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Use of the Fluorescent Probe 1-*N*-Phenylnaphthylamine to Study the Interactions of Aminoglycoside Antibiotics with the Outer Membrane of *Pseudomonas aeruginosa*

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The mode of interaction of the polycationic aminoglycoside antibiotics with the surface of *Pseudomonas aeruginosa* cells was studied with the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN). The addition of the aminoglycoside gentamicin to intact cells in the presence of NPN led to a shift in the fluorescence emission maximum from 460 to 420 nm. At the same time the NPN fluorescence intensity increased fourfold. Gentamicin caused no such effects when added to outer membrane vesicles, suggesting that the increased fluorescence resulted from the interaction of gentamicin with intact cells. Gentamicin-promoted NPN uptake was inhibited by the divalent cations Mg^{2+} and Ca^{2+}, but occurred in the absence of gentamicin transport across the inner membrane. Low concentrations of gentamicin (2 μg/ml) caused NPN fluorescence to increase over a period of 4 min in a sigmoidal fashion. At higher concentrations (50 μg/ml) the increase occurred within a few seconds. The final fluorescence intensity was almost independent of the gentamicin concentration. A centrifugation technique was used to demonstrate that gentamicin caused actual uptake of NPN from the supernatant. The initial rate of NPN uptake varied according to the gentamicin concentration in a sigmoidal fashion. Similar data were obtained for seven other aminoglycoside antibiotics. The data, when reanalyzed as a Hill plot, gave a series of lines with a mean slope (the Hill number) of 2.26 ± 0.26, suggesting that the interaction of aminoglycosides with the cell surface to permeabilize it to NPN involved at least three sites and demonstrated positive cooperativity. There was a statistically significant relationship between the pseudoassociation constant K_s from the Hill plots and the minimal inhibitory concentrations for the eight antibiotics. These results are consistent with the concept that aminoglycosides interact at a divalent cation binding site on the *P. aeruginosa* outer membrane and permeabilize it to the hydrophobic probe NPN.

The aminoglycosides are a group of polycationic antibiotics first recognized in the 1940s with the discovery of streptomycin. Since then this family of antibiotics, e.g., gentamicin, tobramycin, and kanamycin, has proved very effective in the treatment of many bacterial infections.

Aminoglycoside uptake into bacterial cells follows a sigmoidal relationship with time and can be divided into three phases (1, 5). First, an ionic binding interaction occurs between the aminoglycoside molecules and the cell, which is followed by two energy-dependent phases called EDPI and EDPII. The initial binding at the cell surface is rapid and reversible and tends to neutralize the cell's net negative surface charge (6). The first energy-dependent phase, EDPI, involves gradual uptake of aminoglycoside (1, 5, 6). EDPII begins when the accumulation of aminoglycoside becomes linear and rapid. It has been suggested that the actual lethal event to the cells precedes or is coincident with the onset of EDPII (6, 9). Both the EDPI and EDPII phases of uptake have been assumed to represent uptake across the energized cytoplasmic membrane.

The uptake of polycationic antibiotics, such as aminoglycosides, across the outer membrane of *Pseudomonas aeruginosa* has been postulated to occur via the self-promoted uptake pathway (6, 14). This uptake mechanism involves the displacement of divalent cations, e.g., Mg^{2+} or Ca^{2+}, from the lipopolysaccharide by the polycationic aminoglycosides. Thus, the divalent cation cross-bridging of lipopolysaccharide is disrupted, causing localized destabilization or distortion of the membrane (6). In support of this hypothesis, the interaction of streptomycin or gentamicin with *P. aeruginosa* causes enhancement of outer membrane permeability to lysozyme and increased uptake of the β-lactam nitrocefin (7). In addition, EDTA, which disrupts Mg^{2+} cross-bridges by chelation rather than displacement, causes similar enhancement of uptake of lysozyme and nitrocefin (7) as well as an increased rate of killing by aminoglycosides (17). Another line of evidence is that outer membrane-altered mutants of *P. aeruginosa*, with an apparent decrease in the number of Mg-binding sites, show cross-resistance to EDTA, polymyxin, and aminoglycosides (13). However, the self-promoted uptake pathway for aminoglycosides has not yet been demonstrated in other gram-negative bacteria, and aminoglycoside uptake may occur in these organisms via the porin protein-mediated pathway (11).

