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Inhibition of Tobramycin Diffusion by Binding to Alginate

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[^{3}H]tobramycin bound to sodium alginate and to exopolysaccharide prepared from two mucoid strains of *Pseudomonas aeruginosa*. Binding to sodium alginate was similar to binding to exopolysaccharide, both in the dependence on tobramycin concentration and in the maximum binding observed at saturation. Incorporation of sodium alginate into agar plates reduced the zone sizes of growth inhibition caused by tobramycin. The reductions in zone sizes were quantitatively accounted for by the binding of tobramycin to sodium alginate during diffusion of the antibiotic away from the well in which it had been placed at the start of the experiment. However, the binding of tobramycin to the exopolysaccharide of *P. aeruginosa*, and the resulting inhibition of diffusion of the antibiotic, did not significantly increase the penetration time of a spherical microcolony with a radius of 125 μm, such as might be found in the respiratory tract of a patient with cystic fibrosis (from a 90% penetration time of 12 s in the absence of exopolysaccharide to one of 35 s with an exopolysaccharide concentration of 1.0% [wt/vol]).

The question of whether bacterial exopolysaccharides reduce the penetration of antibiotics to their target sites (5, 22) is an important one in antibacterial chemotherapy. This is because exopolysaccharide-producing bacteria existing as biofilms are less susceptible to antibiotics than are freely suspended bacteria (6, 17), and mucoid *Pseudomonas aeruginosa* apparently forms microcolonies (12) when causing respiratory tract infections that are refractory to chemotherapy in patients with cystic fibrosis. Moreover, in a recent review (4), the exopolysaccharide material of the biofilm was specifically postulated to exclude antibacterial substances.

Inhibition of the diffusion of aminoglycoside antibiotics occurs in the presence of alginate (21), a polyanionic polysaccharide similar in structure to the exopolysaccharide of mucoid *P. aeruginosa* (13), or in the presence of the exopolysaccharide from *P. aeruginosa* (21). A likely reason for the reduced rate of diffusion of aminoglycosides within the anionic polysaccharide matrix is that any antibiotic-binding sites act as sinks, thereby reducing the free concentration of antibiotic, which is effectively the driving force of diffusion. In apparent conflict with this suggestion, the binding of tobramycin and streptomycin to alginate has been reported (23) as not being detectable in a physiological buffer containing 0.10 M NaCl.

We quantitatively investigated the inhibition of diffusion and assessed the binding of tobramycin to alginate and *Pseudomonas* exopolysaccharide using radiolabeled tobramycin. Here we report that in a physiological buffer solution, tobramycin binds to alginate and to exopolysaccharides isolated from two mucoid strains of *P. aeruginosa* and that the binding to alginate quantitatively accounts for the inhibition of diffusion reported previously (21). However, binding and consequent inhibition of diffusion cannot account for antibiotic resistance within microcolonies and biofilms.

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MATERIALS AND METHODS

Bacterial strains. The organisms used in bioassays of antibiotic diffusion were *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571.

The organisms used for the preparation of *Pseudomonas* exopolysaccharide were two mucoid strains of *P. aeruginosa* isolated from the sputum of patients with cystic fibrosis. These strains were obtained from T. L. Pitt (Central Public Health Laboratory, London, United Kingdom), who designated them *P. aeruginosa* CF6a and CF8.

Preparation of exopolysaccharide. Bacteria were grown with orbital shaking in 400- or 500-ml volumes of brain heart infusion broth (Oxoid Ltd., Basingstoke, United Kingdom) in baffled 1-liter conical flasks either at 37°C for 36 h or at room temperature (20 to 23°C) for 45 h. Then 5 M NaCl and 0.5 M dipotassium EDTA were added to each flask in proportions of 2 ml/100 ml of growth medium. Exopolysaccharide was isolated from these mixtures by the method of Evans and Linker (10), except that drying to constant weight was done in a hot-air oven at 50 or 80°C. At various stages in the preparation procedures, the aqueous polysaccharide solutions were tested for sterility by sampling with a microbiological loop, spreading the sample on blood agar, and incubating the inoculated plate at 37°C overnight.

