Use of the fluorescent probe 1-N-phenyl napthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*

by

Bernadette Anne Loh

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Department of MICROBIOLOGY

The University of British Columbia
1956 Main Mall
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V6T 1Y3

Date October 11, 1984

ABSTRACT

The mode of interaction of the polycationic aminoglycoside antibiotics with the surface of Pseudomonas aeruginosa cells was studied using the hydrophobic fluorescent probe 1-N-phenyl napthylamine (NPN). 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 four fold. 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 minutes 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 reanalysed 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 3 sites and demonstrated

positive cooperativity. There was a statistically significant relationship between the pseudo-association constant K_s from the Hill plots and the minimal inhibitory concentrations for the 8 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.

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I would also like to thank the members of my supervisory committee, Dr. D. Syeklocha and Dr. R.A.J. Warren for their time and patience, and Susan Heming for her skillful typing of this manuscript.

DEDICATION

This thesis is dedicated with gratitude to my parents who have given me great support, inspiration and encouragement over the years.

INTRODUCTION

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. gentamycin, tobramycin, 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 (1,6). 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 (7). The first energy dependent phase, EDPI, involves gradual uptake of aminoglycosides (1,6,7). EDPII begins when 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 (7,10). 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 (7,15). This uptake mechanism involves the displacement of divalent cations e.g. Mg^{++} or Ca^{++} from the LPS by the polycationic aminoglycosides. Thus, the divalent cations crossbridging of lipopolysaccharide (LPS) is disrupted causing localized destabilization or distortion of the membrane (7). 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 ß-lactam, nitrocefin (8). In addition, ethylenediaminetetraacetate (EDTA), which disrupts Mg^{2+} crossbridges by chelation rather than displacement causes similar enhancement of uptake of lysozyme and nitrocefin (8) as well as an increased rate of killing by aminoglycosides (18). 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 (14). 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 (13).

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 (4,5,9,11). Commonly used probes in biomembrane studies are 1,8-anilino-1-napthalene sulphonic acid (ANS) and 1-N-phenyl naphthylamine (NPN). These probes are particularly useful because they fluoresce weakly in aqueous environments but become very strongly fluorescent in non-polar 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 thesis, the results of a study using fluorescent probes to look at the interaction of aminoglycosides with P. aeruginosa is reported. I have obtained further evidence 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. Pseudomonas aeruginosa PAO1 strain H103 (14) was used in all experiments. It was grown in 1% (wt/vol) proteose peptone No. 2 medium (Difco). Experimental cultures were started from an overnight broth culture and grown at 37°C with vigorous shaking to an optical density at 600 nm (OD_{600}) of 0.4 - 0.6.

Preparation of cell suspension. Fifty ml of mid-log phase cells were centrifuged down at 3000 x g for 10 min, and resuspended in 5 mM Na Hepes buffer pH 7.2, with or without 1 mM KCN, at an OD_{600} of 0.5. This cell suspension was left at 23°C for 30-60 min before adding any reagents. Control experiments using P. aeruginosa strain H103 (RP1) containing a plasmid-encoded ß-lactamase in its periplasm, demonstrated that during the time course of the experiments no ß-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), while sisomycin sulfate was obtained from the Schering Corporation (Pointe Claire, Quebec). NPN; 1,6-diphenylhexatriene (DPH); and ANS were purchased from Sigma Chemical Co.

Fluorescence and polarization measurements. ANS was made as a 60mM stock solution in 0.9% (w/v) NaCl and used at a final concentration of 60 μM. Excitation and emission wavelengths for ANS were set at 375 and 475 nm, respectively with slit widths of 5nm. 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. Fluorescence spectra and emission intensities were measured using 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 nm and 420nm respectively, with slit widths of 5nm. Fluorescence intensities are given in arbitrary units.

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 described by Nieva-Gomez et al. (16). Samples (4 ml) were prepared containing cyanide-treated cells (resuspended to OD_{600} = 0.5) and 10 μM NPN. Aliquots (1 ml) of each sample were taken before and after the addition of gentamicin at varying concentrations, and at the end of each experiment. These aliquots were centrifuged at 9,000 rpm for 1 min using 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% (v/v) Triton X-100 and comparison with a standard curve. Standard curves were linear over the range of NPN concentrations used (0 - 10 μM).

Preparation of outer membrane vesicles and liposomes. Outer membranes from strain H103 were prepared as previously described (14) and resuspended at a final protein concentration of 5 mg/ml. Twently μl of outer membrane suspension was diluted in 2 ml Hepes buffer and sonicated for 15-30 sec. Mg^{2+}, gentamicin and fluorescent probe were added as required.

Vesicles were also prepared from commercially obtained lipids such as phosphatidylcholine and phosphalidylethanolamine and from H103 lipopolysaccharide (LPS) [prepared according to method by Darveau, 1983 (3)].

RESULTS

Selection of a fluorescent probe. Preliminary experiments were carried out with 1,8-ANS, a probe commonly used in membrane studies. Gentamicin (GM) addition to intact P. aeruginosa cells in the presence of ANS caused a substantial increase in fluorescence; however, the kinetics of this increase were complex and included an initial immediate increase followed by a second, slower rate of increase (Fig. 1, curve A).

These complex kinetics led me to look at a simpler system using membrane or LPS vesicles and liposomes. It was found that both inner and outer membrane vesicles had intrinsic excitation and emission wavelengths of 350 and 430 nm respectively (Fig. 2). In the presence of ANS, excitation was shown as a broad band between 330-390 nm while the emission wavelength was red-shifted to 520 nm (Fig. 3). With the addition of 50 µg/ml GM, there was an immediate increase in fluorescence intensity (Fig. 1, curve C), with a shift in emission wavelength back to 470 nm (Fig. 4).

Vesicles prepared from phosphatidylcholine (PC) and phosphatidyl ethanolamine (PE) as well as those prepared from H103-lipopolysaccharide (LPS) showed a similar response (Fig. 5). That is, gentamicin addition caused an immediate enhancement of ANS fluorescence and no secondary increase.