To obtain further information about the interaction of aminoglycosides with *P. aeruginosa*, we decided to utilize fluorescent probes as tools. The use of fluorescent probes to study the structure and function of biological membranes is well documented (3, 4, 8, 10, 18). Commonly used probes in biomembrane studies are 1,8-anilino-1-napthalene sulfonic acid (ANS) and 1-*N*-phenylnaphthylamine (NPN). These probes are particularly useful because they fluoresce weakly in aqueous environments, but become very strongly fluorescent in nonpolar or hydrophobic environments. Furthermore, they are extremely sensitive to environmental factors such as solvent, pH, and temperature, and very little material is required in a given assay.

In this paper, we report the results of a study with fluorescent probes to look at the interaction of aminoglycosides with *P. aeruginosa*. We have obtained further evidence

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that aminoglycosides permeabilize the cell at the outer membrane and that the site of interaction is probably a divalent cation-binding site.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* PAO1 strain H103 (13) was used in all experiments. It was grown in 1% (wt/vol) proteose peptone no. 2 medium (Difco Laboratories, Detroit, Mich.). Experimental cultures were started from an overnight broth culture and grown at 37°C with vigorous shaking to an optical density at 600 nm of 0.4 to 0.6.

Preparation of cell suspension. A 50-ml sample of midlog-phase cells was centrifuged down at 3,000 × *g* for 10 min and suspended in 5 mM sodium HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2), with or without 1 mM KCN, at an optical density at 600 nm of 0.5. This cell suspension was left at 23°C for 30 to 60 min before adding any reagents. Control experiments with *P. aeruginosa* strain H103 (RP1), which contains a plasmid-encoded β-lactamase in its periplasm, demonstrated that during the time course of the experiments no β-lactamase leaked out of the cell, suggesting that the outer membrane remained intact.

Antibiotics and chemicals. Gentamicin sulfate, neomycin sulfate, kanamycin sulfate, and streptomycin sulfate were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Tobramycin and netilmycin sulfate were received from Eli Lilly, Inc. Canada (Scarborough, Ontario). Amikacin was a gift from Bristol-Myers Canada (Ottawa, Ontario), whereas sisomycin sulfate was obtained from the Schering Corp. (Pointe Claire, Quebec). NPN, 1,6-diphenylhexatriene, and ANS were purchased from Sigma.

Fluorescence and polarization measurements. ANS was made as a 60 mM stock solution in 0.9% (wt/vol) NaCl and used at a final concentration of 60 μM. Excitation and emission wavelengths for ANS were set at 375 and 510 nm, respectively, with slit widths of 5 nm. NPN was dissolved in acetone at a concentration of 500 μM and used at a final concentration (in cell suspension) of 10 μM. Control experiments showed no significant effect of the added acetone on the results reported here. The fluorescence spectra and emission intensities were measured with a Perkin-Elmer 650-10S fluorescence spectrophotometer equipped with a Haake circulating water bath to maintain the cuvette holding chamber at 30°C. Excitation and emission wavelengths for NPN were usually set at 350 and 420 nm, respectively, with slit widths of 5 nm.

