Inhibition of *Escherichia coli* Growth and Respiration by Polymyxin B Covalently Attached to Agarose Beads†

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ABSTRACT: Polymyxin B was attached to agarose beads by stable covalent bonds and the antimicrobial activity of the immobilized peptide was examined. Polymyxin-agarose inhibited the growth of *Escherichia coli* and *Pseudomonas aeruginosa,* but not *Bacillus subtilis.* In addition, the respiration of *E. coli, E. coli* spheroplasts, and *B. subtilis* protoplasts was inhibited by immobilized polymyxin, whereas the respiration of *B. subtilis* was unaffected by polymyxin-agarose. The activity of polymyxin-agarose was not due to the release of free peptide from the derivative. These data indicate that polymyxin can inhibit the growth and respiration of gram-negative bacteria by interacting with the outer surface of these cells. It is proposed that perturbation of outer membrane structure by polymyxin-agarose indirectly affected the selective permeability of the inner membrane and inhibited respiration. The results of this study emphasize the importance of outer membrane structural integrity for the normal functions of gram-negative bacteria.

The polymyxins are a family of closely related peptide antibiotics containing a cationic cycloheptapeptide ring with a C:8 or C:9 fatty acid attached through an amide bond (Nakajima, 1967). These antibiotics are active against a wide spectrum of microorganisms including both gram-positive and gram-negative bacteria (Newton, 1956). Although the mechanism for the antibiotic activity of the polymyxins has not been fully defined, there is considerable evidence that these peptides disrupt both the structure and function of bacterial membranes (Newton, 1956; Teuber, 1974). Polymyxin interacts strongly with bacterial membranes (Koike and Iida, 1971), bacterial phospholipids (Few, 1955; Storm, 1974; Imai et al., 1975), and lipopolysaccharide aggregates (Lopes and Inniss, 1969; Bader and Teuber, 1973).

Polymyxin treatment rapidly inhibits bacterial respiration and stimulates the release of both periplasmic and cytoplasmic components (Cerny and Teuber, 1971). It has been proposed that polymyxin's effects on the growth and respiration of gram-negative bacteria are due to a direct interaction between the peptide and the plasma membrane (Teuber, 1969; Feingold et al., 1974). This requires that the antibiotic readily pass through the outer-membrane barrier. The possibility that structural damage to the outer membrane by polymyxin may indirectly affect the functions of the plasma membrane has not been directly examined. Nor have the functions of the outer membrane been completely defined, although there is substantial evidence for its permeability and osmotic barrier function (Leive, 1974; Nakae and Nakaido, 1975) and the presence of bacteriophage (Lindberg and Hellequist, 1974) and group B colicin receptors (Braun et al., 1973) in outer membranes.

In order to examine these questions more carefully, polymyxin B was covalently attached to agarose beads to limit contact to the outer surface of gram-negative bacteria. Polymyxin-agarose is shown to inhibit the respiration and growth of two gram-negative bacteria, *E. coli* and *P. aeruginosa,* but not of *B. subtilis.* These results indicate that polymyxin can affect the respiration of gram-negative bacteria indirectly by interacting with the outer membrane and emphasize the importance of outer membrane structural integrity for normal functions of gram-negative bacteria.

Experimental Procedure

Materials. Lysozyme and polymyxin B sulfate were obtained from Sigma Chemical Co. Bacitracin was purchased from Commercial Solvents. Affi-Gel 10 was obtained from Bio-Rad Laboratories. $H_3{}^{32}PO_4$ was purchased from New England Nuclear. EM 49 (lot No. SQ 21 286) as well as *E. coli* strains SC 9251, SC 9252, and SC 9253 were kindly supplied by Squibb Institute for Medical Research. *P. aeruginosa* PaG 13 was obtained from W. Toscano and I. C. Gunsalus. *B. subtilis* GSY 201 was supplied by G. Ordal. Nutrient media contained 1% Bactopeptone, 0.1% yeast extract, and 0.5% NaCl at pH 7.0. Synthetic media consisted of 50 mM KH_2PO_4, 0.12 mM $MgCl_2$, 1 mM $(NH_4)_2SO_4$, 20 mM sorbitol, and 0.1% casein hydrolysate at pH 7.0. Minimal salts media contained 40 mM K_2HPO_4, 20 mM KH_2PO_4, 0.41 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, and 10 mM sorbitol at pH 7.0. Phosphate-buffered saline consisted of 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, and 140 mM NaCl at pH 7.0.