The protein content of the preparations was determined by the method of Lowry et al. (14) by using bovine serum albumin as a standard. Nucleic acid contamination was estimated by measuring the A_{260} and A_{280} (8). Lipopolysaccharide contamination was estimated by measuring 2-keto-3-deoxyoctonate by the modified (18) 2-thiobarbituric acid method (26), following hydrolysis in 10 mM H_2SO_4 for 20 min at 100°C. The dry weight of lipopolysaccharide was calculated by assuming an average M_r of 9,000 and two 2-keto-3-deoxyoctonate residues per molecule (19). In two polysaccharide preparations from *P. aeruginosa* CF8, the protein contents were 2.3 and 1.2% (wt/wt), the nucleic acid contents were 0.55 and 0.20% (wt/wt), and the lipopolysaccharide contents were 2.7 and 1.8% (wt/wt). In one polysaccharide preparation from *P. aeruginosa* CF6a, the protein content was 2.3% (wt/wt), the nucleic acid content was

0.21% (wt/wt), and the lipopolysaccharide content was 3.2% (wt/wt).

Equilibrium dialysis. The solution (hereafter referred to as Ringer phosphate) for equilibrium dialysis was prepared from 0.154 M NaCl, 0.154 M KCl, 0.154 M KH_2PO_4, and 0.1 M sodium phosphate buffer (pH 7.4) in volume ratios of 100:4:1:21, and 2 M $MgCl_2$ was added to a final concentration of 2 mM. This solution is similar to Krebs original Ringer phosphate (8), except that it lacked Ca^{2+}; inclusion of $CaCl_2$ in the solution caused exopolysaccharide to precipitate, presumably as the calcium salt.

Equilibrium dialysis was carried out in specially made acrylic blocks which clamped single sheets of Visking dialysis membrane (cut from size 3-20/32-in. [1.6-cm] tubing) between two chambers, each of which had a volume of about 2 ml. Alginate or exopolysaccharide solution (1.0 or 1.3% [wt/vol]) in the Ringer phosphate was placed in one chamber, and [^{3}H]tobramycin solution with various concentrations of unlabeled tobramycin was placed in the other chamber. The volume added to each chamber was 1.50 ml. There were nine measuring assemblies set up, and one control assembly was set up which contained no polysaccharide in one chamber and [^{3}H]tobramycin at 14.6 μg of base ml^{-1} in the other chamber. The chemical concentration of tobramycin in the nine test chambers at the start of the experiment ranged between 2.06 μg and 6.54 mg of base ml^{-1}. A constant amount of radioactivity was added to each chamber, namely, 90.7 nCi. The assemblies were shaken at 37°C, and samples of 10 μl were taken at 24-h intervals from the chamber to which [^{3}H]tobramycin was added. Each 10-μl sample was placed in 0.49 ml of water, and then 3.5 ml of an aqueous scintillation fluid (made up as described previously [16] but containing 500 ml of Triton X-100 per liter of toluene) was added. Samples were counted for 10 min on a scintillation counter (Rackbeta; LKB Instruments, Inc., Rockville, Md.).

Diffusion bioassays. The principles of assessing diffusion phenomena from the zone sizes of bacterial growth inhibition have been reviewed by Cooper (2, 3).

Experiments were carried out by using plates measuring 25 by 25 cm. These were filled with Iso-Sensitest agar to a depth of about 6 mm. The set, dried agar was flooded with a 1/2,000 dilution in water of a 16-h culture of test organism grown at 37°C in brain heart infusion broth. The plate was then drained and dried, and 6-mm-diameter circular wells were cut (18 per plate). Wells, three replicates for each concentration, were filled with antibiotic solution in water (prewarmed to 37°C), either immediately or following a timed preincubation period at 37°C. Plates were then incubated at 37°C for up to 24 h, and zones of growth inhibition were measured as diameters of the clear circles. Radii were expressed as one-half the diameter; that is, they included the 3-mm radius of the well.

Chemicals. [^{3}H]tobramycin was obtained from Amersham International plc (Amersham, United Kingdom), and its radiochemical purity was checked periodically by descending paper chromatography; the developer was propan-1-ol–pyridine–acetic acid–water (10:5:3:12). Sodium alginate, protease (pronase E; type XIV), DNase I (DN-25), and RNase-A (type I-AS) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Tobramycin was generously provided by Lilley Industries Ltd.