Furthermore, divalent cations like Mg^{2+} and Ca^{2+} caused a similar increase in ANS fluorescence (Fig. 1, curve B; 5).

Figure 1. Effects of gentamicin and magnesium on the fluorescence intensity of ANS in the presence of P. aeruginosa intact cells or outer membrane vesicles. At 0 min 5 μM ANS was added to the cells (curves A and D) or vesicles (B and C). Shortly thereafter (as indicated by the arrow) the following additions were made: Curve A - 20 μg/ml gentamicin added to intact cells; Curve B - 10 μM $MgCl_2$ added to outer membrane vesicles; Curve C - 20 μg/ml gentamicin added to outer membrane vesicles; Curve D - no further additions to intact cells (similar results to curve D were obtained when no addition was made to outer membrane vesicles).

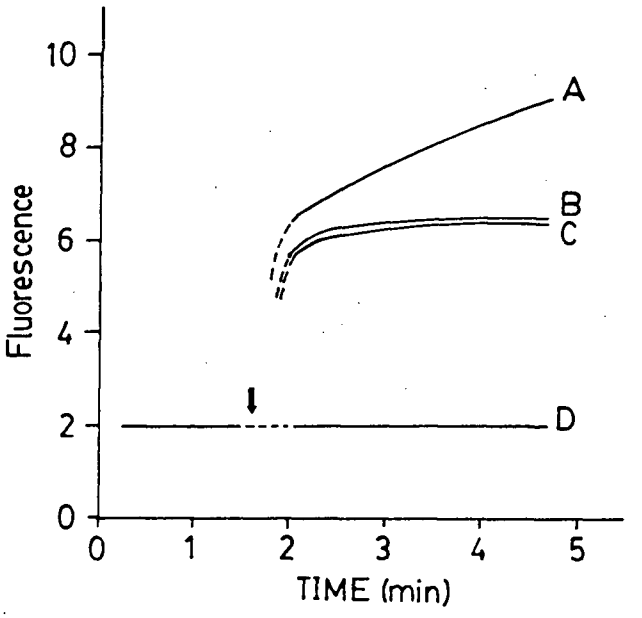
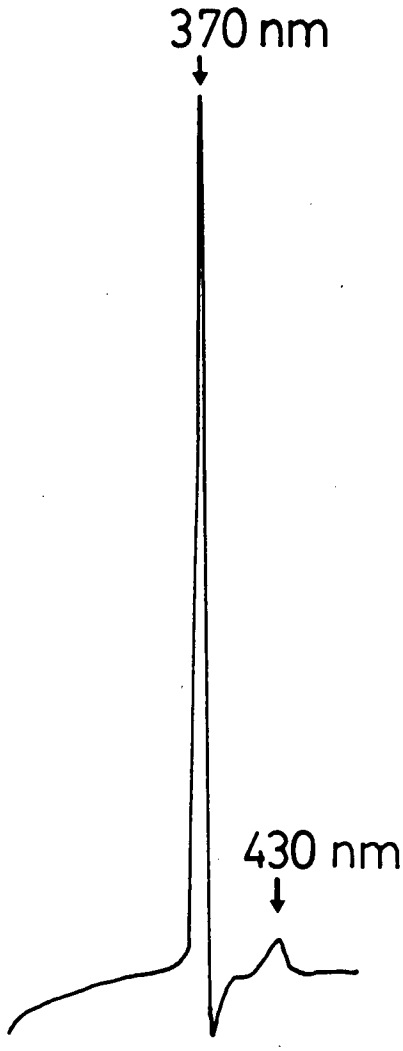
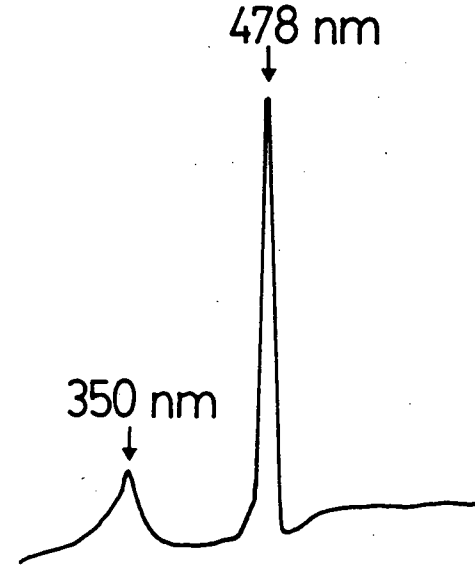

Fluorescence
10
8
6
4
2
0
0
1
2
3
4
5
TIME (min)
A
B
C
D

Figure 2. Intrinsic emission and excitation spectra of outer membrane vesicles of P. aeruginosa H103. Outer membrane vesicles were resuspended to a final concentration of 0.1 mg/ml protein in 2 ml of 5 mM Hepes and sonicated for 30 sec. Excitation and emission wavelengths were set at 370 nm and 478 nm respectively for emission and excitation scans. In the absence of added probe, the emission and excitation maxima were 430 nm (A) and 350 nm (B) respectively. Similar values were obtained with inner membrane vesicles.



A



B

Figure 3. Excitation and emission spectra of ANS fluorescence in the presence of outer membrane vesicles. Outer membrane vesicles were resuspended to a final concentration of 100 µg/ml in 2 ml of 5 mM Hepes. ANS was added to 60 µM and excitation and emission wavelengths were set at 375 and 480 nm respectively. Emission and excitation maxima were 520 nm (A) and a broad band of 330-390 nm (B) respectively. Similar scans were obtained with inner membranes.