Covalent Attachment of Polymyxin B to Agarose. Polymyxin B, bacitracin, and EM 49 were covalently attached to agarose beads using Affi-Gel 10 (Bio-Rad). Affi-Gel 10 is an agarose derivative with added aliphatic arms 10 Å long, terminated by active carboxy-*N*-hydroxysuccinimide esters (Cuatrecasas and Parikh, 1972). One of the major advantages of Affi-Gel 10 is that the aliphatic arm is attached to agarose by means of an ether linkage. This coupling procedure is superior to those using cyanogen bromide activation of agarose because of the stability of the ether bond. The peptides were attached to the spacer arms by means of an amide bond. The antibiotics were dissolved in 12.5 mL of 0.1 M $NaHCO_3$, pH 7.5, and 0.5 g of Affi-Gel 10 was added. When polymyxin B, bacitracin, or EM 49 was coupled, they were used in 15 molar excess over the Affi-Gel 10 carboxy-*N*-hydroxysuccinimide esters in order to minimize multipoint attachment. The mixture was incubated with mild agitation for 3 h at 4 °C. The deriv-

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atized beads were washed with 85 mL of 1.0 M NaCl, 50 mL of distilled water, 50 mL of water-ethanol (1:1), and 250 mL of absolute ethanol. The efficacy of the washing procedure was followed by monitoring A_{260} and testing the final aqueous washes for antibiotic activity. Free antibiotic and *N*-hydroxysuccinimide were completely removed by this washing procedure. One batch of Affi-Gel 10 was treated with 0.1 M $NaHCO_3$, pH 8.0, for 12 h in the absence of an added ligand to hydrolyze the carboxy-*N*-hydroxysuccinimide to the acid form. These beads were then washed by the procedure described above. Underivatized agarose beads and hydrolyzed Affi-Gel 10 beads were used as controls. The derivatized beads were dried and analyzed for C, H, and N. On the basis of this analysis, it was determined that 1 mg of polymyxin-agarose contained 150 μg of covalently bound polymyxin.

Inhibition of Bacterial Growth by Polymyxin-Agarose. E. coli, P. aeruginosa, or *B. subtilis* were grown to midlog phase in nutrient media at 37 °C. These cultures were then diluted appropriately with nutrient media and 0.1-mL inoculations were introduced into 25-mL side-arm Erlenmeyer flasks containing 10 mL of nutrient media. The starting concentrations of bacteria ranged from 2×10^4 to 2×10^6 cells/mL. Viable cell count was determined by serial dilution and plating on nutrient agar. Varying amounts of hydrated, derivatized agarose beads (0.0–1 mg/mL) were then added and growth was monitored as a function of time by light scattering using a Klett Summerson spectrophotometer (Figure 1). Underivatized agarose beads or hydrolyzed Affi-Gel 10 beads had no effect on the growth of any of the bacterial strains. The growth lag period was the difference in time required for the control and polymyxin-agarose treated samples to reach 50% of their maximum growth. The controls were equivalent samples containing no polymyxin-agarose.

Preparation of E. coli spheroplasts. Spheroplasts were prepared by the EDTA[1]-lysozyme method described by Kaback (Kaback, 1971). The final preparation was washed in minimal salts media containing 20% sucrose and suspended in the same media for respiration studies. Spheroplast formation was monitored by morphological examination under a phase contract microscope. Spheroplast preparations were readily lysed in distilled water. *B. subtilis* protoplasts were prepared by an analogous procedure, except that EDTA was omitted.

Inhibition of Respiration by Polymyxin-Agarose. E. coli SC 9251 or *B. subtilis* GSY 201 were grown to midlog phase in minimal media and suspended in a 2/5 dilution of minimal media to give 10^8 bacteria/mL. Five milliliters of the bacterial or spheroplast suspension was saturated with O_2 by gently bubbling air through the solution. O_2 uptake was monitored in a stirred cell with a Clark O_2 electrode (Yellow Springs Instrument Co.). The rate of O_2 uptake was monitored for several minutes until a linear rate was established and then varying amounts of hydrated polymyxin-agarose were added and the rate of O_2 uptake was continually monitored up to 40 min. Inhibition of the respiration rate is expressed as the change in slope relative to the initial slope in the absence of added antibiotic. The extent of inhibition of respiration is reported at various times following addition of polymyxin-agarose. These data were corrected for the small dilution which occurred when the hydrated beads were added. The kinetics for inhibition of respiration were first-order and the half-lives for the rates of respiration inhibition are also reported. Hydrolyzed Affi-Gel 10 beads and underivatized agarose up to 1 mg/mL were totally without effect on respiration.

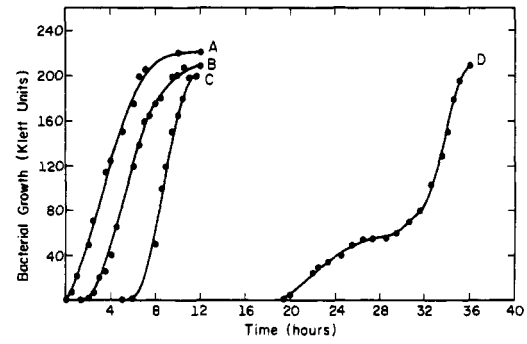


FIGURE 1: Inhibition of *E. coli* growth in the presence of polymyxin B covalently attached to agarose beads. An inoculum of *E. coli* SC 9251 (2.3×10^6 bacteria/mL) was grown in nutrient media at 37 °C in the absence or presence of polymyxin B covalently attached to agarose. (A) No polymyxin-agarose or 0.5 mg/mL of underivatized agarose; (B) 0.1 mg/mL polymyxin-agarose; (C) 0.25 mg/mL polymyxin-agarose; (D) 0.50 mg/mL polymyxin-agarose. A sample containing 1.0 mg/mL polymyxin-agarose did not show any growth when monitored up to 96 h.

