). Phospholipid phosphate was determined by first extracting phospholipid by the Bligh and Dyer method (Bligh and Dyer, 1959) and then assaying for phosphate (Ames, 1966).

Results

Scanning Electron Microscopy of EM 49 Treated E. coli. Transmission electron micrographs of *E. coli* Y-10 treated with EM 49 and polymyxin B showed the formation of numerous blebs on the outside surface of treated cells which were absent in untreated controls (Meyers et al., 1975). EM 49, in contrast to polymyxin B, caused the release of small membrane vesicles from *E. coli* into the surrounding media. This phenomena was examined using scanning electron microscopy in order to visualize antibiotic-induced morphological changes. A number of previous studies have substantiated the utility of scanning electron microscopy for examining the surface morphology of microorganisms (Bartlett, 1967; Barnes et al., 1971; Klainer and Perkins, 1971) and this technique has been used to study antibiotic-induced changes in the surface of microorganisms (Greenwood and O'Grady, 1969). The critical point drying technique was used in this study because it provides the most artifact-free method for sample preparation with maintenance of cell size and dimensions. Particulate material was seen with both controls and EM 49 treated samples. However, the untreated *E. coli* cells had a much smoother surface compared to those treated with EM 49 at 50 μg/ml (Figure 3). The morphological changes caused by EM 49 were extensive and a significant fraction of the cell's surface was affected by the antibiotic. These electron micrographs and those reported by Meyers et al. suggested that particulate material was released from *E. coli* upon EM 49 treatment. The origin of the released particles could not be determined by electron microscopy; therefore, this phenomena was examined by biochemical methods described below.

Release of ^{32}P-Labeled Phospholipids from EM 49 Treated E. coli. In order to detect the release of membrane phospholipids caused by EM 49, *E. coli* Y-10 grown in the presence of $H_3{}^{32}PO_4$ was treated with EM 49 or polymyxin B, and the resulting supernatants were analyzed for ^{32}P-labeled material released from the cells. The results of this experiment are summarized in Table I. Concentrations of EM 49 and polymyxin as low as 4 μg/ml stimulated the release of ^{32}P-labeled material from the bacterial cells. EM 49, at a concentration of 400 μg/ml, caused the release of approximately 10% of the total cellular ^{32}P. Most of this released material was water soluble. Only 5% of the liberated ^{32}P-labeled material was extracted into chloroform–methanol (2:1). Although the water-soluble ^{32}P-labeled compounds were not characterized, they were low molecular weight and dialyzable. This material was probably phosphorylated compounds (e.g., nucleotides) released from the cytoplasm or periplasmic space because of membrane damage affected by EM 49. The lipids released by EM 49 were analyzed by thin-layer silica gel chromatography. Greater than 90% of this material was a mixture of phosphatidylethanolamine (87.6%), phosphatidylglycerol (10.4%), and cardiolipin (1.0%). This phospholipid composition is quite similar to the outer-membrane phospholipid composition. In contrast to EM 49, less than 0.1% of the total ^{32}P released by polymyxin B was extractable into chloroform–methanol. These data suggested that the released vesicular material seen in electron micrographs of EM 49 treated *E. coli* were outer-membrane fragments. It is significant, in terms of results presented in a later section, that a mixture of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ completely inhibited the EM 49 stimulated release of ^{32}P-labeled material from *E. coli* Y-10 (Table I).

Lipopolysaccharide Release by EM 49. The previous results suggested that EM 49 may perturb outer-membrane structure and release outer membrane fragments from *E. coli*. Lipopolysaccharide (LPS) release was examined, since LPS is found

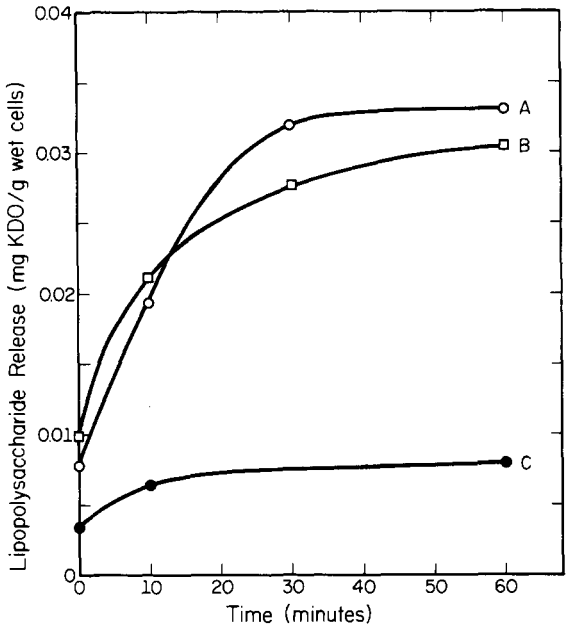

FIGURE 4: Kinetics of lipopolysaccharide release. *E. coli* SC 9251 was grown to midlog phase, harvested, and washed once with 0.1 M NH_4HCO_3. The bacteria were divided into 12 equal fractions (0.50 g/sample) and suspended in 10 ml of 0.1 M NH_4HCO_3. Eight of these samples were treated with 50 μg/ml of EM 49 for various periods of time (0, 10, 30, and 60 min). The cells were then pelleted at 10 000*g* for 2 min and the supernatants were withdrawn and analyzed for KDO. Zero time in this kinetic plot is actually 2 min after the addition of EM 49 because of the centrifugation step. One set of supernatants obtained from EM 49 treated samples was treated with DNase and Mg^{2+} for 3 h at 23 °C to check for deoxyribose contamination. All samples were dialyzed overnight against 4 l. of distilled water, evaporated to dryness, and assayed for KDO as described under Methods. Control (C); EM 49 treated cells (B); EM 49 and DNase treated (A).