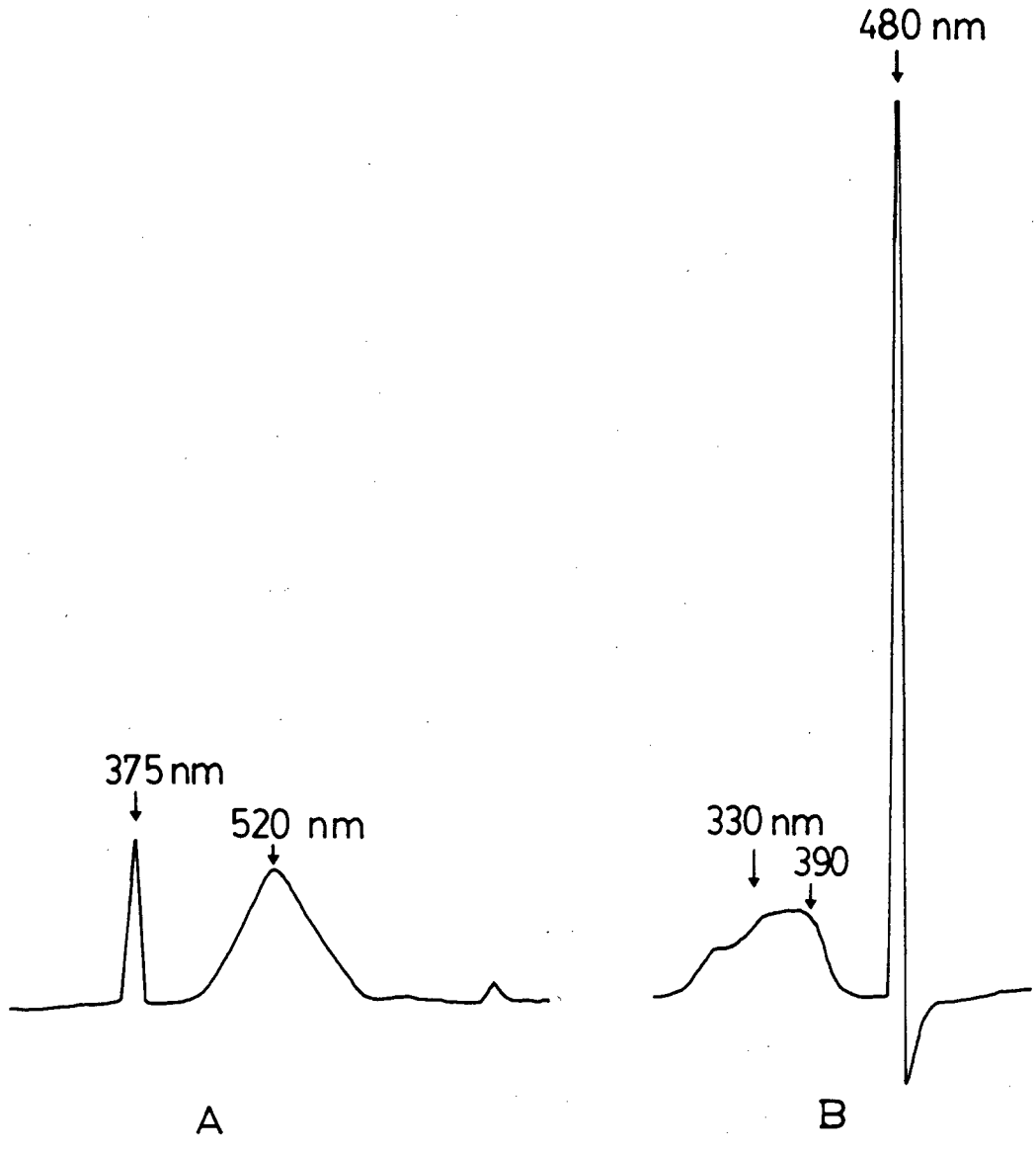

480 nm
375 nm
520 nm
330 nm
390
A
B

Figure 4. Excitation (A) and emission (B) fluorescence spectra of ANS added to outer membrane vesicles and the effect of added gentamicin. Excitation and emission wavelengths were set at 375 and 475 nm respectively. Outer membranes were resuspended to a final concentration of 100 µg/ml in 2 ml of 5 mM Hepes. ANS was added to a concentration of 60 µM and gentamicin to 50 µg/ml. Emission and excitation maxima were 470 and 385 nm respectively. Inner membrane vesicles gave similar results.