RESULTS

Binding of [^{3}H]tobramycin to alginate and *Pseudomonas* exopolysaccharide. The concentration dependence of the binding measured by equilibrium dialysis is depicted in Fig. 1a. The binding to alginate and to the exopolysaccharides from two mucoid strains of *P. aeruginosa* were similar both in the maximum amount of binding and in the concentration dependence of the binding. The data for three experiments on binding of [^{3}H]tobramycin to algal alginate were analyzed in terms of a Langmuir adsorption isotherm equation, $b = b_{max} C/(K + C)$, where b is the amount of tobramycin bound (in micrograms per milligram of sodium alginate), b_{max} is the maximum value of b at saturation, C is the free tobramycin concentration (in micrograms per milliliter), and K is a dissociation constant for the adsorption process. Values of b_{max} and K were estimated from the straight-line Scatchard (20) plot of b/C against b (Fig. 1b). The equation yielded by the linear least-squares regression line (Fig. 1b) was $b = 71.31C/(365.8 + C)$. The curve of this equation is also shown in Fig. 1a.

Zones of inhibition produced by tobramycin in the presence and absence of alginate. We measured the zones of growth inhibition produced by tobramycin in the presence and

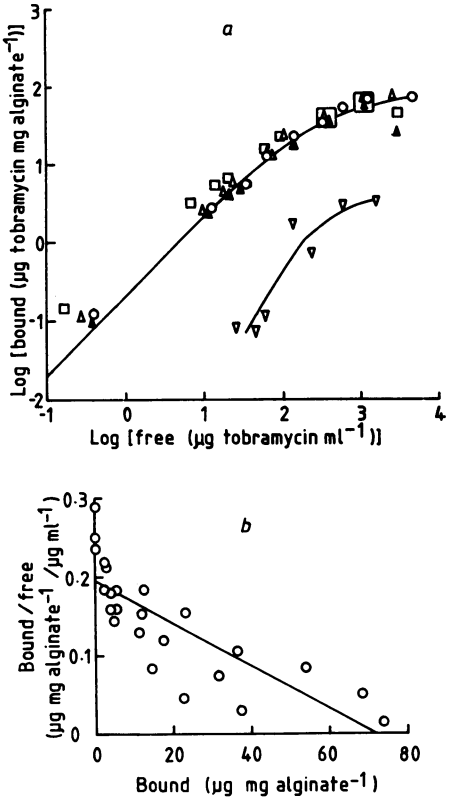

FIG. 1. Measurement by equilibrium dialysis of tobramycin binding to alginate and to the exopolysaccharide produced by mucoid *P. aeruginosa*. (a) Log-log plot of the concentration dependence of tobramycin binding to algal alginate (○), two preparations of exopolysaccharide from *P. aeruginosa* CF8 (△,▲), one preparation of polysaccharide from *P. aeruginosa* CF6a (□), and human serum albumin (▽). The line drawn through the polysaccharide points is that calculated by using the sodium alginate binding constants derived from panel b. (b) Scatchard (20) plot of bound tobramycin/free tobramycin against bound tobramycin for three experiments with algal sodium alginate. The linear least-squares regression line is drawn from which an adsorption dissociation constant (K) of 365.8 μg ml^{-1} (0.78 mM) and an adsorption maximum (b_{max}) of 71.31 μg of tobramycin mg of sodium $alginate^{-1}$ were calculated.

absence of alginate using sodium alginate from the alga *Macrocystis pyrifera* because large quantities were required for incorporation into agar plates. The concentration dependence of binding of tobramycin to this sodium alginate was similar to the concentration dependence of binding to *Pseudomonas* exopolysaccharide (Fig. 1a). Moreover, we have reported previously (21) that the diffusion of the aminoglycoside netilmicin is retarded in the presence of the exopolysaccharide from a mucoid strain of *P. aeruginosa*.

When sodium alginate (1.0% [wt/vol]) was incorporated into Iso-Sensitest agar, we observed reduced zones of growth inhibition surrounding wells which contained tobramycin solutions at the start of the experiment. For example, when the concentration of tobramycin was 330 μg ml^{-1}, the mean zone radii in the absence of alginate were 1.56 ± 0.005 cm (standard error of the mean; $n = 3$) and 1.69 ± 0.010 cm ($n = 6$) for *E. coli* and *S. aureus*, respectively, whereas in the presence of 1.0% (wt/vol) sodium alginate, the radii were 0.931 ± 0.011 cm ($n = 6$) and 1.07 ± 0.018 cm ($n = 6$), respectively. Zone radii for tobramycin at a concentration of 33 μg ml^{-1} were 1.21 ± 0.004 cm ($n = 3$) for *E. coli* and 1.32 ± 0.008 cm ($n = 6$) for *S. aureus* in the absence of alginate and 0.670 ± 0.005 cm ($n = 3$) and 0.814 ± 0.005 cm ($n = 6$), respectively, in the presence of 1.0% (wt/vol) sodium alginate.