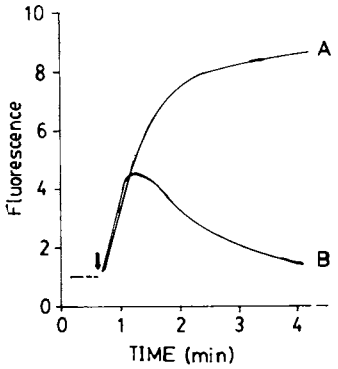

FIG. 1. Effect of KCN treatment on the gentamicin-promoted rise in NPN fluorescence. At 0 min, 5 μM NPN was added to intact *P. aeruginosa* cells. At the arrow 20, μg of gentamicin per ml was added. Curve A shows the data from cells suspended in 1 mM KCN in HEPES buffer and held for 10 min before NPN addition. Curve B shows the data from cells suspended in HEPES buffer.

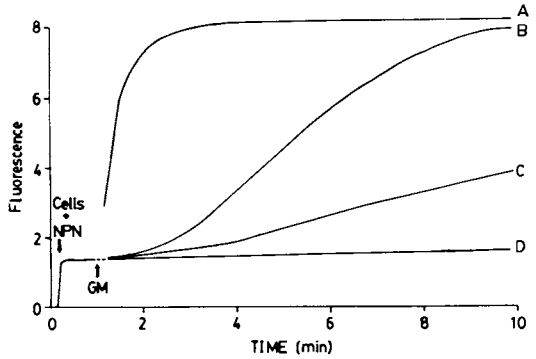

FIG. 2. Time course of increase in NPN fluorescence intensity in the presence of intact *P. aeruginosa* cells and different concentrations of gentamicin or Mg^{2+}. At the arrow labeled GM the following additions were made: A, 20 μg of gentamicin per ml; B, 2 μg of gentamicin per ml; C, 2 μg of gentamicin per ml and 100 μM $MgCl_2$; D, no gentamicin added (results were identical whether or not $MgCl_2$ was added in the absence of gentamicin). Cells were pretreated with 1 mM KCN as described in the Fig. 1 legend.

Measurement of cell-bound NPN. The amount of NPN bound to cells before and after gentamicin treatment was determined by a modification of the centrifugation technique of Nieva-Gomez et al. (15). Samples (4 ml) were prepared containing cyanide-treated cells (suspended to an optical density at 600 nm of 0.5) and 10 μM NPN. Subsamples (1 ml) of each sample were taken before and after the addition of gentamicin at various concentrations and at the end of each experiment. These subsamples were centrifuged at 9,000 rpm for 1 min in an Eppendorf minifuge. Control experiments without added cells or gentamicin were also performed. The NPN concentration of the supernatant was determined by measuring the fluorescence in the presence of 3% (vol/vol) Triton X-100 and comparison with a standard curve. Standard curves were linear over the range of NPN concentrations used (0 to 10 μM).

Preparation of outer membrane vesicles. Outer membranes from strain H103 were prepared as previously described (13) and suspended at a final concentration of 5 mg of protein per ml. A 20-μl sample of outer membrane suspension was diluted in 2 ml of HEPES buffer and sonicated for 15 to 30 s. Mg^{2+}, gentamicin, and the fluorescent probe were added as required.

RESULTS

Selection of a fluorescent probe. Preliminary experiments were carried out with ANS, a probe commonly used in membrane studies. However, the effects of the polycation gentamicin in enhancing ANS fluorescence in intact cells were complicated by charge neutralization (4, 10) of the negatively charged ANS, thus making kinetic analysis difficult. To avoid this, we utilized the neutral probe NPN.

The addition of gentamicin to intact *P. aeruginosa* cells in the presence of NPN caused a time-dependent increase in fluorescence (Fig. 1, curve A). In contrast, although NPN was rapidly taken up by outer membrane vesicles, no further fluorescence enhancement was observed upon gentamicin addition. This suggested that NPN was specifically reporting on an interaction of gentamicin with the outer surface of the

outer membrane of intact cells. Therefore, NPN was chosen for most subsequent studies.