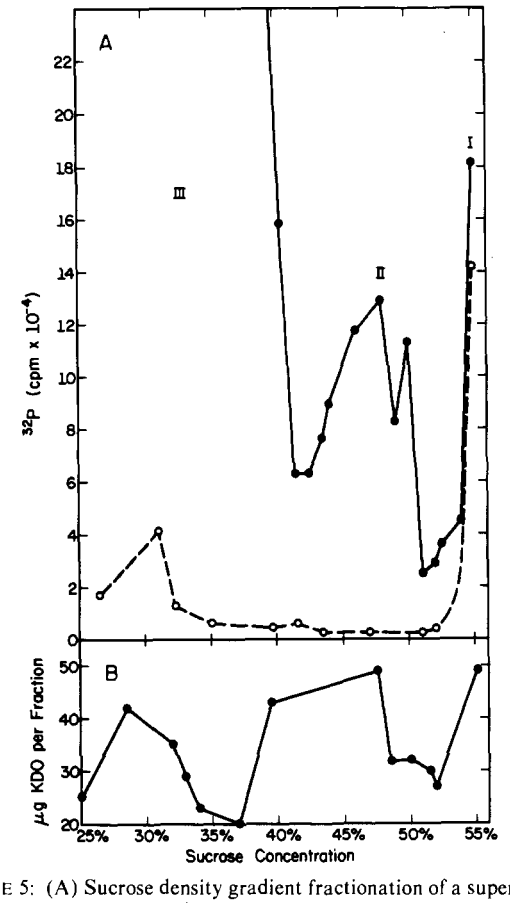

FIGURE 5: (A) Sucrose density gradient fractionation of a supernatant obtained from EM 49 treated *E. coli*. *E. coli* SC 9251 was grown to midlog phase in enriched media containing 5 mCi of $H_3{}^{32}PO_4$. The cells were harvested, washed once in 1 mM $NaHCO_3$, and resuspended in 1 mM $NaHCO_3$ containing either 0 (- - ○ - -) or 100 μg/ml of EM 49 (- ● -) for 30 min at 25 °C. The bacterial suspensions were centrifuged at 3000*g* for 15 min and the supernatants were isolated and concentrated by ultrafiltration using an Amicon XM 50 filter. The samples were then applied to a discontinuous sucrose gradient ranging from 30 to 55% and centrifuged for 22 h at 25 000 rpm in a SW 25.2 rotor. The gradient was fractionated into 2-ml aliquots and 100-μl fractions were counted for ^{32}P. Results are plotted as total cpm ^{32}P/0.5% increment of sucrose. (B) Lipopolysaccharide content of sucrose density gradient fractions. *E. coli* SC 9251 was treated as described above. Sucrose gradient fractions were dialyzed individually to remove the sucrose. The fractions were evaporated to dryness and assayed for KDO, as described under Methods.

almost exclusively in the outer membrane of gram-negative bacteria (Osborn, et al., 1972). KDO was used as a marker for LPS as described under Methods. These experiments demonstrated that EM 49 stimulated the release of LPS from *E. coli* relative to controls treated identically in the absence of antibiotic (Figure 4). The percentage of LPS released by EM 49 was linear with an EM 49 concentration up to 100 μg/ml. The maximum amount of LPS released by EM 49, at a concentration of 50 μg/ml, was 10–15% of the total cellular LPS.

LPS release by EM 49 could be a secondary effect resulting from autolysis of antibiotic-killed bacteria. Therefore, the kinetics for LPS release were examined as described under Methods (Figure 4). Zero time for this experiment was actually 2 min after addition of EM 49 because of the time required for sedimentation and removal of the supernatant. The data in Figure 4 illustrate that EM 49 stimulated the release of LPS from *E. coli* in 2 min or less after treatment with the peptide. The kinetics of LPS release were relatively rapid compared to the kinetics for killing of *E. coli* by EM 49. At comparable concentrations of the antibiotic, viable cell count was not significantly reduced until 10–15 min after EM 49 treatment (Meyers et al., 1973b). It has been shown that outer-membrane material is released from gram-negative bacteria when protein synthesis is inhibited by chloramphenicol (Rothfield and Pearlman-Kothencz, 1969). However, release of outer-membrane material following inhibition of protein synthesis is relatively slow compared to the EM 49 stimulated release of LPS. These data indicate that LPS release by EM 49 was not a secondary or late event arising from incipient lysis or inhibition of protein synthesis. On the contrary, the kinetic data illustrate that LPS release probably precedes death of the organism. In order to ensure that the material given off was not DNA, one sample was treated with DNase and compared to an identical sample not treated with DNase. Both samples were dialyzed extensively to remove small-molecular-weight material. The kinetic curves in Figure 4 show that these two samples gave very similar results, indicating that deoxyribose was not interfering with the assay for KDO.