Predicted effect of adsorption to alginate on tobramycin diffusion. The predicted effect of binding to alginate on tobramycin diffusion was obtained by following the derivation of Crank (7) for diffusion with simultaneous adsorption. Crank (7) considers the simplest case of the adsorption isotherm being linear, i.e., $B = RC$, where B is the amount of substance adsorbed in a unit volume (in micrograms per milliliter), C is the free concentration (in micrograms per milliliter), and R is the constant of proportionality. When adsorption reaches equilibrium instantaneously, the effective diffusion coefficient (D') in the presence of adsorbent is related to the diffusion coefficient in the absence of the binding sink by the following equation (7):

$$D' = D\frac{1}{R+1} \quad (1)$$

The adsorption isotherm was not linear but fitted the Langmuir equation (see above); however, note that the Langmuir equation is approximately linear when $C << K$. A plot of B [=713.1 C/(365.8 + C) for 1% (wt/vol) sodium alginate] against C in the range of 0 to 33 μg of tobramycin ml^{-1} was linear by eye, with a proportionality constant of R = 1.83. In other words, to a first approximation, in 1.0% (wt/vol) sodium alginate and at low tobramycin concentrations, $B = 1.83C$. Substitution of this value of 1.83 for R into equation 1 yielded a predicted ratio of D/D' of 2.83 (Table 1). R was determined graphically over the range of 0 to 33 μg of tobramycin ml^{-1} because the concentrations of tobramycin used in the diffusion experiments (described below) included concentrations of 33 μg ml^{-1} and higher. The value of R at a more clinically relevant tobramycin concentration of 3.3 μg ml^{-1} was 713.1/369.1 = 1.93. The consequent two predicted D/D' ratios of 2.83 (Table 1) and 2.93 differed by only 3.5%.

Measured effect of alginate on the tobramycin diffusion coefficient. Experimental values of D and D' were measured by methods following the analyses of Cooper (2) that were based on the theoretical work of Brimley (1) and Vesterdal (25). The equation used by Vesterdal (25) was recently shown (9) to yield acceptable results with a large number of organisms. Equation 1 of Vesterdal (25), given as equation 16 by Cooper (2), yields the following relationship:

$$r^2 = 4Dt_0 \ln C_0 + \text{constant} \quad (2)$$

where r is the radius of the zone of inhibition of growth, C_0 is the concentration of antibiotic in the well at the start of the experiment, and t_0 is the critical time at which the edge of the inhibition zone forms. The straight-line nature of equation 2 was used to determine diffusion coefficients (2). Squared radii of zones of inhibition (r^2) were plotted against $\ln C_0$ for a series of plates that were preincubated at 37°C for various periods of time before the addition of tobramycin (Fig. 2a). Straight lines were obtained, and the slopes were calculated by linear least-squares regression of r^2 on $\ln C_0$. From equation 2, the slopes (S_1) of these lines are given by the following equation (2):

$$S_1 = 4D(t_0 - h) \quad (3)$$

where h is the preincubation time. Rearranging equation 3 yields $h = t_0 - S_1/4D$. Thus, plots of preincubation time h against the slope S_1 gave straight lines with slope $S_2 = -1/4D$ and intercept t_0. Examples of these plots with the data from Fig. 2a are shown in Fig. 2b. The slopes of the two lines shown in Fig. 2b were −18.56 h cm^{-2} for *E. coli* and −18.49 h cm^{-2} for *S. aureus*, which yielded values for the tobramycin diffusion coefficient of 3.74×10^{-6} and 3.76×10^{-6} cm^2 s^{-1}, respectively. Other values for the diffusion coefficient of tobramycin in the presence and absence of sodium alginate were obtained similarly and are summarized in Table 1.

DISCUSSION

Binding of tobramycin to alginate and the exopolysaccharide produced by mucoid *P. aeruginosa*. The results in Fig. 1a demonstrate that tobramycin binds to alginate and to the exopolysaccharide prepared from two mucoid strains of *P. aeruginosa*.

Although 1.0 or 1.3% alginate or exopolysaccharide concentrations were used in the binding studies, the Langmuir equation obtained applies to any alginate solution, regardless of its concentration. The alginate/tobramycin ratios in the equilibrium dialysis studies were not relevant to the way the data were analyzed, because the quoted tobramycin concentrations were free concentrations (e.g., see Fig. 1b).