Cyanide pretreatment of cells. To ensure that the observed results were not complicated by either the effects of gentamicin on the cytoplasmic membrane during transport or by postuptake effects on cell metabolism (6), we pretreated cells with 1 mM potassium cyanide, which blocks gentamicin transport (both EDPI and EDPII) and killing (1, 5). The initial rate of increase of fluorescence was identical in the presence or absence of cyanide (Fig. 1). This was confirmed under a variety of the conditions (e.g., at different gentamicin concentrations, in the presence of Mg^{2+}, etc.) tested below. However, cyanide-treated cells demonstrated a continuing fluorescence increase until a steady state was achieved, whereas in nontreated cells the initial increase was followed by a decline in fluorescence to baseline levels. Another energy inhibitor, sodium azide, also prevented the decline in fluorescence without influencing the initial rates of the gentamicin-promoted increase in NPN fluorescence. Similar phenomena (i.e., a fluorescence increase followed by a steady decrease) have been observed in *Escherichia coli*; an energized secretion of NPN, which would be blocked by cyanide or azide in this case, was postulated to be responsible for these effects (2). All subsequent experiments were performed with cyanide due to the simpler kinetics and identical initial rates in the presence or absence of cyanide.

Gentamicin enhancement of NPN fluorescence and inhibition by divalent cations. Control experiments were performed to demonstrate that 10 μM NPN was the ideal concentration for the visualization of the effects of gentamicin on cells. The addition of 10 μM NPN to cyanide-treated *P. aeruginosa* strain H103 cells caused an immediate, small increase in fluorescence intensity above the background level of the cells (Fig. 2, curve D). At this stage the excitation wavelength maximum was 340 nm, and the emission wavelength maximum was 460 nm, similar to the maxima observed with NPN added to aqueous solution. When gentamicin was added, the emission maximum shifted to 420 nm, and the fluorescence intensity at this wavelength increased in a time-dependent process. The excitation wavelength maximum shifted to 350 nm.

When cells in the presence of 10 μM NPN were excited by light of 350 nm wavelength and the emission at 420 nm was followed over time, the kinetics of fluorescence increase varied with the concentration of gentamicin added. At low gentamicin concentrations (2 μg/ml—below the minimal inhibitory concentration for strain H103 cells grown under these conditions), the enhancement of NPN fluorescence was biphasic (Fig. 2, curve B). At higher concentrations of gentamin, e.g., 20 μg/ml, the increase of NPN fluorescence was rapid and plateaued within 20 s after a fourfold increase in fluorescence emission (Fig. 2, curve A). The fluorescence increase in the presence of 100 μg of gentamicin per ml was almost instantaneous, and fluorescence did not decay over time (data not shown). The concentration of gentamicin added influenced only the kinetics of fluorescence increase and not the steady-state level achieved (Fig. 2, curves A and B).

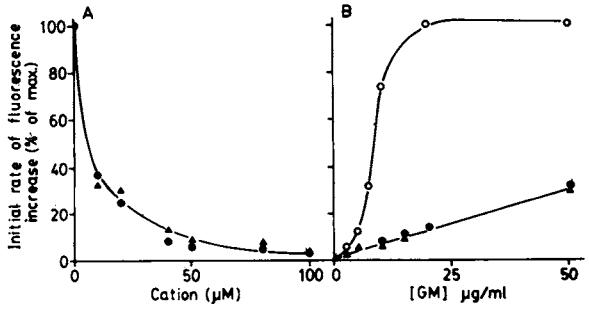

FIG. 3. Effect of divalent cations on the initial rate of the gentamicin-promoted increase in NPN fluorescence of *P. aeruginosa* cells. In panel A increasing concentrations of Mg^{2+} (▲) and Ca^{2+} (●) were added to separate cuvettes containing cells and NPN as described in the text. Immediately afterward, 20 μg of gentamicin per ml was added to each cuvette, and the kinetics of fluorescence increase were followed over time. In panel B either 50 μM Mg^{2+} (▲), 40 μM Ca^{2+} (●), or no cation (○) was added before the addition of various amounts of gentamicin to the final concentration given on the *x* axis. All experiments were performed on cyanide-treated cells.