Isolation of Outer-Membrane Fragments Released by EM 49. Since outer-membrane phospholipids and LPS were released by EM 49, it suggested that perhaps an entire outer-membrane fragment containing these lipids was released. In order to verify this, the supernatants obtained from cells treated with buffer or EM 49 at concentrations of 50, 100, or 200 μg/ml were submitted to sucrose density gradient centrifugation as described under Methods (Figure 5). Three major ^{32}P-containing peaks were obtained on this gradient. Peak I was observed with control and EM 49 treated samples and its density suggest that it was either whole cells or large cell

TABLE II: Composition of the Membrane Fragment Released by EM 49.

	EM 49 Released[a] Particle	Isolated Outer Membranes
Density	1.22 g/cm	1.22 g/cm[b]
Phospholipid-LPS-protein (wt ratios)	0.34:0.53:1	0.30:0.32–0.98:1[b]
Phospholipid composition		
PE	87.6%	87.9%
PG	11.4%	10.5%
CL	1.0%	1.7%

[a] Membrane particles released by EM 49 and isolated by sucrose density gradient fractionation as described in Figure 5. [b] Osborn et al., 1972.

fragments. Peak II occurred with EM 49 treatment but not with the control sample. Peak III was seen in both the control and EM 49 treated samples, although much more of this material was released in the presence of the antibiotic. The density of this material suggests that it is probably small membrane fragments, LPS, or phospholipids. The fractionation profile shown in Figure 5 was obtained in six independent experiments, regardless of whether samples were concentrated by lyophilization or ultrafiltration. Centrifugations up to 22 h did not change the gradient profile. Peak II contained LPS and a phospholipid composition identical to isolated outer membranes (Table II). In addition, the ratio of phospholipid-LPS-protein in peak II was 0.34:0.53:1, which compares favorably with the ratio obtained with isolated outer membranes (0.30:0.32–0.98:1) (Osborn et al., 1972). The apparent density of peak II was 1.22 g/ml, which approximates the reported densities of outer membranes from gram-negative organisms (Osborn et al., 1972). Isolated outer membranes submitted to this sucrose density gradient centrifugation had a mobility identical to peak II.

Outer membranes prepared by the method of Mizushima and Yamada (Mizushima and Yamada, 1975) and fraction II from the sucrose gradient were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 6). The two gels shown in Figure 6 were not of identical length; therefore, protein bands having identical mobilities relative to bromphenol blue were labeled 1–9 for comparison. Polypeptide bands 1–8 were observed in both the outer membranes and the EM 49 released membrane fragments. However, the relative amounts of each polypeptide differed. Bands 5, 6, and 7 (mol wts; 44 000, 42 500, and 33 000) were present in about the same relative amounts in both samples. In contrast, band 3 (66 000 daltons) was barely detectable in the EM 49 released particles. These sodium dodecyl sulfate gel profiles are consistent with the proposal that outer-membrane fragments released by EM 49 contained the major outer-membrane polypeptides. However, the composition of the released proteins, in quantitative terms, was not identical to the outer membranes. This suggests that either these proteins are not uniformly distributed in the outer membrane and EM 49 released specific membrane domains, or that differential absorption or solubilization of specific polypeptides occurred during isolation of these two samples. It should also be emphasized that it is difficult to isolate absolutely pure outer membranes without some contamination by inner membranes and other cellular components (Osborn et al., 1972). Furthermore, the sodium dodecyl sulfate gel patterns for outer membranes isolated by several different procedures are comparable, but not identical,