Preparation of ^{32}P-Labeled E. coli Phospholipids. Two liters of *E. coli* SC 9251 were grown to late-log phase in nutrient media containing 10 mCi of $H_3{}^{32}PO_4$. The cells were harvested by centrifugation and washed two times with phosphate-buffered saline. The cell pellet was dispersed in 40 mL of chloroform-methanol (2:1) and homogenized for 10 min in a dounce homogenizer. This homogenate was filtered through a Buchner funnel and back-extracted with 1 M NaCl. The extract was then washed with methanol-water (1:1) and dried over sodium sulfate. The lipid solution was evaporated to dryness. Lipid was dispersed in 0.1 M Tris-HCl, pH 7.5, by sonicating for 0.5 h at 4 °C under N_2. The specific activity of the phospholipid preparation was 2.8×10^6 cpm/μmol. Unlabeled phospholipid dispersions were prepared by an identical procedure using *E. coli* SC 9251 grown in the absence of $H_3{}^{32}PO_4$. Phosphate was determined by the method of Ames (1966).

Binding between E. coli and Polymyxin-Agarose. E. coli SC 9251 was grown to late-log phase in 10 mL of minimal media limited to 10 mM phosphate containing 250 μCi of $H_3{}^{32}PO_4$. The bacteria were harvested and washed repeatedly with minimal media. The cells were carefully resuspended in minimal media and 0.1-mL aliquots of the ^{32}P-labeled bacteria (7×10^7 cells, 1.2×10^5 cpm of ^{32}P) were added to 5 mL of minimal media containing varying amounts of polymyxin-agarose (0.0 to 1.0 mg/mL). After 20 min of incubation at 37 °C, the suspension was centrifuged at 10*g* for 5 min to sediment beads, but not the bacteria, and an aliquot was counted for ^{32}P. The 10*g* supernatant was then centrifuged at 5000*g* for 10 min to sediment whole cells and an aliquot of this supernatant was counted for ^{32}P.

Binding of ^{32}P-Labeled Phospholipids by Polymyxin-Agarose. Varying amounts of ^{32}P-labeled *E. coli* phospholipids (0.0–0.150 μmol/mL) were dispersed by sonication into 0.1 M Tris-HCl, pH 7.0, and filtered through Whatman No. 1 filter paper to remove particulates. Polymyxin-agarose was present at 0.25 mg/mL. The mixture was incubated with mild agitation for 15 min at 37 °C and then centrifuged at 10*g* for 5 min to sediment the polymyxin-agarose beads. An aliquot of the supernatant was then counted for ^{32}P. Parallel samples containing 0.25 mg/mL of underivatized agarose were used as controls to correct for agarose absorption of phospholipids. Values for phospholipid bound by polymyxin-agarose are

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

TABLE I: Inhibition of *E. coli* and *P. aeruginosa* Growth by Immobilized Polymyxin B.

Bacterial Strain	Inoculum Size[a] (bacteria/mL)	Polymyxin-Agarose[b] (mg/mL)	Growth Lag[c] (h)
E. coli SC 9251	2.3×10^6	0.00	0.0
	2.3×10^6	0.10	1.8
	2.3×10^6	0.25	6.0
	2.3×10^6	0.50	31.0
	2.3×10^6	1.00	>96
	2.3×10^5	0.10	10.6
	2.3×10^4	0.10	13.6
P. aeruginosa PaG 13	9.1×10^5	0.00	0.0
	9.1×10^5	0.10	0.0
	9.1×10^5	0.25	2.5
	9.1×10^5	0.50	6.6
	9.1×10^5	1.00	16.8
B. subtilis GSY 201	3.0×10^5	0.10	0
	3.0×10^4	0.10	0
	3.0×10^4	0.25	0
	3.0×10^4	0.50	0
	3.0×10^4	1.00	0

[a] Bacteria were grown in nutrient media at 37 °C with shaking. [b] Polymyxin was covalently attached to Agarose as described under Methods. [c] Growth lag is the difference in time required for the control and a polymyxin-agarose treated sample to reach 50% of their maximum growth.

corrected for binding by underivatized agarose. All determinations were done in triplicate.

Results

Inhibition of E. coli and P. aeruginosa Growth by Polymyxin-Agarose. Polymyxin-agarose inhibited the growth of *E. coli* SC 9251 (Figure 1). The growth lag period was extended with increasing concentrations of polymyxin-agarose. For example, at 2.3×10^6 bacteria/mL the increases in the growth lag period relative to untreated controls were 1.8, 6.0, and 31.0 h in the presence of 0.10, 0.25, and 0.50 mg/mL polymyxin-agarose. At a polymyxin-agarose concentration of 1 mg/mL, no growth of *E. coli* occurred even after 96 h. The increase in the growth lag period was dependent upon the concentration of polymyxin-agarose and the number of bacteria present (Table I). At a polymyxin-agarose concentration of 0.10 mg/mL, the growth lag times were 1.8, 10.6, and 13.6 h in the presence of 2.3×10^6, 2.3×10^5, and 2.3×10^4 bacteria/L. Underivatized agarose, hydrolyzed Affi-Gel 10 beads, and bacitracin-agarose were totally without effect on *E. coli* growth. After the initial growth lag period, growth of *E. coli* in the presence or absence of polymyxin-agarose was exponential, followed by the normal stationary phase. However, the rate of growth during the exponential phase was actually greater for *E. coli* treated with polymyxin-agarose compared to controls. The doubling time for *E. coli* SC 9251 during exponential growth was 52 min for untreated cells and 33 min when treated with polymyxin-agarose.