TABLE 1. Influence of gentamicin concentration on the total uptake of NPN and enhancement of NPN fluorescence in *P. aeruginosa* cells

Gentamicin concn (μg/ml)	Cell-bound NPN in arbitrary units (μmol of NPN taken up)[a]	Total fluorescence of cell-bound NPN in arbitrary units[b]	Calculated fluoresence enhancement of cell-bound NPN[c]
2	10.3 ± 0.6 (1.7)	17.7 ± 4.6	1.7
5	12.4 ± 4.5 (2.0)	23.3 ± 4.4	1.9
10	12.0 ± 0.8 (1.9)	26.3 ± 2.4	2.2
20	14.0 ± 1.8 (2.1)	23.4 ± 2.4	1.7
50	17.7 ± 4.6 (2.5)	29.7 ± 2.0	1.7

[a] Cell-bound NPN was calculated by determining the free NPN in the absence of gentamicin (after removal of cells by centrifugation and addition of 3% Triton X-100) and subtracting the determination of the NPN remaining in the supernatant 10 min after the addition of gentamicin. The results (means ± standard deviations) in arbitrary fluorescent units were converted to micromoles of NPN taken up by reference to a standard curve constructed by the addition of 3% Triton X-100 to different amounts of NPN.

[b] Total fluorescence was the actual increase in fluorescence measured in the 10 min after addition of gentamicin to cells and is the fluorescence intensity of the cell sample after gentamicin addition minus the fluorescence intensity before gentamicin addition. (Before gentamicin addition, 4 fluorescent units [0.3 μmol of NPN] was associated with cells on the average.)

[c] Calculated as the ratio of total fluorescence to cell-bound NPN. This is the increase in the fluorescence of cell-bound NPN over and above the expected fluorescence in 3% Triton X-100.

The addition of the divalent cations Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, and Sr^{2+} at low levels (e.g., 50 μM Mg^{2+}; Fig. 2, curve C) inhibited the enhancement (by gentamicin) of NPN fluorescence. Increasing concentrations of 0 to 50 μM Mg^{2+} or Ca^{2+} produced corresponding decreases in the initial rate of NPN fluorescence enhancement (Fig. 3A). Increasing the gentamicin concentration in the presence of a fixed amount of Ca^{2+} or Mg^{2+} led to a gradual increase in the initial rate of fluorescence (Fig. 3B). As mentioned above, Ca^{2+} and Mg^{2+} at the concentrations used in the experiments represented in Fig. 3 did not themselves cause enhancement of NPN fluorescence.

Uptake of NPN. The increase of NPN fluorescence upon gentamicin addition could have two explanations; it could represent NPN being taken up into the cells from the supernatant, or it could be due to an alteration in the environment of the NPN resulting in fluorescence enhancement. Our data (Table 1) suggested that a combination of these two effects was responsible for the observed fluorescence increase. The amount of NPN bound to cells before and after gentamicin treatment was determined by a centrifugation technique with Triton X-100. Triton X-100 was previ-

ously demonstrated to enhance the fluorescence of cell-free NPN, but had no effect on cell bound NPN (15). In the absence of added gentamicin, approximately 4 fluorescent units of NPN was associated with cells. After the addition of gentamicin and sufficient time to allow fluorescence to reach a steady-state level, cell-associated fluorescence had increased four- to sevenfold. The maximum level of cell-bound NPN was relatively independent of the gentamicin concentration since over a 25-fold range of gentamicin concentrations, cell-bound fluorescence changed only 1.7-fold. The total increase in fluorescence after gentamicin addition could not be accounted for by the cell-bound NPN (Table 1). Thus, part of the increase must have been due to the NPN being incorporated into an environment in which it was more highly fluorescent than in Triton X-100 solutions (Table 1; this fluorescence enhancement apparently only occurred as a consequence of uptake—thus the total fluorescence increase reflects NPN uptake). The increase in fluorescence might have been caused by an increased lifetime of the probe (8). The increase in fluorescent yield of the cell-bound NPN was almost independent of the gentamicin concentration, suggesting that the NPN partitioned into a similar environment in all experiments.