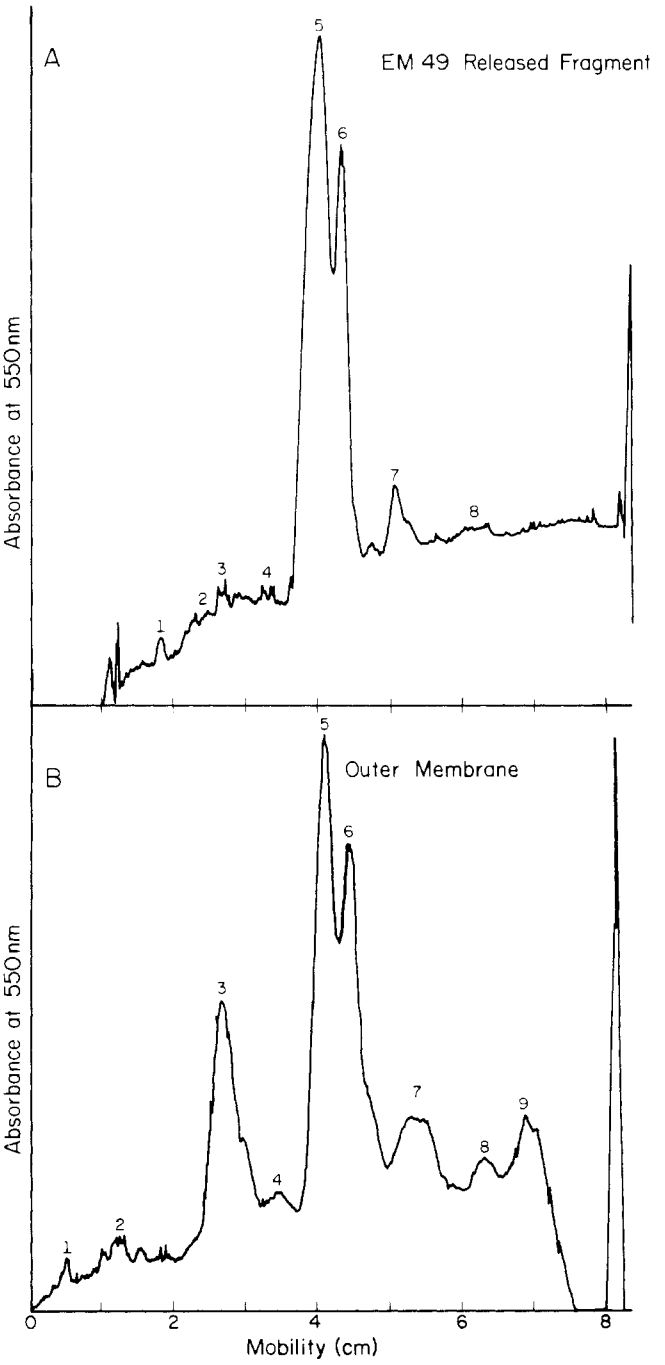

FIGURE 6: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane particles released by EM 49 and isolated outer membranes. Pooled fractions of peak II, Figure 5(A), and outer membranes, Figure 5(B), isolated by the method of Mizushima and Yamada (Mizushima and Yamada, 1975), were prepared for electrophoresis as described under Methods. Known protein standards were run for molecular weight standardization. Seventy micrograms of membrane protein was run in each case. The two gels were not of identical length; therefore, protein bands having identical molecular weights are numbered 1 through 9 for the sake of comparison. Coomassie blue stained gels were scanned at 550 nm using a linear-transport system.

to each other (Mizushima and Yamada, 1975; Schnaitman, 1974). During the normal growth of *E. coli*, small amounts of outer-membrane material are released into the media (Rothfield and Pearlman-Kothencz, 1969). However, the compositional analysis cited above is for membrane particles released by EM 49 which were not seen on the sucrose gradients with control samples treated identically in the absence of EM 49.

TABLE III: Phospholipid, Fatty Acid, and KDO Composition of Polymyxin Resistant *E. coli* Mutants.

E. coli strain	SC 9251[e]	SC 9252	SC 9253
MIC,[a] Polymyxin B	1.17	100	>200
MIC,[a] EM 49	1.65	0.3	<0.01
Phospholipid Composition (%)[b]			
Phosphatidylethanolamine	72.3 ± 1	66.3 ± 0.4	77.9 ± 0.4
Phosphatidylglycerol	25.3 ± 1	25.7 ± 0.5	15.7 ± 0.3
Cardiolipin	2.5 ± 0.1	8.3 ± 0.2	6.6 ± 0.1
Fatty Acid Composition (%)[c]			
14:0	1.23	1.35	4.10
16:0	37.01	39.30	40.30
16.1	10.50	9.26	26.74
18.0	9.10	4.27	5.96
18:1 (oleate)	38.43	38.89	20.51
18:1 (vaccenate)	3.72	6.94	2.41
KDO (mg of KDO/g of wet cells)[d]	0.91 ± 0.04	0.85 ± 0.10	0.83 ± 0.02

[a] Minimal inhibitory concentrations, μg/ml. [b] Whole cell phospholipid composition determined as described under Methods, average of three determinations with range indicated. [c] Whole cell fatty acid composition determined as described in Methods, one determination. [d] KDO extractable from whole cells by 1-butanol. LPS determined as described under Methods. Average of three determinations with range indicated. [e] Strains SC 9252 and 9253 were spontaneous mutants obtained from the parental strain SC 9251.

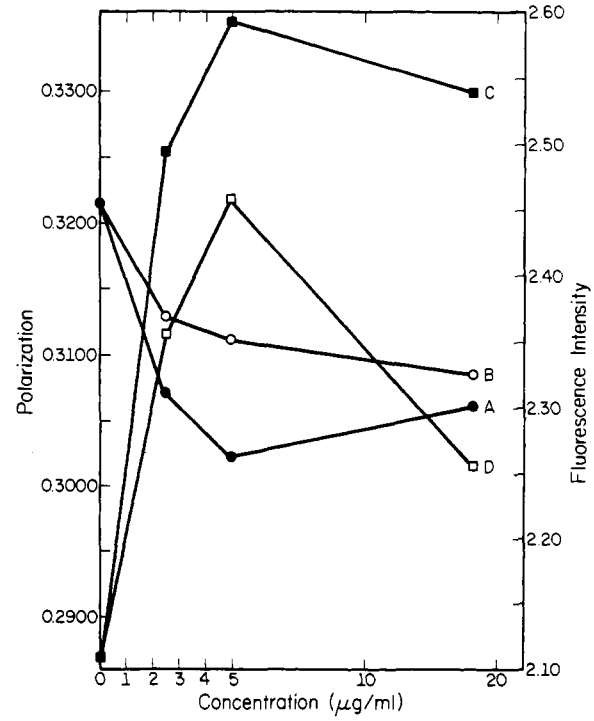

FIGURE 7: Effect of EM 49 and polymyxin B on the fluorescence intensity and fluorescence polarization of *E. coli* outer membranes containing DPH. Membranes were treated with DPH as described under Methods. Two-milliliter membrane samples containing 105 μg of protein/ml were treated with varying concentrations of EM 49 polymyxin or an equal volume of buffer containing no antibiotic. Fluorescence measurements were made 10 min after antibiotic treatment. Fluorescence polarization of EM 49 treated membranes (●-●); fluorescence intensity of EM 49 treated membranes (■-■); fluorescence polarization of polymyxin treated membranes (○-○); fluorescence intensity of polymyxin treated membranes (□-□).