Some lipopolysaccharide copurified with the *Pseudomonas* exopolysaccharide (see above), and lipopolysaccharide binds aminoglycosides (19). A cationic spin probe, 4-dodecyl dimethyl ammonium-1-oxyl-2,2,6,6-tetramethyl piperidine (abbreviated CAT_{12}), binds to lipopolysaccharide from *P. aeruginosa* in a ratio of 2.2 molecules per molecule of lipopolysaccharide (19). This assumption that tobramycin

TABLE 1. Comparison between experimentally determined diffusion coefficients and those predicted from the binding of tobramycin to alginate

Diffusion coefficient[a]	n	Mean ± SEM
D (cm^2 s^{-1})[b]	5	$3.84 \pm 0.06 \times 10^{-6}$
D' (cm^2 s^{-1})[c]	4	$1.52 \pm 0.08 \times 10^{-6}$
Measured D/D'		2.53
Predicted D/D'		2.83

[a] D was measured from three experiments with *S. aureus* and two with *E. coli*; D' was measured from two experiments with each indicator organism.
[b] In the absence of alginate.
[c] In the presence of alginate.

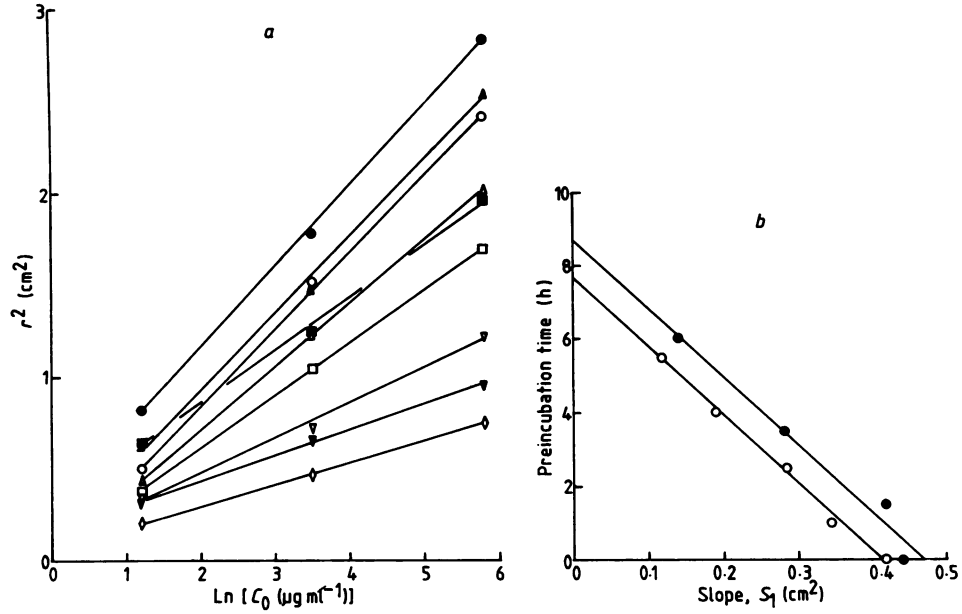

FIG. 2. Determination by bioassay of the time when the inhibition zone formed (t_0) and the diffusion coefficient (D) of tobramycin. (a) The squared radius of the inhibition zone plotted against the natural logarithm of the concentration of antibiotic in the well at the start of the experiment. In these two experiments the medium was Iso-Sensitest agar; i.e., there was no sodium alginate present. Empty symbols indicate data for *E. coli*, with preincubation times at 37°C (i.e., h, time after the plate was seeded before the antibiotic was put in the wells) of 0.0, 1.0, 2.5, 4.0, and 5.5 h (○, △, □, ▽, and ◇, respectively). Filled symbols indicate data for *S. aureus*, with preincubation times of 0.0, 1.5, 3.5, and 6.0 h (●, ▲, ■, and ▼, respectively). Each point is the mean of three replicate observations; the lines are the linear least-squares regressions. (b) Preincubation time (h) plotted against the slope (S_1) determined as described above for panel a. Symbols: ○, *E. coli*; ●, *S. aureus*. Linear least-squares regression lines are drawn and are extrapolated to zero S_1. The intercepts on the preincubation time (h) axis are the two values of t_0 from these two experiments. The slopes of the lines were used to determine two of the diffusion coefficients that are collated in Table 1.