Kinetics of gentamicin-promoted NPN uptake. A series of experiments with gentamicin concentrations between 1 and 20 μg/ml were performed to evaluate how the rate of NPN uptake was affected by the gentamicin concentration present. We obtained a family of sigmoidal curves of fluorescence increase over time after gentamicin addition (e.g., Fig. 2, curves A and B). Due to the complexity these interactions, we attempted only to analyze the initial rates of the NPN fluorescence increase. A plot of these initial rates against gentamicin concentration produced a sigmoidal plot (Fig. 4A). From this, it appeared that a cooperative interaction

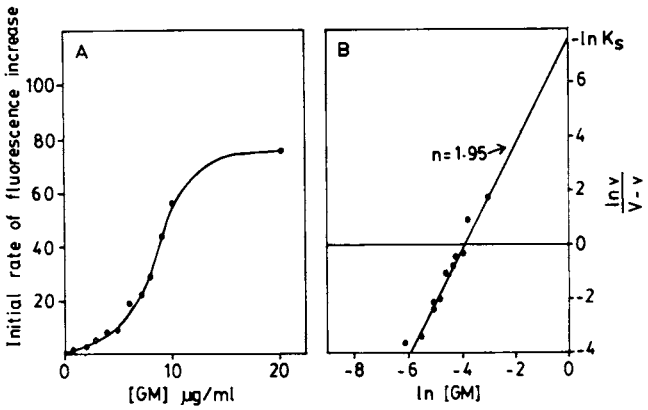

FIG. 4. Kinetics of gentamicin-promoted increase in NPN fluorescence of intact cells. In panel A the initial rate of fluorescence increase (V, expressed in arbitrary fluorescence units per minute per milligram of cell dry weight), taken from a series of experiments like those depicted in curves A and B of Fig. 2, was plotted against the concentration of gentamicin [GM] in micrograms per milliliter. All experiments were performed with cyanide-treated cells. This data were reanalyzed according to a Hill plot (B), in which the gentamicin concentration was converted to molar concentrations and ϕ is the ratio of the rate of fluorescence increase at the given gentamicin concentration to the maximal rate of fluorescence increase extrapolated from panel A. The Hill number (the slope of the Hill plot) is given in panel B. The y-axis intercept is equal to $-\ln K_s$. The correlation coefficient for the linear regression of the data for this and all other Hill plots was greater than 0.99.

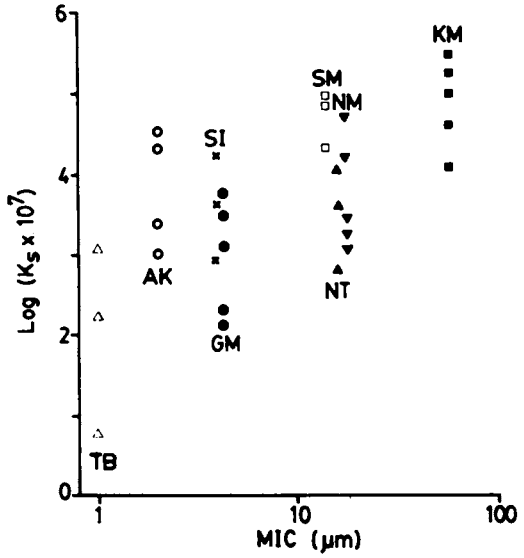

FIG. 5. Relationship between the MICs of the different aminoglycoside antibiotics and the $\log_{10}$ of the pseudoassociation constants K_s, extrapolated from Hill plots such as those shown in Fig. 4B. The aminoglycoside antibiotic abbreviations and their symbols were as follows: tobramycin, TM (△); amikacin, AK (○); sisomycin, SI (×); gentamicin, GM (●); streptomycin, SM (□); netilmycin, NT (▲); neomycin, NM (▼); kanamycin, KM (■).