The polymyxin-agarose beads used in this experiment contained 150 μg of polymyxin per mg of polymyxin-agarose. If it is assumed that the bacteria can only interact with the surface of the beads, then only a minor percentage (no more than 1%) of the polymyxin was available for interaction with the bacteria. It is impossible to make accurate estimates for the amount of polymyxin B accessible on the outer surface of the beads. However, considering the size of the beads (100 μm) and the length of the arm to which polymyxin was attached (10 Å), it can be calculated that no more than 1% of the polymyxin was on the outside surface. A concentration of 1 mg/mL of polymyxin-agarose, which completely inhibited the growth of *E. coli* SC 9251, would correspond to 1.5 μg/mL or less of accessible polymyxin. The minimal inhibitory concentration of free polymyxin was 1.2 μg/mL. Thus, the potency of immobilized polymyxin was equivalent to or greater than the free antibiotic.

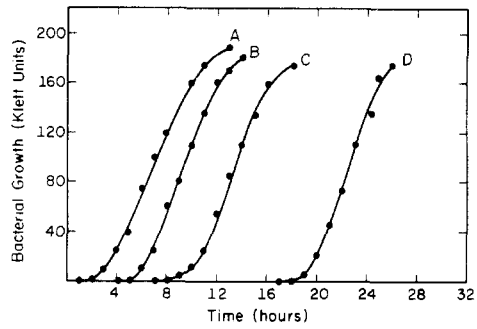
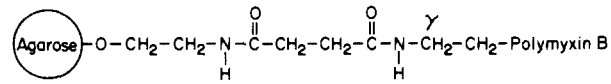


FIGURE 2: Inhibition of *P. aeruginosa* growth in the presence of polymyxin B covalently attached to agarose beads. An inoculum of *P. aeruginosa* PaG 13 (9.1×10^5 bacteria/mL) was grown in nutrient media at 37 °C in the absence or presence of polymyxin B covalently attached to agarose. (A) No polymyxin-agarose or 0.5 mg/mL underivatized agarose; (B) 0.25 mg/mL polymyxin-agarose; (C) 0.5 mg/mL polymyxin-agarose; (D) 1.0 mg/mL polymyxin-agarose.

The effect of polymyxin-agarose on the growth of two polymyxin resistant strains, *E. coli* SC 9252 and SC 9253, was also examined. These two strains are spontaneous mutants obtained from *E. coli* SC 9251 (Rosenthal et al., 1976). The minimal inhibitory concentrations of free polymyxin for *E. coli* SC 9251, 9252, and 9253 were 1.17, 100, and >200 μg/mL. Polymyxin-agarose had no effect on the growth of *E. coli* SC 9252 or SC 9253 up to 1 mg/mL. If free polymyxin was not released from the agarose beads (discussed below), then the differential sensitivity of these three strains must be due to interactions between polymyxin and the outer-membrane system. The differential sensitivity of these three strains to free polymyxin was lost when their corresponding spheroplasts were compared (Rosenthal and Storm, manuscript in preparation).

P. aeruginosa PaG 13 was also sensitive to polymyxin-agarose (Figure 2, Table I). However, *P. aeruginosa* was less sensitive to immobilized polymyxin than *E. coli.* For example, at a concentration of 1.0 mg of polymyxin-agarose/mL and 9.1×10^5 cells/mL of *P. aeruginosa*, there was a 16.8-h growth lag relative to untreated controls. At a comparable inoculum of *E. coli,* there was complete inhibition of bacterial growth in the presence of 1 mg/mL polymyxin-agarose. *B. subtilis* growth was unaffected by polymyxin-agarose (Table I) and this organism is quite sensitive to free polymyxin. The minimal inhibitory concentration of free polymyxin B for *B. subtilis* was 0.6 μg/mL.

Stability of Polymyxin-Agarose in the Presence of E. coli. Polymyxin was attached to agarose by stable covalent bonds. The structure of the chemical linkage between agarose and polymyxin B is shown below:



The only derivatizable amino groups of polymyxin B were the γ amino groups of the diaminobutyric acid residues. Therefore, polymyxin B was covalently attached to the spacer arm

TABLE II: Inhibition of *E. coli* Respiration by Immobilized Polymyxin B.

Bacterial Strain	Polymyxin-Agarose[a] (mg/mL)	Inhibition of Respiration I_{10}[b] (%)	I_{30} (%)	I_∞ (%)	$t_{1/2}$[c] (min)
E. coli SC 9251	0.0	0.0	0.0	0.0	
	2.5	19	42	90	38.5
	5.0	50	83	90	8.5
	10.0	70	90	90	4.2
E. coli SC 9251 spheroplasts	5.0	50	89	100	8.2
B. subtilis	5.0	0.0	0.0	0.0	
B. subtilis protoplasts	5.0	100	100	100	0.2

[a] Polymyxin B was covalently attached to agarose as described under Methods. [b] Inhibition of respiration rate 10 min after introduction of polymyxin-agarose. Rates of respiration were determined as detailed under Methods. The concentration of bacteria was 10^8 bacteria/mL. [c] Time following addition of polymyxin-agarose which gave 50% of maximum inhibition.

through an amide bond between the γ amino group of one or more diaminobutyric acid residues and the carboxyl group at the end of the spacer arm. The ether bond is extremely stable, and spontaneous hydrolysis of the amide bonds under the conditions of these studies would not be expected. Although the amide bonds involved in coupling polymyxin B to agarose should not be susceptible to attack by bacterial proteases, it was important to prove that free polymyxin, in an active form, was not released in the presence of bacteria.