binds to lipopolysaccharide with the same stoichiometry as CAT_{12} resulted in maximum amounts of lipopolysaccharide-dependent binding of 3.7, 3.1, and 2.0 μg of tobramycin (mg of exopolysaccharide)$^{-1}$ for the three preparations containing, respectively, 3.2, 2.7, and 1.8% (wt/wt) lipopolysaccharide. This lipopolysaccharide-dependent binding may explain why binding of tobramycin, at free concentrations below 100 μg ml^{-1}, was slightly higher for exopolysaccharide from *P. aeruginosa* CF6a than it was for the algal alginate (Fig. 1a).

That the binding shown in Fig. 1a was not due to large amounts of binding to contaminating lipopolysaccharide is also evident from the fact that, although the lipopolysaccharide content varied between 1.8 and 3.2% (wt/wt), the three binding isotherms were similar (Fig. 1a). Similar arguments hold for contaminating protein and nucleic acid. The levels of each of these were different in each exopolysaccharide preparation; nevertheless, the tobramycin-binding isotherms were similar. Thus, neither protein nor nucleic acid could account for the tobramycin binding observed either.

Binding was a saturable function of the tobramycin concentration, and constants obtained for the adsorption isotherm with algal sodium alginate (Fig. 1b) gave a line that ran close to the experimental points obtained with three preparations of *Pseudomonas* exopolysaccharide (Fig. 1a).

Our observation that tobramycin binds to exopolysaccharide from mucoid *P. aeruginosa* apparently conflicts with the results of an earlier study (23). The binding depicted in Fig. 1 was not due to the effects of Donnan potentials or low ionic strength because the Ringer phosphate medium that we used contained 120 mM NaCl, 4.9 mM KCl, and 16.6 mM phosphate buffer. Moreover, binding of tobramycin to human serum albumin was extremely low (Fig. 1a), which is also consistent with the proposal that Donnan potential or ionic strength effects were not responsible. We make these points because in the earlier study (23), tobramycin did bind to *P. aeruginosa* exopolysaccharide dissolved in water and adjusted to pH 7.4 (i.e., at low ionic strength), but binding was not detected in physiological buffer containing 101 mM NaCl.

In fact, our quantitative binding data are in complete agreement with the findings of Tannenbaum et al. (23); their bioassay method of measuring binding would not have detected the level of binding reported here. The evidence for this is as follows. We used the binding measurements with sodium alginate for the calculations, because these were similar to those obtained with the *Pseudomonas* exopolysaccharides (Fig. 1a). Tannenbaum et al. (23) placed 5 mg of polysaccharide in 2 ml of water or buffer inside a dialysis sac that was then immersed in 9 ml of a solution of 10 μg of tobramycin ml^{-1}. In the control experiment, in which the bag contained 2 ml of solvent only, the equilibrium concentration outside the bag was expected to have been 90/11, or 8.18 μg of tobramycin ml^{-1}. Tannenbaum et al. (23) measured the tobramycin concentration in the medium outside the bag by bioassay with *S. aureus*; from our *S. aureus* data (Fig. 2) the diameter of the growth inhibition zone for this concentration was expected to be about 2.19 cm. Our Langmuir adsorption equation predicted that in the presence of 5 mg of exopolysaccharide in high-ionic-strength buffer, equilibrium would occur at a free tobramycin concentration of 7.53 μg of tobramycin ml^{-1} and an amount of bound

tobramycin of 1.44 μg of tobramycin mg of polysaccharide^{-1}. This free tobramycin concentration should give a zone diameter for *S. aureus* of about 2.16 cm (from the data given in Fig. 2a). The difference between these zone diameters (0.03 mm) was not large enough to be detected in bioassay experiments. We therefore conclude that the level of binding of tobramycin to *Pseudomonas* exopolysaccharide that occurs in physiological buffer solutions was lower than that which could have been detected by the method of Tannenbaum et al. (23).

Diffusion inhibition. The degree of binding of tobramycin to *Pseudomonas* exopolysaccharide quantitatively accounted for the reduction in zone sizes of growth inhibition reported previously (21). The diffusion coefficients of tobramycin, measured from the radii of zones of growth inhibition produced in the presence and absence of algal alginate, agreed well with what was predicted from the binding measurements (Table 1). We propose that the reduction in aminoglycoside inhibition zones that occurs in the presence of alginate or *Pseudomonas* exopolysaccharide (21) can be fully explained by filling of binding sites in the polyanionic matrix causing reduction of the local free concentrations of aminoglycosides and, therefore, reducing the driving force for diffusion.