had occurred between gentamicin and cells giving rise to the uptake of NPN, i.e., the interaction of one molecule of gentamicin with the cell surface enhanced subsequent interactions. The data were replotted as a Hill plot (Fig. 4B), which allows one to distinguish simple, multiple, or cooperative interactions. The slope of the Hill plot is referred to as the Hill number. This number is usually interpreted as the approximate minimum number of binding sites. For gentamicin, the Hill number was 1.95 ± 0.3 (average of five experiments), indicating a cooperative interaction with a minimum of two or three interaction sites. Similar linear Hill plots were obtained with each of eight different aminoglycoside antibiotics. Interestingly, the Hill numbers derived from Hill plot analyses of these data were very similar among the eight aminoglycosides. A mean and standard deviation of 2.26 ± 0.26 for the 30 analyses was calculated.

In addition to this interaction coefficient, one can calculate from the Hill plot a K_s value (pseudoassociation constant) for the interaction of gentamicin with cells, since the y-axis intercept is $-\ln K_s$. The calculated K_s values were used for relative comparisons of the different aminoglycosides since they varied over 4 orders of magnitude. A plot of log K_s against the logarithm of the MICs for the different aminoglycosides is shown in Fig. 5. There were significant variations for each antibiotic in individual determinations of the K_s despite the facts that these numbers derived from Hill plots in which the correlation coefficients (r) were greater than 0.99 and, as noted above, the slopes of the individual lines (the Hill numbers) were remarkably constant. One possible reason for this might be differential contamination of individual batches of cells by divalent cations since the initial rate of NPN uptake was strongly affected by divalent cations (Fig. 3). Nevertheless, a highly significant correlation between log MIC and log K_s was demonstrated (r = 0.68; df = 29, $P < 0.001$ for a straight line with a slope of 1.33 by least-squares analysis). The affinity of a substrate for its interac-

tion site in a cooperative interaction is sometimes expressed as the $S_{0.5}$ or substrate concentration at half-maximal velocity (given by the x axis intercept of the Hill plot = log $S_{0.5}$). We were also able to demonstrate a significant correlation between $S_{0.5}$ and the MIC for different aminoglycosides.

DISCUSSION

In this paper we have demonstrated the effectiveness of fluorescent probes in studying the interactions of aminoglycosides with *P. aeruginosa* cells. The uptake of NPN that we measured probably reflects outer membrane permeabilization to NPN by aminoglycosides. As evidence for this, we could only demonstrate enhancement of NPN uptake by gentamicin in intact bacterial cells. Gentamicin did not stimulate uptake of NPN into bacterial outer membrane vesicles, nor into inner membrane vesicles (in the presence or absence of added carbon sources or electron acceptors) or synthetic phospholipid liposomes (B. Loh, M.Sc. thesis, University of British Columbia, 1984). In fact, these vesicles became immediately saturated with NPN that had fluorescence spectral properties similar to the NPN taken up by gentamicin-treated cells. This suggests that gentamicin is disorganizing the cell surface in such a way that NPN can partition into the outer (and probably also the inner) membrane. Presumably the outer membrane structure is also disorganized by the French press treatment used to make outer membrane vesicles. In further agreement with this concept, the rate of NPN fluorescence increase was related to the amount of gentamicin added (Fig. 3 and 5), although the final amount of NPN associated with cells was relatively constant (Table 1). This suggests that the NPN enters the cell via a limited number of access areas (presumably this number is determined by the gentamicin concentration), but that NPN uptake, once initiated, proceeds until the sites with which NPN associates are saturated.