Phospholipid, Fatty Acid, and KDO Composition of Polymyxin-Resistant E. coli Mutants. The release of outer membrane fragments by EM 49 implies a direct interaction between the peptide and outer membranes, possibly by virtue of specific interactions with membrane lipids. Two polymyxin-resistant *E. coli* strains (SC 9252 and SC 9253), which were hypersensitive to EM 49, were available (Table III). SC 9252 and SC 9253 were spontaneous mutants obtained from SC 9251. The genetics of these *E. coli* strains have not been characterized and it is not known whether or not sensitivity of these strains to the antibiotics is the only genetic difference between the strains. The lack of cross-resistance between EM 49 and polymyxin suggested that perhaps the two peptides interacted with different membrane components identifiable by examining the membrane composition of these strains. Therefore, the KDO, phospholipid, and the fatty acid compositions of SC 9251, SC 9252, and SC 9253 were examined to determine if polymyxin or EM 49 sensitivity could be correlated with any compositional differences between these strains.

The phospholipid compositions in Table III are the average of three determinations. The only compositional changes correlating with EM 49 sensitivity and polymyxin resistance were a decrease in the percentage of phosphatidylglycerol and an increase in cardiolipin. However, the binding constants between these purified phospholipids and polymyxin B or EM 49 were comparable (discussed below). This suggests that the differential sensitivity of these strains to EM 49 and polymyxin B was probably not due to changes in the relative amount of phosphatidylglycerol. Although SC 9251, SC 9252, and SC 9253 differed somewhat in fatty acid composition, there were no obvious correlations between sensitivity to these two antibiotics and fatty acid composition. The amount of KDO found in these three strains was quite similar, suggesting that variation in the amount of LPS was not responsible for differences in sensitivity to these antibiotics. Thus, there is no evidence from this compositional data that EM 49 or polymyxin B interacts preferentially with a particular phospholipid or fatty acid.

Effect of EM 49 on Outer-Membrane Fluorescence Polarization. Direct interaction between EM 49 and isolated outer membranes was demonstrated using outer membranes labeled with a fluorescent dye. Changes in fluorescence polarization and fluorescence intensity of a hydrophobic fluorescent dye molecule incorporated into membranes can give information concerning the hydrophobicity and fluidity of the dye environment (Weber, 1966). This technique was employed to detect outer-membrane structural changes induced by EM 49. The fluorescent dye 1,6-diphenyl-1,3,5-hexatriene, an asymmetric, hydrophobic molecule, was used as a fluorescence probe for monitoring the fluidity and hydrophobicity of membrane lipid layers (Shinitsky and Inbar, 1974). Upon treatment of DPH containing outer membranes with EM 49 or polymyxin B (5 μg/ml), the fluorescence polarization values decreased (Figure 7). With increasing concentrations of these peptides, the polarization values either leveled off or increased slightly. Fluorescence intensity increased sharply with antibiotic concentration up to 5 μg/ml and then decreased with increasing concentrations of EM 49 or polymyxin B.

The peptides could complex DPH and remove the dye from the membranes, thereby, affecting the fluorescence polarization and intensity. It is expected that the dye–peptide complex would fluoresce, whereas the free dye in aqueous solution does not. Therefore, fluorescent-labeled membrane suspensions, treated with EM 49 or polymyxin, were centrifuged at

TABLE IV: Binding Constants for the Interaction of EM 49 and Polymyxin B with *E. coli* Phospholipids.

	Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Cardio-lipin
EM 49[a]	1.2×10^5	2.1×10^5	5.3×10^5
Polymyxin B	2.2×10^5	3.9×10^5	1.8×10^5
Bacitracin A	*b*	*b*	*b*
CTAB	*b*	*b*	*b*
Sodium dodecyl sulfate	*b*	*b*	*b*

[a] Binding constants (M^{-1}) determined by the method of Hummel and Dreyer as described under Methods. [b] No interaction detected using the Hummel and Dreyer technique in the concentration range examined (10^{-6} to 10^{-3} M).

100 000*g* to pellet membranes. No fluorescence was observed with these supernatants, indicating that a peptide–dye complex was not present. However, interaction between the peptides and the dye within the membrane could not be ruled out.

The change in fluorescence intensity of DPH observed with EM 49 or polymyxin B is directly proportional to the change in fluorescence lifetime of the dye excited state, indicating a change in the hydrophobicity of the dye environment. At concentrations of EM 49 up to 5 μg/ml, the microenvironment of the dye increased in relative hydrophobicity. Although the uncorrected flourescence polarization data would indicate otherwise, correcting this data for lifetime changes indicated that, in the concentration range 0–5 μg/ml, both EM 49 and polymyxin caused a decrease in membrane fluidity. Although it is difficult to interpret these data in molecular terms, it clearly illustrates that EM 49 caused detectable structural changes in outer membranes. It is also evident from these data that different structural changes may occur depending upon the antibiotic concentration range employed.