The following experiment was designed to detect the presence of free, active polymyxin in the presence of *E. coli*. Dialysis tubing containing 50 mL of nutrient media was suspended in an Erlenmeyer flask containing 100 mL of nutrient media, stoppered, and autoclaved. The sterilized media inside and outside of the dialysis bag was inoculated with 1.0×10^6 bacteria/mL and 1.00 mg/mL polymyxin-agarose was introduced inside the dialysis bag. After 18 h at 37 °C, there was no growth in the dialysis bag, but growth occurred normally outside. Similarly, when polymyxin-agarose was placed outside the dialysis bag, bacteria grew inside but not outside. Free polymyxin readily diffused through the dialysis tubing. Underivatized agarose beads were totally without effect on *E. coli* growth. Furthermore, polymyxin-agarose beads (0.5 mg/mL) were incubated with *E. coli* SC 9251 (10^6 bacteria/mL) for 6 h and then centrifuged at 3000*g* to sediment both bacteria and immobilized polymyxin. The supernatant was then reinoculated with *E. coli* (10^6 bacteria/mL), and no inhibition of growth occurred.

The data described above indicate that free polymyxin was not released when polymyxin-agarose was exposed to *E. coli*. Although unlikely, it might be argued that the peptide was released from the agarose bead through the action of a bacterial protease at the cell surface and was directly taken up by the cells. Radioactively labeled polymyxin B was not available. However, [^{3}H]EM 49 was. EM 49 is quite similar to polymyxin B structurally and also disrupts the structure of outer membranes (Rosenthal et al., 1976). Radioactively labeled EM 49 was covalently attached to Affi-Gel 10 through an amide bond between a γ amino group of diaminobutyric acid and the carboxyl group at the end of the Affi-Gel 10 spacer arm. The coupling procedure was completely analogous to that used to attach polymyxin B to agarose and EM 49-agarose also inhibited the growth and respiration of *E. coli* (Table IV). The ^{3}H-labeled EM 49-agarose (5 mg/mL) was incubated with *E. coli* SC 9251 (10^8 bacteria/mL) for 20 min or 3.0 h. The sample was then centrifuged at 10*g* to separate the beads from the bacteria and the supernatant was counted. The bacteria remained in the supernatant. No [^{3}H]EM 49 was found in the supernatant after 20 min or 3 h, indicating that the peptide was not released either free or bound to bacterial cells. The specific activity of the labeled peptide (0.14 μCi/μmol) would allow the detection of 0.3 μg of EM 49/mL. The minimal inhibitory concentration of EM 49 for *E. coli* SC 9251 was 1.7 μg/mL. It is concluded that free peptide, in any form, was not released in the presence of *E. coli*, and that the inhibition of bacterial growth was due to an interaction between immobilized polymyxin or immobilized EM 49 with the outer surface of *E. coli*.

Other evidence supporting the above conclusion is the observation that polymyxin-agarose was not active against *B. subtilis*, an organism sensitive to free polymyxin. Finally, bacitracin coupled to agarose by an analogous linkage was not active against *E. coli*. Free bacitracin inhibited the growth of *E. coli*.

Inhibition of E. coli Respiration of Polymyxin-Agarose. It has been demonstrated that polymyxin inhibits the respiration of bacteria (Cerny and Teuber, 1971) and that the polymyxin concentration dependence for inhibition of respiration correlates well with the minimal biocidal concentrations for a number of bacterial strains (Rosenthal and Storm, 1975). Furthermore, at biocidal concentrations of polymyxin, bacterial respiration is inhibited within 15 s or less after addition of the peptide. Therefore, the effects of polymyxin-agarose on *E. coli* respiration were examined.

The rate of *E. coli* O_2 uptake was inhibited by polymyxin-agarose (Figure 3, Table II). The kinetics for inhibition of respiration by polymyxin-agarose were first order and dependent upon the concentration of polymyxin-agarose. The half-lives for the kinetics of respiration inhibition were 38.5, 8.2, and 4.2 min at polymyxin-agarose concentrations of 2.5, 5.0, and 10.0 mg/mL, respectively. With whole *E. coli* cells, the maximum inhibition of respiration was 90%.

The respiration of *E. coli* SC 9251 spheroplasts stabilized in 20% sucrose was also inhibited by polymyxin-agarose (Figure 4). The sensitivity of the spheroplasts to polymyxin-agarose was comparable to the whole cells, except that respiration could be completely inhibited by polymyxin-agarose. Presumably, the effects of polymyxin-agarose on spheroplast respiration were due to direct interactions between immobilized polymyxin and the cytoplasmic membrane. *B. subtilis* respiration was unaffected by polymyxin-agarose. However, when the cell wall was removed the resulting protoplasts were extremely sensitive to polymyxin-agarose (Table II).