We have previously proposed that the interaction sites for aminoglycosides on the outer membrane are those sites where divalent cations noncovalently cross-bridge adjacent lipopolysaccharide molecules (13, 14). Presumably aminoglycosides displace Mg^{2+} from these sites, thus distorting outer membrane structure. In agreement with this, we could demonstrate that Mg^{2+} or Ca^{2+} inhibited gentamicin-promoted uptake of NPN (Fig. 2 and 3), a result that could be explained by competition of the polycationic antibiotic gentamicin and the divalent cations for a divalent cation-binding site on the outer membrane.

Evidence has been provided which suggests that both the polycationic antibiotic polymyxin B and the divalent cation chelator EDTA interact with the same divalent cation-binding site as aminoglycosides like gentamicin (14). In further agreement with this, both polymyxin B (12) and EDTA (8) cause an increase in the fluorescence intensity of hydrophobic fluorescent probes added to cells. Helgerson and Cramer (8) postulated that EDTA treatment removed the permeability barrier of the outer membrane of *E. coli* to NPN. More recently, it has been shown that polymyxin B interacts with a divalent cation-binding site on lipopolysaccharide (16). Furthermore, polymyxin B and gentamicin each compete with a cationic spin label probe, CAT_{12}, for a divalent cation-binding site on *P. aeruginosa* LPS (A. A. Paterson, R. E. W. Hancock, and E. J. McGroary, manuscript in preparation).

One of the major observations of this paper is that the initial rates of fluorescence increase vary according to the gentamicin concentration in a fashion that is amenable to kinetic analysis. A plot of the initial rate of fluorescence increase as a function of gentamicin concentrations gave a sigmoidal plot, suggesting positive cooperativity. This was confirmed by replotting the data as a Hill plot. The advantage of this form of kinetic treatment is that the ordinate term [$\log(1 - \phi)/\phi$, where ϕ is the ratio of the rate of fluorescence increase at a given gentamicin concentration to the maximal rate of fluorescence increase at infinite gentamicin concentration] has no units and thus is independent of the arbitrary fluorescent units. Thus, if we make the assumption that the rate of NPN uptake directly reflects the interaction of gentamicin with outer membranes, which seems reasonable, the Hill plot provides kinetic numbers that reflect only this interaction. Similar arguments are valid for each of the eight aminoglycosides subjected to kinetic analysis. In Hill plot analyses with all eight aminoglycosides involving 30 separate sets of data, the Hill number (the slope of the Hill plot) was 2.26 ± 0.26. Since this provides an estimation of the minimum number of interaction sites involved in the cooperation permeabilization of outer membranes to NPN, it can be assumed that at least three or more sites are involved.

Although the Hill numbers were remarkably similar for each of the eight aminoglycosides, the pseudoassociation constant K_s varied substantially. The highly significant ($P < 0.001$) linear relationship between the K_s value and the MIC value for the different aminoglycosides suggests that the measured interaction at the surface of the outer membrane may be an important determinant of the efficiency or rate (or both) of killing. In agreement with this, Mg^{2+} and Ca^{2+}, which strongly antagonize the transport of and killing by aminoglycosides (1), also inhibited the enhancement of NPN uptake by aminoglycosides (Fig. 2 and 3). It should be noted, however, that our experiments were performed in the presence of cyanide, which blocks energy-dependent transport and killing, suggesting that interaction at the outer membrane precedes these energy-dependent events. In this regard it is intriguing that the time course of NPN uptake after the treatment of cells with aminoglycosides strongly mimicked the time course of aminoglycoside uptake (1, 6).

Although we have not formally demonstrated that aminoglycosides interact with outer membranes to promote their own uptake, we have described the enhancement of uptake of the hydrophobic probe NPN as well as the protein lysozyme and the β-lactam antibiotic nitrocefin (7). These data are thus consistent with the self-promoted uptake hypothesis (6, 14), but do not prove it. To further test this hypothesis, it will be necessary to learn more about the events after the initial interaction. For this reason, we are currently attempting to fluorescently tag the aminoglycoside antibiotics themselves.

ACKNOWLEDGMENTS

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