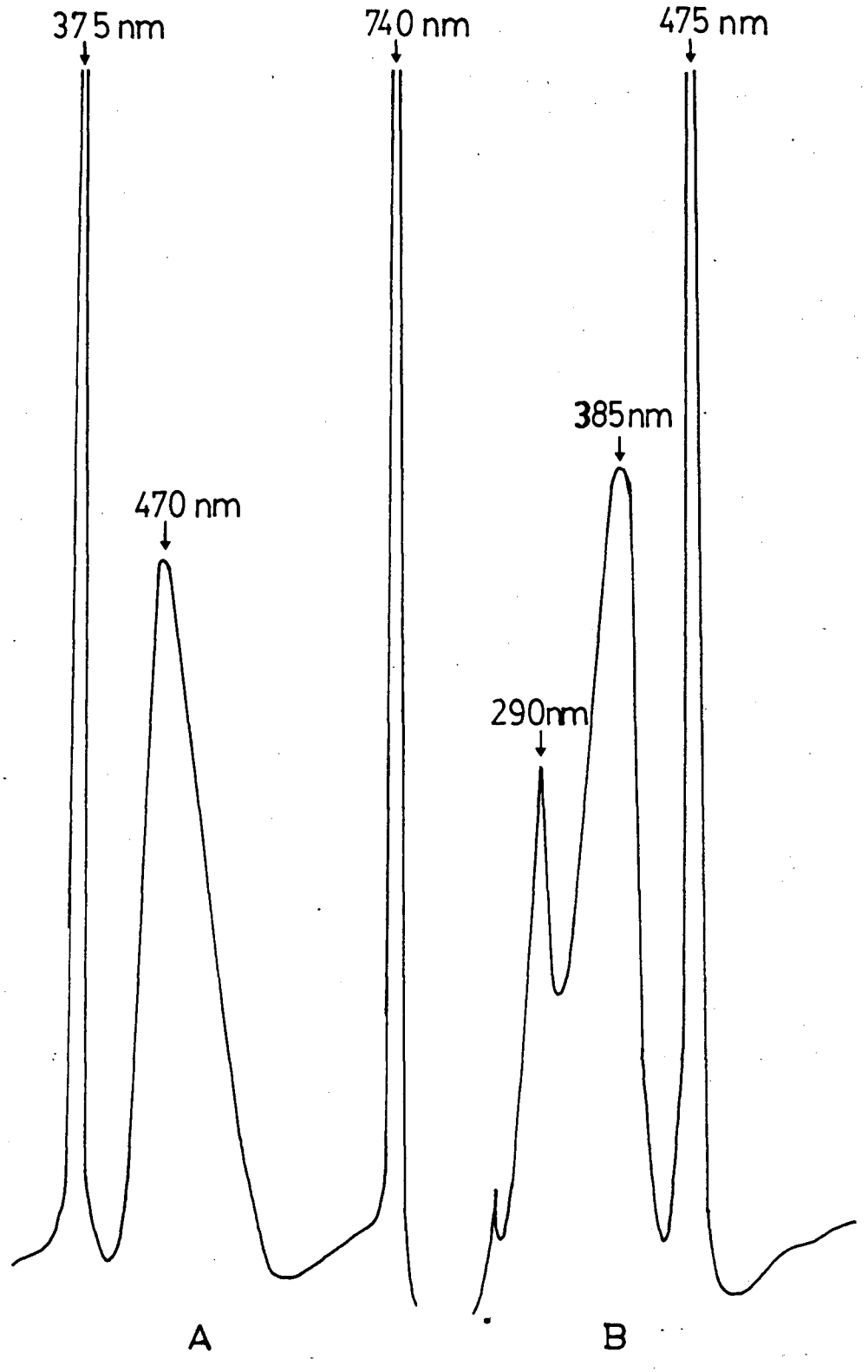
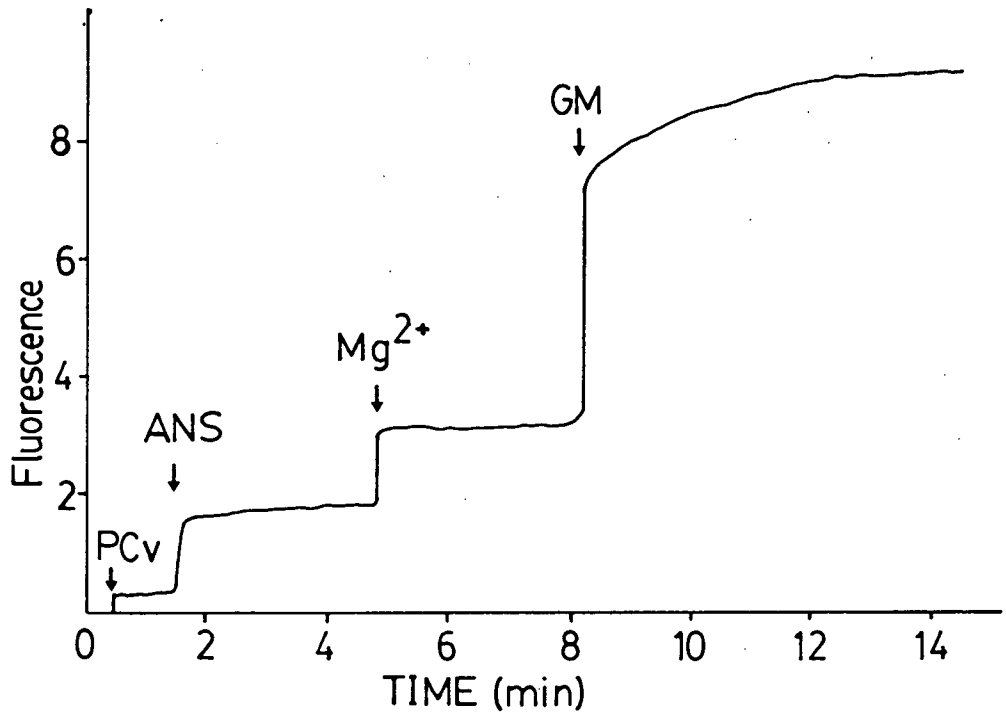

375 nm
740 nm
475 nm
470 nm
385nm
290nm
A
B

Figure 5. Effect of magnesium and gentamicin addition on ANS fluorescence in phospholipid vesicles. Phosphatidylcholine vesicles (PC_v) were resuspended to 100 µg/ml in 2 ml of 5 mM Hepes buffer and sonicated for 30 sec. ANS was added at 60 µM. Subsequently Mg^{2+} ($MgCl_2$) was added to 0.1 mM and gentamicin to 20 µg/ml final concentration. Similar values of fluorescence intensity were seen with phosphatidylethanolamine and LPS vesicles.


Fluorescence
8
6
4
2
PCv
ANS
Mg^{2+}
GM
0
2
4
6
8
10
12
14
TIME (min)

Presumably, the effects of gentamicin enhancement of fluorescence in intact cells were complicated by charge neutralization of negatively-charged ANS by polycationic aminoglycosides allowing further uptake of ANS. Such effects were previously demonstrated for di-and trivalent cations (5,11).

A second fluorescent probe, pyrene, was found to be rapidly taken up by phospholipid vesicles but this fluorescence was not stable and almost instantaneously started to decay (Fig. 6). This suggested that pyrene probably had a very short fluorescence lifetime in this system and was therefore not suitable. Subsequent gentamicin addition did not enhance fluorescence.