Interaction between ^{32}P-Labeled E. coli and Polymyxin-

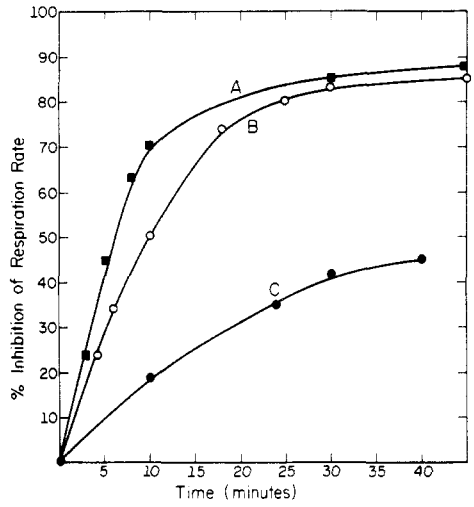


FIGURE 3: Kinetics for inhibition of *E. coli* respiration by immobilized polymyxin B. The rate of O_2 uptake was monitored with an O_2 electrode as described under Methods. The percentage of respiration inhibition by polymyxin-agarose was determined by comparing the rates of O_2 uptake at various times in the presence and absence of immobilized polymyxin. *E. coli* SC 9251 was present at 3×10^8 bacteria/mL. (A) Ten milligrams of polymyxin-agarose/mL; (B) 5 mg of polymyxin-agarose/mL; C, 2.5 mg of polymyxin-agarose/mL.

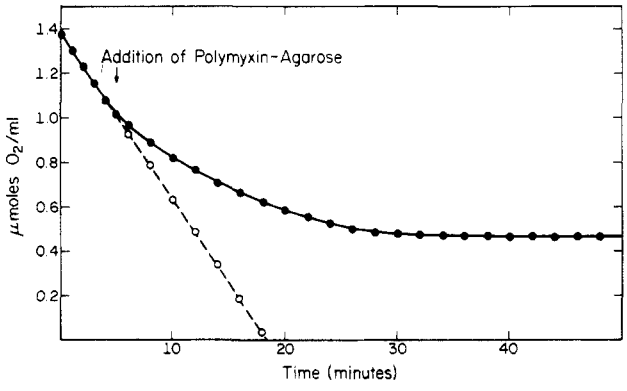


FIGURE 4: Inhibition of *E. coli* spheroplast respiration by polymyxin-agarose. The rate of O_2 uptake by spheroplasts stabilized in 20% sucrose was monitored as described under Methods. The initial rate of respiration was established and then 5 mg/mL hydrated polymyxin-agarose was added as indicated. The rate of O_2 uptake in the absence of added polymyxin-agarose is represented by the dashed line.

Agarose Beads. Polymyxin is a cationic peptide and the surface of *E. coli* and *P. aeruginosa* contains negatively charged phosphate groups from lipopolysaccharide and phospholipids. The effects of polymyxin-agarose on the growth and respiration of these organisms might be due to binding of the bacteria to the polymyxin-agarose beads. For example, absorption of bacteria to a zirconium hydroxide matrix decreased their rate of respiration (Kennedy et al., 1976). In order to determine if *E. coli* was bound by polymyxin-agarose beads, ^{32}P-labeled bacteria were incubated with varying amounts of polymyxin-agarose (Table III). The beads were sedimented at low speed to separate them from the bacteria and the supernatant was counted. No binding of *E. coli* to polymyxin-agarose in a 15-min incubation period was observed at concentrations up to 1.0 mg/mL polymyxin-agarose. However, treatment with immobilized polymyxin did stimulate the release of ^{32}P labeled material nonsedimentable at 5000*g*. The released labeled material was not characterized. These data indicate that immobilized polymyxin did not bind *E. coli* strongly, but did perturb the permeability of the inner- or outer-membrane system.

TABLE III: Interaction between ^{32}P-Labeled *E. coli* and Polymyxin-Agarose Beads.

Polymyxin-Agarose (mg/mL)	Total Supernatant Counts[a] 10*g* (cpm)	Total Supernatant Counts 5000*g* (cpm)	% ^{32}P Released
0.0	1.12×10^5	1.3×10^4	11
0.1	1.11×10^5	1.3×10^4	11
0.5	1.17×10^5	1.6×10^4	14
1.0	1.16×10^5	2.3×10^4	20

[a] *E. coli* SC 9251 was grown in the presence of $H_3{}^{32}PO_4$, harvested, and washed as described under Methods. Ten milliliters of bacteria (1.3×10^9 cells/mL), in nutrient media was incubated for 15 min at 37 °C in the absence or presence of polymyxin-agarose. The suspension was centrifuged at 10*g* to sediment beads but not bacteria, and an aliquot of the 10*g* supernatant was counted. No bacteria, absorbed to the beads, were brought down in the 10*g* centrifugation. The 10*g* supernatant was then centrifuged at 5000*g* to sediment whole cells, and an aliquot of this supernatant was counted to detect ^{32}P-labeled material released by polymyxin-agarose.