Interaction between EM 49 and Phospholipid Dispersions. Interaction between EM 49 and phospholipid dispersions was examined using the Hummel and Dreyer technique (Hummel and Dreyer, 1962). It was discovered that both EM 49 and polymyxin B extracted phospholipids from phospholipid micelles. The three phospholipids studied were in an aggregated form in aqueous solutions and were excluded from Sephadex G-25. In the absence of the peptide antibiotic, all of the phospholipid ran in the void volume of the column. As shown in Figure 2, addition of EM 49 to the top of the column resulted in perturbations from the baseline of the elution profile, a trough followed by a peak of equivalent area. Analysis for peptide demonstrated that all of the antibiotic appeared in the peak of radioactivity. These findings indicate that the peptide interacted strongly with the phospholipids, extracting some phospholipid from the micelle in the form of a peptide–phospholipid complex which was small enough to be included in Sephadex G-25. Since the exclusion limit for Sephadex G-25 is approximately 5000, this suggests that the included phospholipid must be in a form having no more than 4 or 5 phospholipid molecules/molecule of peptide.

The apparent association constants for interaction of EM 49 and polymyxin B with *E. coli* phospholipids are given in Table IV. The method for calculating these binding constants is defined under Methods. These are not true binding constants for the interaction between the phospholipid in monomeric form and the antibiotics, since interactions between phospholipids in the micelle must be overcome during the extraction process. Clearly, EM 49 and polymyxin interacted very

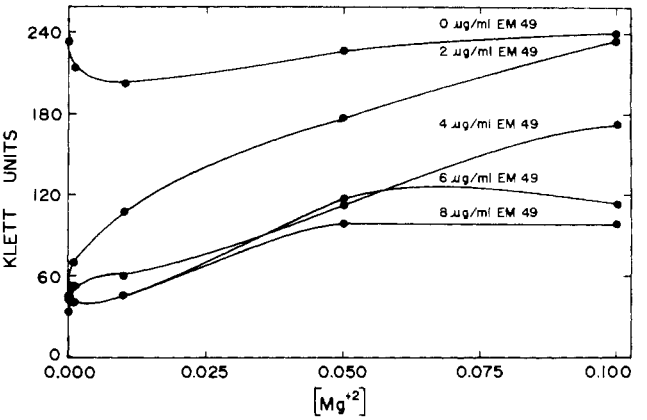

FIGURE 8: Effect of Mg^{2+} on the antibiotic activity of EM 49. *E. coli* SC 9251 was grown in enriched media at EM 49 concentrations of 0, 2, 4, 6, and 8 μg/ml. The cultures were preincubated with Mg^{2+} for 1 h and then the antibiotic was added. After 14 h of growth, the turbidity of the cultures was measured with a Klett colorimeter.

strongly with phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. Bacitracin, which specifically interacts with C_{55}-isoprenyl pyrophosphate of bacterial membranes, did not interact strongly with these phospholipids using this assay (Storm, 1974). Neither EM 49 nor polymyxin B demonstrated strong specificity for any particular phospholipid class, although EM 49 did show slightly stronger binding with phosphatidylglycerol and cardiolipin compared to phosphatidylethanolamine. These binding studies do indicate that EM 49 has a strong affinity for *E. coli* phospholipids, which may explain, at least in part, its ability to disrupt outer-membrane structure. It should be emphasized that simple detergents, such as sodium dodecyl sulfate and quaternary ammonium salts, did not show this phenomena at concentrations of the detergents comparable to the antibiotics. The disruptive effect of EM 49 and polymyxin B on phospholipid micelles was probably not a simple detergent action and undoubtedly involved much more specific interactions. EM 49 and polymyxin B exhibit antimicrobial activity at concentrations much lower than simple detergents (Rose et al., 1966). These two antibiotics are structurally similar, yet their antimicrobial spectra are significantly different. The effect of minor structural changes on the activities of these peptides not consistent with a nonspecific detergent-like effect.

Effect of Divalent Cations on EM 49 Antibiotic Activity. The polycationic nature of EM 49 and the inhibition of EM 49 stimulated phospholipid release by $MgCl_2$ and $CaCl_2$ (Table I) suggested that the antibiotic might interfere with the normal electrostatic interactions between components of the outer membrane. It has been proposed that Mg^{2+} and Ca^{2+} play an important role for the structural integrity of outer membranes by functioning as metal ion bridges between the phosphates of lipopolysaccharide and phospholipid molecules (Leive, 1974). Compositional analysis has shown that a relatively high percentage of Ca^{2+} and Mg^{2+} is found in bacterial cell envelopes (Leive, 1968). For these reasons, the effect of divalent cations on EM 49 antibiotic activity was examined. At the minimal inhibitory concentration of EM 49 (2 μg/ml), *E. coli* SC 9251 was completely protected from EM 49 at 100 mM $MgCl_2$ and partial protection was observed at concentrations as low as 1 mM $MgCl_2$ (Figure 8). At higher concentrations of EM 49 (>4 μg/ml), Mg^{2+} afforded only partial protection. Calcium ion gave a similar effect but other divalent and monovalent cations did not protect *E. coli* from EM 49. These results, coupled with the data in Table I, suggest that

EM 49 may compete with Mg^{2+} and Ca^{2+} for binding sites in the outer membrane. It is unlikely that the peptide functions as a chelator of divalent cations, like EDTA, because of its polycationic nature.