In contrast to these data, the fluorescence of the neutral probe NPN, was enhanced upon the interaction of intact cells with gentamicin (Fig. 8, curve A) and after plateauing remained stable for 10-20 min. However, although NPN itself was rapidly taken up by outer membrane and phospholipid vesicles, very little or no fluorescence enhancement was observed upon addition of gentamicin, Mg^{2+} or Ca^{2+} (Fig. 7). This suggested that NPN was specifically reporting on an interaction of gentamicin with the surface of intact cells. Therefore, NPN was chosen for all subsequent studies.

In addition, the sequential addition of NPN to outer membrane vesicles caused an increase in fluorescence from 1-5 μm NPN, a peak or plateau at 7-8 μm NPN, and slight lower fluorescence emission at higher NPN concentrations to 10 μm (Fig. 9).

Figure 6. Fluorescence of phospholipid vesicles in the presence of pyrene, and the effect of gentamicin addition. Phosphatidylcholine vesicles (PC_v) were resuspended to 100 µg/ml in 2 ml of 5 mM Hepes buffer and sonicated for 30 sec. Pyrene was added at 2.5 µm and subsequently gentamicin was added at 20 µg/ml. Phosphatidylethanolamine vesicles (PE_v) gave similar results.

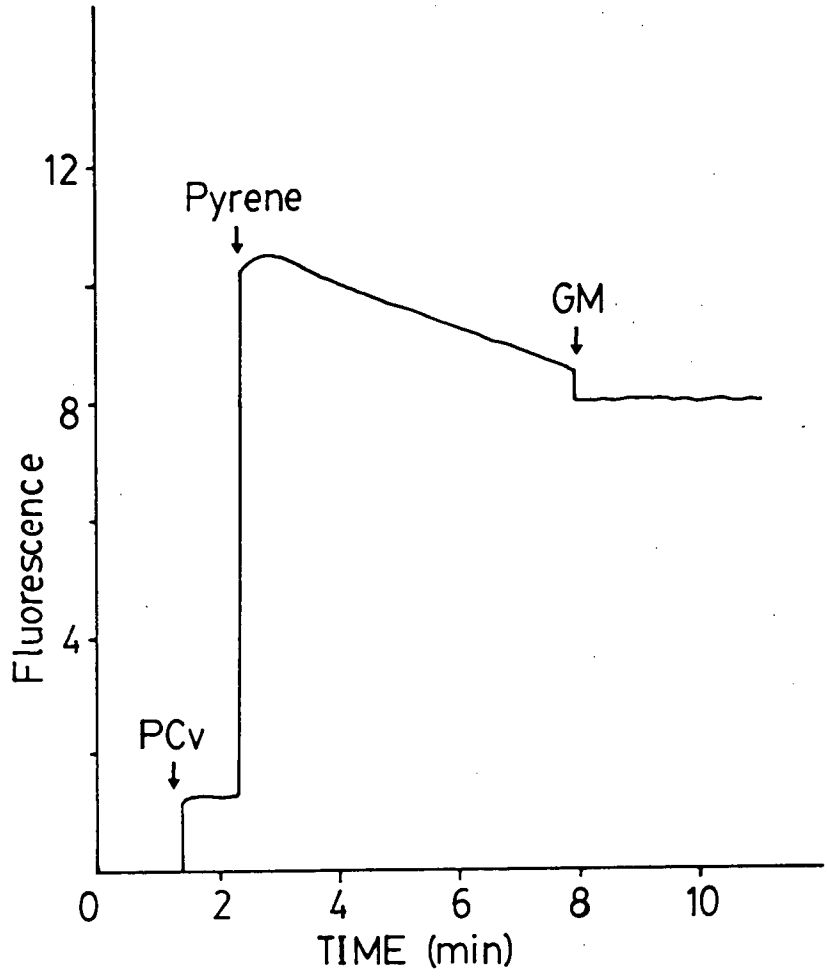

12
8
4
Fluorescence
Pyrene
GM
PCv
0
2
4
6
8
10
TIME (min)

Figure 7. Effect of gentamicin or magnesium addition on NPN fluorescence in membrane vesicles. Inner or outer membranes were resuspended in 2 ml of 5 mM Hepes buffer in the presence of 10 μM NPN. Magnesium was added to 1 mM and gentamicin to 20 μg/ml as required.

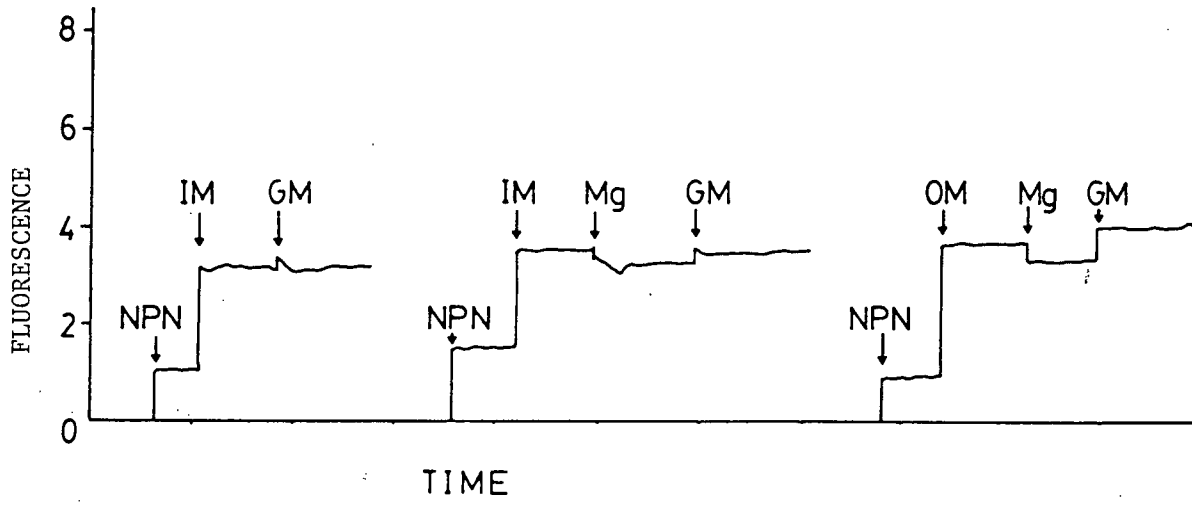

8
6
4
2
0
FLUORESCENCE
NPN
IM
GM
NPN
IM
Mg
GM
NPN
OM
Mg
GM
TIME

Figure 8. 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 labelled GM the following additions were made: Curve A - 20 µg/ml of gentamicin; Curve B - 2 µg/ml of gentamicin; Curve C - 2 µg/ml of gentamicin and 100 µM $MgCl_2$; Curve 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 below (see Fig. 11).