Protection of E. coli from Polymyxin-Agarose by Phospholipids. Covalent attachment of the polymyxin to agarose limited its interactions to the outer membrane of *E. coli*. Free polymyxin interacts strongly with membranes and phospholipids (Koike and Iida, 1971; Few, 1955; Storm, 1974; Imae et al., 1975), and the antimicrobial activity of polymyxin is eliminated in the presence of added phospholipids (Few, 1955). Therefore, the effects of *E. coli* phospholipids on the antimicrobial activity of polymyxin-agarose were examined. The characteristic growth lag period caused by the immobilized polymyxin was progressively shortened as the concentration of phospholipid was increased. At an inoculum of 1×10^6 bacteria/mL and 0.20 mg/mL polymyxin-agarose, the growth lag periods were 4.0, 3.3, 0.7, and 0.0 h in the presence of 0.0, 0.10, 0.25, and 0.5 μmol/mL of phospholipid, respectively. Thus, the biological activities of both the free and immobilized polymyxin were eliminated by added phospholipids, presumably by binding to polymyxin preventing its interaction with membranes.

Binding between Polymyxin-Agarose and E. coli Phospholipids. The data described above suggested that the phospholipids were bound by the immobilized polymyxin. Therefore, ^{32}P-labeled phospholipids were prepared and examined for binding with polymyxin-agarose. The data were corrected for phospholipid binding to underivatized agarose at an equivalent concentration. Phospholipid was bound very strongly by immobilized polymyxin. For example, at 0.25 mg/mL polymyxin-agarose in the presence of 0.1 μM phospholipid, 10% of the lipid was bound by the derivatized beads. It can be estimated from these data that the binding constant for polymyxin and phospholipids was approximately 10^5 to 10^6 M^{-1}. These binding data indicate that the protection of *E. coli* from polymyxin-agarose was probably due to phospholipid binding to immobilized polymyxin.

Discussion

The growth and respiration of two gram-negative bacteria, *E. coli* and *P. aeriginosa*, but not *B. subtilis* were inhibited by polymyxin-agarose. It was demonstrated that free polymyxin, in an active form, was not released in the presence of bacteria. The immobilized polymyxin was biostatic at low concentra-

tions and biocidal at higher concentrations. Free polymyxin and immobilized polymyxin shared several common properties. For example, both inhibited the respiration of whole *E. coli* cells, *E. coli* spheroplasts, and *B. subtilis* protoplasts. The polymyxin-resistant strains, *E. coli* SC 9252 and SC 9253, were insensitive to polymyxin–agarose. In addition, phospholipid dispersions inhibited the antimicrobial activity of both free and immobilized antibiotics.

Polymyxin has been absorbed to cellulose and cellulose carbonate, and the insolubilized complex possessed antimicrobial activity (Kennedy and Cho Tun, 1973). However, the observed activity was due to release of the antibiotic from the matrix. In the present studies, polymyxin was coupled to agarose by means of an amide bond. It is possible to derivatize one of the amino groups of polymyxin B and still retain antibiotic activity; however, modification of two or more amino groups depresses the activity of polymyxin B (Teuber, 1970). The coupling procedure used in this study employed polymyxin in a 15 M excess over the activated arms on Affi Gel 10 in order to minimize multipoint attachment. Thus, on the average, polymyxin was covalently attached to agarose by one amide bond.

The molecular basis for the antimicrobial activity of polymyxin–agarose is not known; however, these studies suggest several conclusions concerning the mechanism of polymyxin action and the importance of gram-negative outer membranes. Polymyxin does not have to enter the bacterial cell to exert its effects upon respiration. In the case of gram-positive bacteria such as *B. subtilis,* the intact cell is insensitive to immobilized polymyxin; however, protoplast respiration was inhibited by polymyxin–agarose. The sensitivity of the gram-negative strains to polymyxin–agarose must have been due to interactions with their outer membranes. The sensitivity of spheroplast and whole-cell respiration to polymyxin–agarose indicate that the peptide can inhibit respiration either by direct interactions with the inner membrane, which is the site for the electron-transport chain and oxidative phosphorylation, or indirectly by perturbation of the outer-membrane structure.

It is of considerable interest that interactions between immobilized polymyxin and outer membranes can inhibit respiration, a function which takes place on the inner membrane. With whole cells, direct interaction between polymyxin and the inner membrane was not possible since the peptide was covalently attached to agarose by a 10-Å spacer arm. It has been shown that polymyxin interacts strongly with phospholipids and lipopolysaccharide, and disrupts outer-membrane structure (Few, 1955; Storm, 1974; Imae et al., 1975; Bader and Teuber, 1973; Cerny and Teuber, 1971; Lounatmaa et al., 1976). For example, treatment of *E. coli* with polymyxin results in numerous projections or blebs from the outer surface (Koike et al., 1969; Meyers et al., 1974). Low levels of EDTA, which are sufficient to complex Mg^{2+} and Ca^{2+} crucial for outer-membrane structure (Leive, 1974) but not affect inner-membrane functions, made *E. coli* osmotically fragile (Birdsell and Cota-Robles, 1967) and resulted in lysis of *P. aeruginosa* (Eagon and Carson, 1965). It is possible that the outer membrane of *E. coli,* which is attached to the peptidoglycan through the Braun protein (Braun and Wolff, 1970; Inouye et al., 1972), functions with the peptidoglycan as a mechanical barrier to prevent lysis. If the latter is true, then damage to the outer membrane by polymyxin–agarose could indirectly affect the selective permeability of the inner membrane and inhibit respiration. Regardless of the detailed mechanism, these results indicate that the outer membrane, peptidoglycan, and inner membrane cannot be considered as separate functional entities and that polymyxin can affect bacterial respiration solely by interactions with the outer membrane. In this regard, it has been proposed that the inner and outer membranes become continuous at certain adhesion sites which allow for the export of newly synthesized lipopolysaccharide and injection of phage nucleic acid (Bayer, 1974).