Discussion

It is well known that many antibiotics that are highly active against gram-positive bacteria are much less active against gram-negative species. These differences in sensitivity are attributed to the unique cell envelope of gram-negative bacteria, which includes the outer membrane, a permeability barrier which is thought to hinder the access of certain antibiotics. For example, Leive has shown that various strains of *E. coli, Aerobacter aerogenes,* and *Salmonellae antum* and *aerogenes,* which are normally insensitive to actinomycin D, become completely sensitive to actinomycin D after a brief EDTA treatment (Leive, 1968). EDTA damages outer-membrane structure and releases 30–50% of the surface LPS by complexing Ca^{2+} and Mg^{2+}, which are crucial for outer-membrane structure (Leive, 1974). The outer membrane is probably a general permeability barrier for many antibacterial reagents, since EDTA increases sensitivity to a number of antibiotics, including penicillin (Weisser et al., 1968), rifampicin (Reid and Speyer, 1970), and streptogramin (Ennis, 1967). Therefore, in order for an antibiotic to be effective against gram-negative bacteria, it must overcome the outer-membrane permeability barrier by one means or another.

A number of experimental observations have established that EM 49 damages *E. coli* outer-membrane structure and releases outer-membrane fragments from the bacteria. EM 49, in contrast to polymyxin B, released a mixture of phospholipids into the media having the same composition as the outer-membrane phospholipids. The antibiotic also stimulated the release of LPS and the kinetics for this phenomena were fast enough to exclude the possibility that LPS release was a secondary effect resulting from autolysis of antibiotic-killed bacteria. In addition, a membrane fragment selectively released by EM 49 was isolated by sucrose density gradient centrifugation and characterized. This membrane fragment had a phospholipid–LPS–protein ratio comparable to outer membranes, a phospholipid composition identical to outer membranes, and a density equivalent to outer membranes. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the released membrane fragments demonstrated that the polypeptide profile was quite similar to, but not identical to, outer membranes. Finally, both scanning and transmission electron microscopy revealed that EM 49 caused extensive damage to the outer surface of *E. coli* cells, with release of membrane particles.

Release of outer-membrane fragments from *E. coli* by EM 49 is a unique property shared by no other antibiotic. Although the mechanism for perturbation of outer membranes by EM 49 is not known, several possibilities are suggested by data presented in this report. This antibiotic has a relatively strong affinity for bacterial phospholipids and fluorescence studies revealed that detectable structural changes occurred when outer membranes were treated with EM 49. Interaction between EM 49 and membranes is not unexpected, since the peptide contains a covalently attached C:11 fatty acid. This fatty acid side chain may be inserted into the hydrophobic core of the membrane. Such an interaction would position the positively charged peptide moiety at the membrane–aqueous interface allowing polar interactions between the peptide and polar head groups of the phospholipids or LPS. Mg^{2+} and Ca^{2+} protected *E. coli* from EM 49 and inhibited the release of phospholipids stimulated by EM 49. This suggests that EM 49 may compete with these cations for negatively charged phosphate groups of phospholipids or LPS. Leive has proposed that Mg^{2+} and Ca^{2+} play a specific structural role in outer membranes as metal ion bridges between phosphate groups of phospholipids and LPS (Leive, 1974). Displacement of these cations by the ammonium functions of EM 49 and insertion of the fatty acid into the core of the membrane may distort the normal packing between phospholipids and LPS in the outer membrane. The effectiveness of EM 49, in this respect, would be due to its polycationic charge and its fatty acid side chain, which could anchor the antibiotic in the membrane. There is presently no conclusive evidence that the peptide interacts exclusively with any specific phospholipid class or fatty acid.

The molecular basis for EM 49 antibiotic activity against gram-negative bacteria is not established. It is conceivable that extensive structural damage to the outer membrane could be lethal for *E. coli.* The structural and functional relationships between the inner membrane, peptidoglycan, and outer membrane have not been completely defined, although it is clear that these structures cannot be considered as separate and independent layers of the cell envelope (Braun, 1975). It is notable in this respect that mutations in the core of LPS are lethal and mutations totally lacking LPS have not been obtained (Osborn, et al., 1974). However, damage to the outer membrane cannot be the common denominator for the antimicrobial activities of this peptide against a wide variety of organisms including gram-positive and -negative bacteria, fungi, yeast, and protozoa. Damage to the gram-negative outer membrane does, however, allow the antibiotic to penetrate to the inner membrane, which is most likely the primary killing site. EM 49 has been shown to affect the respiration of *E. coli, Bacillus subtilus,* as well as the yeast *Candida albicans* (Rosenthal and Storm, 1975). With *E. coli* and *B. subtilis,* EM 49 enhanced the rate of respiration at minimal inhibitory concentrations of the antibiotic and completely inhibited respiration at biocidal concentrations of EM 49. These effects of EM 49 on respiration are similar to those manifested by classical uncouplers of oxidative phosphorylation (Scholes and Mitchell, 1970). These data imply a direct or indirect action on inner-membrane function. In conclusion, the properties of this new antibiotic suggest that it may be a useful tool for studying the structure and function of bacterial membranes, as well as lipid–peptide interactions.

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References

Ames, B. N. (1966), *Methods Enzymol. 8,* 115–118.
Barnes, W. G. A., Flesher, A. E., Berger, A. E., and Arnold, J. D. (1971), *J. Bacteriol. 106,* 276.
Bartlett, G. A. (1967), *Science 158,* 1318.
Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37,* 911.
Braun, V. (1975), *Biochim. Biophys. Acta 415,* 335.
Brown, M. R. W., and Watkins, W. M. (1970), *Nature (London) 227,* 1360.
Ennis, H. L. (1967), *J. Bacteriol. 93,* 1881.
Ferguson, K. A., Glaser, M., Boyer, W. H., and Vageolos, P.