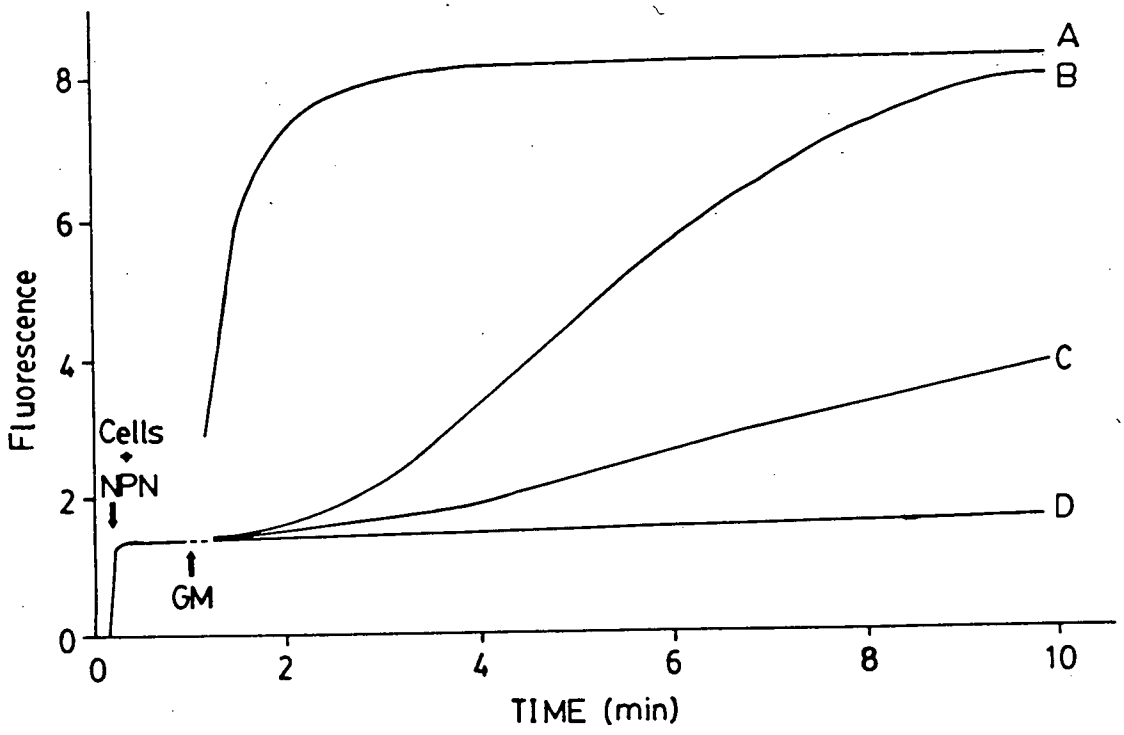
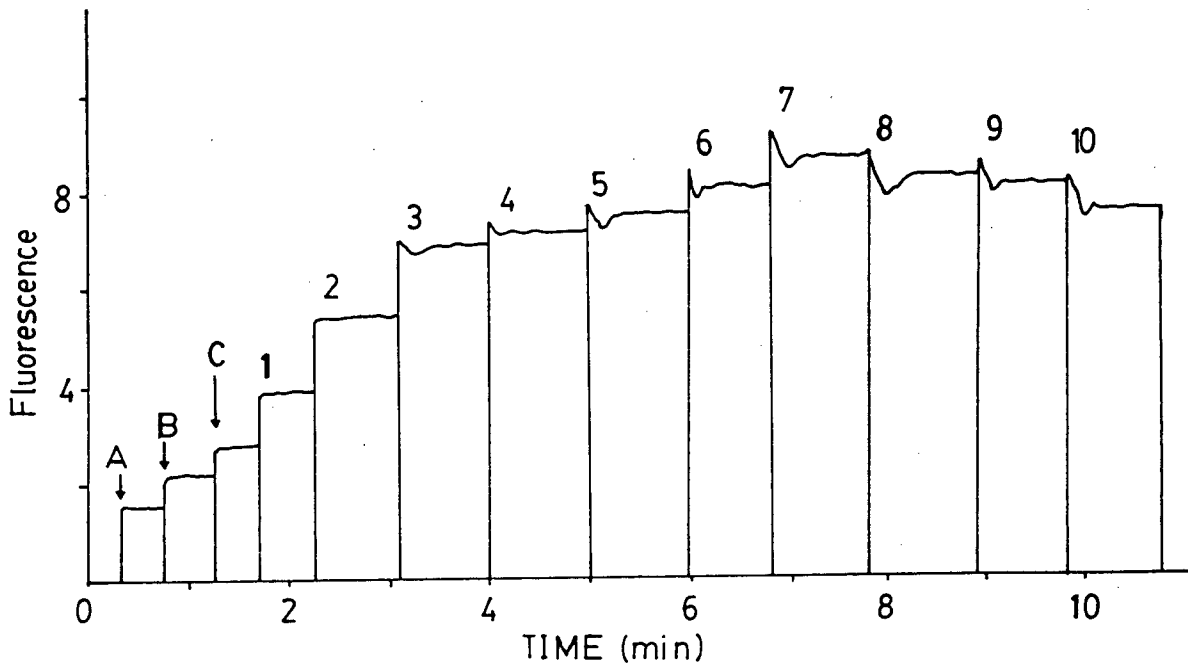

A
B
C
D
Cells
NPN
GM
Fluorescence
TIME (min)
0
2
4
6
8
10

Figure 9. Effect of sequential addition of NPN on fluorescence of outer membrane vesicles in the presence of gentamicin. Outer membranes were resuspended in 2 ml of 5 mM Hepes buffer (arrow A). Gentamicin was added at 20 µg/ml (arrow B). NPN was then added sequentially, starting at 0.5 µM (arrow C) and continuing from 1-10 µM.