TABLE IV: Inhibition of *E. coli* Growth by Immobilized EM 49.

Inoculum Size[a] (bacteria/mL)	EM 49-Agarose[b] (mg/mL)	Growth Lag[c] (h)
1.6×10^6	0.00	0.0
1.6×10^6	0.10	0.0
1.6×10^6	0.25	1.8
1.6×10^6	0.50	8.0
1.6×10^6	0.75	22.5
1.6×10^6	1.00	38.2
1.6×10^5	0.10	0.9
1.6×10^4	0.10	1.4

[a] *E. coli* SC 9251 was grown in nutrient media at 37 °C with shaking. [b] EM 49 was covalently attached to agarose as described under Methods. [c] Growth lag is the difference in time required for the control and an EM 49–agarose treated sample to reach 50% of their maximum growth.

The hypothesis presented above was tested by covalently attaching another outer membrane perturbing antibiotic to agarose and examining its effect on *E. coli* growth and respiration. EM 49 is a peptide antibiotic which disrupts the structure of *E. coli* outer membranes and releases outer-membrane fragments from the bacteria (Rosenthal et al., 1976). EM 49 covalently coupled to agarose inhibited *E. coli* growth and respiration (Table IV). On the other hand, bacitracin and vancomycin covalently attached to agarose did not affect *E. coli* growth. It might be argued that polymyxin–agarose and agarose–EM 49 can actually reach the inner membrane because both peptides disrupt outer-membrane structure. However, considering the fact that the agarose beads were approximately 100 times larger than the bacteria and the peptide was attached by a 10-Å arm, this would be physically impossible unless the majority of the outer membrane was removed. Furthermore, even though the peptidoglycan layer of gram-negative bacteria is relatively thin (Bayer, 1974), it is still thicker than the length of the arm used for immobilization of polymyxin and EM 49.

The ability to covalently attach certain antibiotics to large polymers with retention of their antimicrobial activity is clearly a powerful tool for studying their interactions with components of the cell surface. Furthermore, this general technique may be useful clinically because it may be possible to permanently sterilize cotton fiber used in bandages. In addition, covalent immobilization may be used with antimicrobials which cannot be used topically because of absorption and secondary toxicity.

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A Protein Cofactor That Stimulates the Activity of DNA-Dependent RNA Polymerase I on Double-Stranded DNA†

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ABSTRACT: Partially purified rat liver RNA polymerase I chromatographed on ribosomal RNA-Sepharose loses most (96%) of its activity assayed on native calf-thymus DNA templates, but loses little (8%) of its activity assayed on poly(deoxycytidylic acid) template. Polymerase I is not stimulated by polymerase II protein factor, or by bovine serum albumin. However, it is stimulated by histones, polylysine, and spermine. Addition of a protein fraction eluted by high ionic strength from the rRNA-Sepharose also restores activity on native calf-thymus DNA. Further purification yields a fraction containing two proteins of 11 000 and 12 000 molecular weight. Both proteins are distinct from histones by electrophoresis in sodium dodecyl sulfate and in acid urea. Both proteins are basic, insensitive to heat, bind to DNA, and stimulate polymerase I activity. The degree of stimulation of polymerase I is dependent upon both the enzyme/DNA and the factor/DNA ratio. The protein factors also stimulate polymerase II activity about half as effectively as polymerase I.

Eucaryotic RNA polymerases I and II are each composed of two high molecular weight and several lower molecular weight subunits (Weaver et al., 1971; Kedinger et al., 1974; Sklar et al., 1975; Hager et al., 1977). The molecular weights of the corresponding subunits of I and II are largely different; thus the molecules are distinct entities. The overall structure is analogous to that of the *Escherichia coli* core polymerase (Burgess, 1969) and implies a similarity of mechanism. This would suggest that eucaryotic RNA polymerases may require factors similar to the sigma factor (Burgess et al., 1969).

Several types of factors affecting transcription have been described in the literature. They may be divided into three groups: (a) nonspecific factors; (b) factors isolated independently that stimulate activity on double-stranded DNA but not on single-stranded DNA; and (c) factors that initially co-purify with the enzyme. Factors in the first group include DNase, which stimulates eucaryotic polymerase by the introduction of single-stranded breaks in the template and production of a greater number of initiation sites (Chambon et al., 1970; Keller and Goor, 1970), and bovine serum albumin, presumably a nonspecific protein, which stimulates both polymerases I and II to varying degrees (Rutter et al., 1973). Factors in the sec-

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