Importance in the antibiotic resistance of biofilms and microcolonies. Aggregations of bacteria embedded within polyanionic polysaccharide glycocalyces are more resistant to antibiotics than are single planktonic cells of the same bacteria (6, 17). We believe that it is important to determine the mechanisms whereby cells within the matrix are protected from antibiotics and biocides; the investigation reported here was carried out for this purpose. The question that we can now answer is that of whether the glycocalyx per se contributes to the prevention of access of antibiotics to cells within a biofilm or microcolony.

Microcolonies. The microcolony is the mode in which mucoid *P. aeruginosa* survives in the respiratory tracts of patients with cystic fibrosis (12). Our approach is to consider a spherical microcolony, as previously (22). Because the alginate concentration in a mucoid microcolony is not known, we assumed an approximate value of 1% (wt/vol). This is because, in our hands, a 1% (wt/vol) solution of alginate or exopolysaccharide possessed a viscosity similar to that of a colony of mucoid *P. aeruginosa* grown on a solid medium.

The physical model allows calculation of how long it will take for a single bacterium at the center of a sphere of exopolysaccharide with radius a to be exposed to 90% of the bathing concentration outside the sphere; we call this the t_{90}. The concentration of antibiotic in the sphere is assumed to be zero at time zero. The concentration (C_0) of antibiotic bathing the sphere is assumed to be constant over the period during which diffusion occurs. The concentration (C) of antibiotic at the center of the sphere is given by Crank (7) in his Fig. 6.1 from which data were replotted to give a curve of C/C_0 versus Dt/a^2. The value of Dt/a^2 thus obtained at $C/C^0 = 0.9$ was 0.307. Therefore, for a sphere with a diameter of 250 μm (about the size of a human alveolus; i.e., $a = 0.0125$ cm), in the absence of any binding of tobramycin to the exopolysaccharide (i.e., $D = 3.84 \times 10^{-6}$ cm^2 s^{-1}), the t_{90} would be 12.5 s. If the concentration of polysaccharide were 1.0% (wt/vol) and the diffusion coefficient theoretically modified for binding were used (equation 1), the t_{90} would be 35.4 s. For a bigger sphere with a diameter of 2 mm, the equivalent t_{90} values would be 13.3 and 37.7 min, respectively. The model described above is an oversimplification, but it does allow order-of-magnitude estimates of time courses of diffusion into spheres of exopolysaccharide material.

Biofilms. We also analyzed a simplified model of diffusion of antibiotics into biofilms, again to compare order-of-magnitude estimates of penetration times in the presence and absence of exopolysaccharide-dependent binding of tobramycin. In this case we took the physical model of diffusion into a plane sheet, thickness l, from a constant bathing concentration of antibiotic of C_0. The biofilm was taken to exist on an inert surface, so that diffusion was from one face of the film only. The concentration of antibiotic at the base of the biofilm, next to the inert surface, was called C. The solution of this diffusion problem is given by equation 2.67 of reference 7, from which we plotted the curve of C/C_0 versus Dt/l^2. For $C/C_0 = 0.9$, Dt/l^2 was 1.04. Thus, t_{90} values for tobramycin diffusion across a 0.1-mm-thick biofilm were calculated to be 27.1 s in the absence of exopolysaccharide and 76.6 s in the presence of exopolysaccharide.

We propose that the differences in times of 90% equilibration caused by exopolysaccharide mean that binding of aminoglycoside to glycocalyx material is not a major mechanism of antibiotic resistance of bacteria in either microcolonies or biofilms. By extrapolation, we suggest that this is also true for other antibiotics and biocides, unless a much greater degree of binding of the agent to the glycocalyx occurs.

The question remains as to the alternative mechanisms that can account for the antibiotic resistance of bacteria in biofilms. Two speculative possibilities occur to us. The first is that bacterial cells in the outer layers of a biofilm or microcolony adsorb enough antibacterial substance to increase t_{90} to a protective level for cells deeper in the matrix. Second, cells embedded within the matrix might have a different physiology, and with it a different inherent single-cell antibiotic susceptibility, from the same bacteria growing in free suspension. Certainly, single-cell antibiotic susceptibility is affected by growth rate (15, 24) and other aspects of cell physiology (11). For these and other potential mechanisms of resistance, further studies are required to evaluate their contributions to the reduced antibiotic susceptibility of bacteria growing in glycocalyx-constituted microcolonies and biofilms.