Fluorescence
8
4
A
B
C
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
TIME (min)

Effects of different growth media and buffers. Resuspension of an overnight culture of strain H103 in PP2 broth resulted in a very high level of intrinsic fluorescence (presumably due to the production of endogeneous fluorophores). No increase in fluorescence was observed with addition of NPN 20 µm or gentamicin at 20 µg/ml.

Cells grown and resuspended in nutrient broth or BM2 (minimal media with low Mg^{2+}) showed a fairly high but unstable fluorescence in the presence of NPN. Gentamicin addition resulted in a moderate NPN fluorescence increase in NB-grown cells, but a decrease in fluorescence of BM2 grown cells.

Of the different media used for growing and resuspending cells, growth in PP2 both overnight and resuspension in 5 mM Hepes buffer after centrifugation proved to be the best combination for convenience and consistency. Control experiments gave comparable results from day to day and allowed repeated results to be analyzed.

Microstirrer Apparatus and Aeration Effect. The fluorescence spectrophotometer was equipped with a microstirrer apparatus (Lawrence Instruments, Ltd.) which consisted of a tiny magnetic stirrer unit attached under the cuvette holder. Our objective was to find out if continuous stirring of the sample would ensure more effective contact between probe molecules and bacterial cells and produce more consistent results among different experiments. Unexpectedly, this caused prolonged fluctuations in fluorescence level and a generally higher baseline fluorescence in any particular sample. Many factors could be responsible

for this including aeration of the sample and light scatter from the magnetic flea bar in the cuvette.

In addition, manual agitation/aeration (shaking) of the cell suspension after reaching the maximum fluorescence emission caused the fluorescence to decrease to its base level. The same effect was observed if a carbon source such as potassium succinate or glucose (1 mM) was added (Fig. 10). Again, these phenomena were reminiscent of similar studies in E. coli (2).

Cyanide pretreatment of cells. In order to ensure that the observed results were not complicated by either the effects of gentamicin on the cytoplasmic membrane during transport or by post-uptake effects on cell metabolism (7), we pretreated cells with 1 mM potassium cyanide which blocks (both EDPI and EDPII) gentamicin transport and killing (1,5). As shown in Fig. 11, the initial rate of increase of fluorescence was identical in the presence or absence of cyanide. This was confirmed under a variety of the conditions tested below. However, cyanide-treated cells demonstrated a continuing fluorescence increase until a steady state was achieved, whereas in non-treated cells, the increase was followed by a decline in fluorescence to baseline levels. Another energy inhibitor, sodium azide (1 mM) also prevented the decline in fluorescence without influencing the initial rates of the gentamicin-promoted increase in NPN fluorescence (Fig. 12A). Similar phenomena (i.e. a fluorescence increase followed by a steady decrease) have been observed in E. coli, and an energized secretion of NPN, which would be blocked by cyanide or azide in

Figure 10. Effect of potassium succinate (or glucose) addition and aeration of the cell suspension on NPN fluorescence. At the arrow labelled 1, intact cells of P. aeruginosa H103 were resuspended in 2 ml of 5 mM Hepes buffer with 10 μM NPN. At arrow 2, potassium succinate (or D-glucose) was added to 2 mM. At arrow 3, the cuvette was shaken manually to aerate the cell suspensions. At later times the fluorescence rose again until it achieved a pre-aeration level of fluorescence.