R. (1975), *Biochemistry 14,* 146.
Greenwood, D., and O'Grady, F. (1969), *Science 163,* 1076.
Hayat, M. A. (1970), *Princ. Techn. Electron Microsc. 1,* 339.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.
Klainer, A. S., and Perkins, R. L. (1971), *J. Am. Med. Assoc. 215,* 1655.
Leive, L. (1968), *J. Biol. Chem. 243,* 2373.
Leive, L. (1974), *Ann. N.Y. Acad. Sci. 235,* 109.
Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951), *J. Biol. Chem. 193,* 265.
Meyers, E., Brown, W. E., Principe, P. A., Rathnum, M. L., and Parker, W. L. (1973a), *Jpn. J. Antibiot. 26,* 444.
Meyers, E., Pansy, F. E., Basch, H. I., McRipley, R. J., Slusarchyck, D. S., Graham, S. F., and Trejo, W. H. (1973b), *Jpn. J. Antibiot. 26,* 457.
Meyers, E., Parker, W. L., Brown, W. E., Linnett, P., and Strominger, J. L. (1974), *Ann. N.Y. Acad. Sci. 235,* 493.
Mizushima, S., and Yamada, H. (1975), *Biochim. Biophys. Acta 375,* 44.
Nakajima, K. (1967), *Chem. Pharm. Bull. 15,* 1219.
Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972), *J. Biol. Chem. 247,* 3962.
Osborn, M. J., Rick, P. D., Lehmann, U., Rupprecht, E., and Singh, M. (1974), *Ann. N.Y. Acad. Sci. 235,* 52.
Parker, W. L., and Rathnum, M. L. (1973), *Jpn. J. Antibiot. 26,* 449.
Parker, W. L., and Rathnum, M. L. (1975), *Jpn. J. Antibiot. 28,* 379.
Reid, P., and Speyer, J. (1970), *J. Bacteriol. 104,* 376.
Rose, M. J., Jr., Aron, A., and Janicki, B. W. (1966), *J. Bacteriol. 91,* 1863.
Rosenthal, K., and Storm, D. R. (1975), 15th Interscience Conference on Antimicrobial Agents and Chemotherapy, Abst. 422.
Rothfield, L., and Pearlman-Kothencz, M. (1969), *J. Mol. Biol. 44,* 477.
Schnaitman, C. A. (1974), *J. Bacteriol. 118,* 454.
Scholes, P., and Mitchell, P. (1970), *Bioenergetics 1,* 61.
Sezikwa, J., and Fukui, S. (1973), *Biochim. Biophys. Acta 307,* 104–117.
Shinitsky, M., and Inbar, M. (1974), *J. Mol. Biol. 85,* 603.
Skipski, V. P., and Barclay, M. (1969), *Methods Enzymol. 14,* 530.
Storm, D. R. (1974), *Ann. N.Y. Acad. Sci. 235,* 387.
Storm, D. R., and Strominger, J. L. (1973), *J. Biol. Chem. 248,* 3940.
Storm, D. R., and Strominger, J. L. (1974), *J. Biol. Chem. 249,* 1823.
Weber, G. (1966), in Fluorescence and Phosphorescence Analysis, Hercules, D. M., Ed., New York, N.Y., Interscience.
Weissbach, A., and Hurwitz, J. (1959), *J. Biol. Chem. 234,* 705.
Weisser, R., Asscher, A. W., and Winpenny, J. (1968), *Nature (London) 219,* 1365.

Natural Abundance Carbon-13 Nuclear Magnetic Resonance Studies of Bovine White Matter and Myelin†

Eliot C. Williams and E. H. Cordes*

ABSTRACT: Whole bovine white matter yields a poorly resolved natural abundance ^{13}C nuclear magnetic resonance (NMR) spectrum. The spectrum principally reflects carbon atoms of the constituent membrane lipids: several resonances could be specifically assigned but no resonances attributable to cholesterol are detectable. Except for the methyl group at the terminus of fatty acyl chains, lipid carbons giving rise to the ^{13}C NMR spectrum have values of spin–lattice relaxation time between 140 and 500 ms, indicating significant restrictions on segmental and rotational mobilities but consistent with a generally fluid structural organization. The ^{13}C NMR spectrum of myelin isolated from bovine white matter is similar to that for the whole white matter itself. In both white matter and isolated meylin, the integrated intensities for several carbon atoms are considerably less than those for the same carbon atoms in total lipid extracts. The data for white matter and myelin are consistent with a model in which observed line broadening is due to restrictions in the amplitude of chain flexing rather than to severe restrictions on chain segmental motion. Failure to detect resonances of cholesterol ring system carbon atoms may reflect marked anisotropy of rotational reorientation.

Both ^{1}H and ^{13}C NMR[1] studies of myelin-containing preparations derived from peripheral nerve have been previously undertaken in an effort to derive information concerning the physical state of the lipids of the membranes comprising the myelin sheath (Dea et al., 1972; Williams et al., 1973). In neither of those studies, however, was it possible to detect many resonances arising from the lipids of the myelin itself, reflecting in part the broadness of these resonances and in part the interference from adventitial triglyceride (Williams et al., 1973). The studies on bovine brain white matter and central nervous system myelin reported herein represent an attempt to circumvent the latter problem by using myelin-containing preparations which are triglyceride free. Moreover, these preparations are obtainable in large quantities, thereby

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance; NOE, nuclear Overhauser enhancement.