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LITERATURE CITED

1. **Brimley, R. C.** 1949. Quantitative paper chromatography. Nature (London) **163**:215–216.
2. **Cooper, K. E.** 1963. The theory of antibiotic inhibition zones, p. 1–86. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press, Inc., London.
3. **Cooper, K. E.** 1972. The theory of antibiotic diffusion zones, p. 13–30. *In* F. Kavanagh (ed.), Analytical Microbiology II. Academic Press, Inc., London.
4. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41**:435–464.
5. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Micro-

biol. 35:299–324.
6. **Costerton, J. W., and T. J. Marrie.** 1983. The role of the bacterial glycocalyx in resistance to antimicrobial agents, p. 63–85. *In* C. S. F. Easmon, J. Jeljaszewicz, M. R. W. Brown and P. A. Lambert (ed.), Role of the envelope in the survival of bacteria in infection. Academic Press, Inc., London.
7. **Crank, J.** 1975. The mathematics of diffusion, p. 2–4, 69, 89–92, 326–327. Oxford University Press, Oxford.
8. **Dawson, R. M. C., D. C. Elliot, W. H. Elliot, and K. M. Jones (ed.).** 1986. Data for biochemical research, p. 446 and 541–542. Oxford University Press, Oxford.
9. **Drugeon, H. B., M.-E. Juvin, J. Caillon, and A.-L. Courtieu.** 1987. Assessment of formulas for calculating critical concentration by the agar diffusion method. Antimicrob. Agents Chemother. **31**:870–875.
10. **Evans, L. R., and A. Linker.** 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol. **116**:915–924.
11. **Gilbert, P., M. R. W. Brown, and J. W. Costerton.** 1987. Inocula for antimicrobial sensitivity testing: a critical review. J. Antimicrob. Chemother. **20**:147–154.
12. **Lam, J., R. Chan, K. Lam, and J. W. Costerton.** 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. **28**:546–556.
13. **Linker, A., and R. S. Jones.** 1966. A new polysaccharide resembling alginic acid isolated from pseudomonads. J. Biol. Chem. **241**:3845–3851.
14. **Lowry, O. H., N. H. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Muir, M. E., R. S. Van Heeswyck, and B. J. Wallace.** 1984. Effect of growth rate on streptomycin accumulation by *Escherichia coli* and *Bacillus megaterium*. J. Gen. Microbiol. **130**: 2015–2022.
16. **Nichols, W. W., and S. N. Young.** 1985. Respiration-dependent uptake of dihydrostreptomycin by *Escherichia coli*: its irreversible nature and lack of evidence for a uniport process. Biochem. J. **228**:505–512.
17. **Nickel, J. C., I. Ruseska, J. B. Wright, and J. W. Costerton.** 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. **27**:619–624.
18. **Osborn, M. J.** 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **50**:499–506.
19. **Peterson, A. A., R. E. W. Hancock, and E. J. McGroaty.** 1985. Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. J. Bacteriol. **164**: 1256–1261.
20. **Scatchard, G.** 1949. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–672.
21. **Slack, M. P. E., and W. W. Nichols.** 1981. The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of *Pseudomonas aeruginosa*. Lancet **ii**:502–503.
22. **Slack, M. P. E., and W. W. Nichols.** 1982. Antibiotic penetration through bacterial capsules and exopolysaccharides. J. Antimicrob. Chemother. **10**:368–372.
23. **Tannenbaum, C. S., A. T. Hastie, M. L. Higgins, F. Kueppers, and G. Weinbaum.** 1984. Inability of purified *Pseudomonas aeruginosa* exopolysaccharide to bind selected antibiotics. Antimicrob. Agents Chemother. **25**:673–675.
24. **Tuomanen, E., R. Cozens, W. Tosch, O. Zak and A. Tomasz.** 1986. The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. J. Gen. Microbiol. **132**:1297–1304.
25. **Vesterdal, J.** 1947. Studies on the inhibition zones observed in the agar cup method for penicillin assay. Acta Pathol. Microbiol. Scand. **24**:272–282.
26. **Weissbach, A., and J. Hurwitz.** 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. I. Identification. J. Biol. Chem. **234**:705–709.