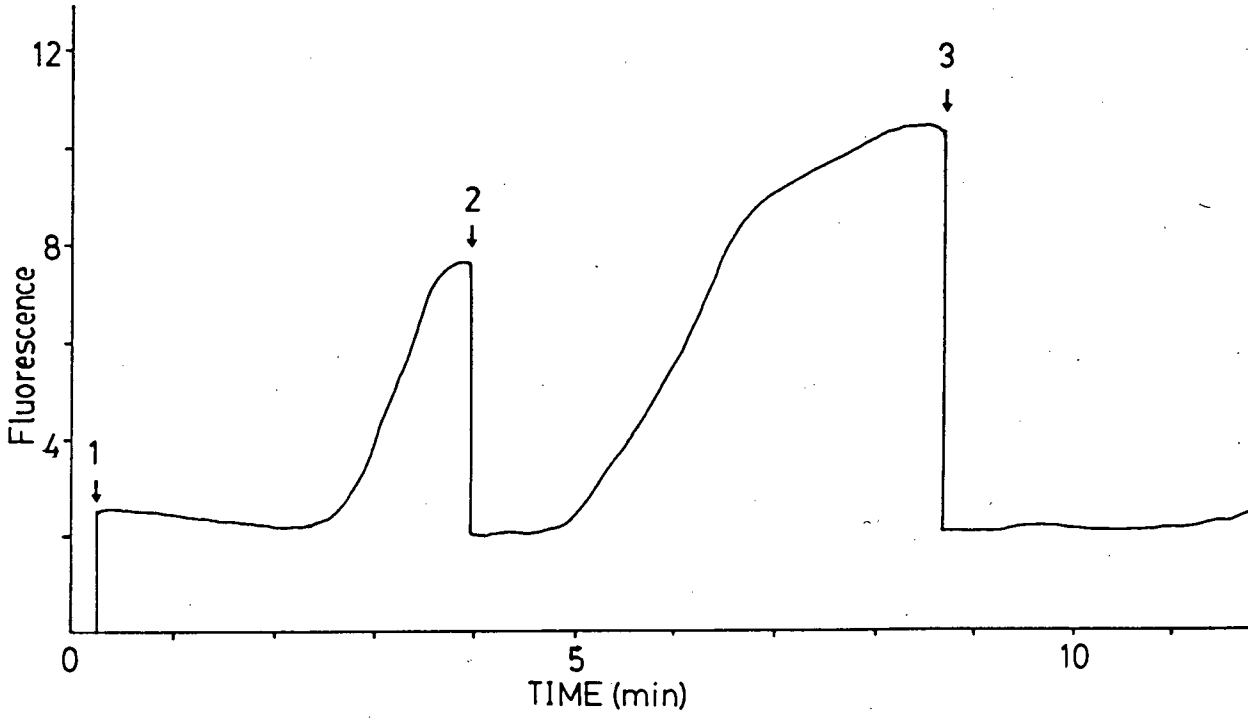

12
8
4
Fluorescence
1
2
3
0
5
10
TIME (min)

Figure 11. 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/ml gentamicin was added. Curve A - cells resuspended in 1 mM KCN in Hepes buffer and held for 10 min prior to NPN addition. Curve B - cells resuspended in Hepes buffer.

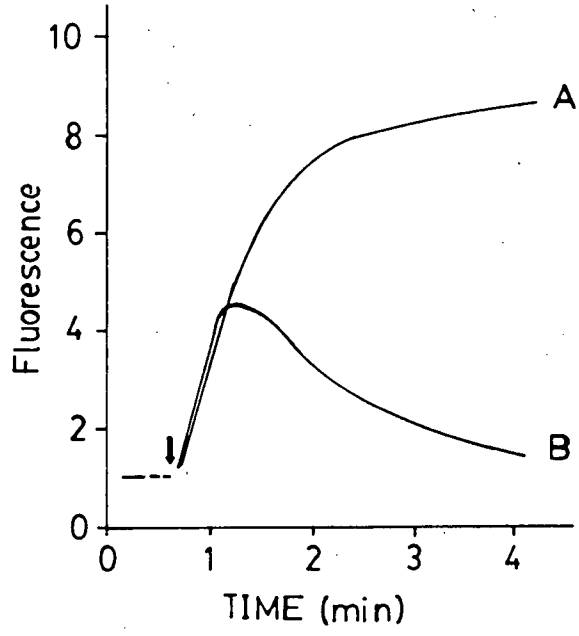
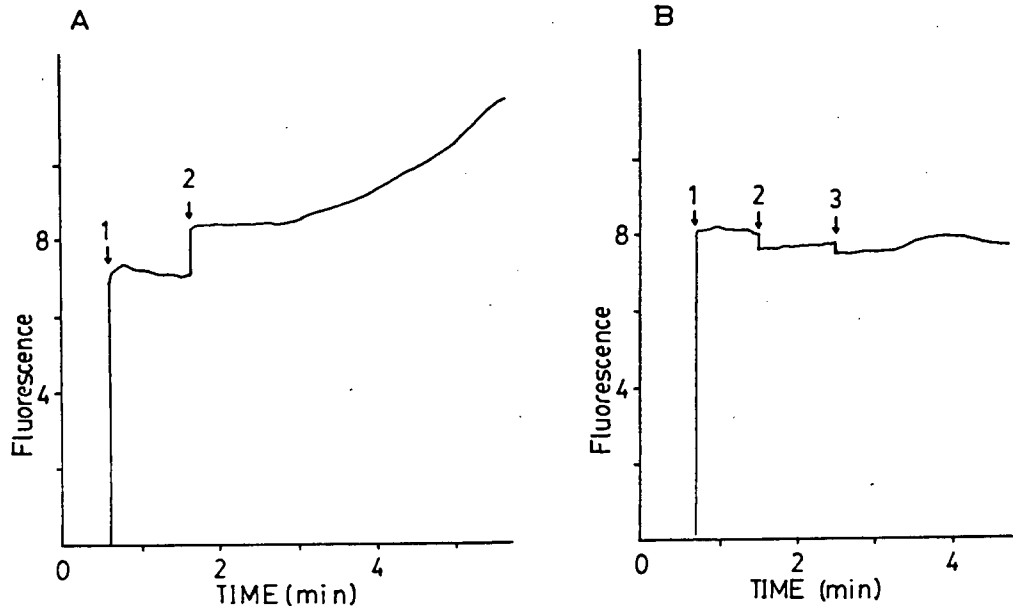

10
8
6
4
2
0
Fluorescence
A
B
0
1
2
3
4
TIME (min)

Figure 12. (A) Gentamicin-promoted increase in NPN fluorescence after addition of NPN to intact cells of P. aeruginosa in the presence of sodium azide. At the arrow labelled 1, H103 cells were resuspended in 2 ml of 5 mM Hepes buffer with 10 μM NPN and 1 mM Na azide. At arrow 2, gentamicin was added to 10 μg/ml.

(B) Inhibition by the addition of divalent cations of the gentamicin-promoted increase in NPN fluorescence of intact cells P. aeruginosa. At the arrow labelled 1, H103 intact cells were resuspended in 2 ml of 5 mM Hepes buffer with 10 μM NPN and 1 mM Na azide. At arrow 2, Mn^{2+}, Mg^{2+} or Ba^{2+} were added to 5 mM. At arrow 3, gentamicin was added to a final concentration of 10 μg/ml.


A
B
1
2
3
8
4
Fluorescence
0
2
4
TIME (min)

this case, was postulated to be responsible for these effects (2). All subsequent experiments were performed wth 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 non-limiting concentration for 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. 8, 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 (Fig. 13). 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 (Fig. 14).

When cells in the presence of 10 μM NPN were excited by light of 350 nm wavelength and the emission at 420 nm 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. 8, curve B). At higher concentrations of gentamicin eg. 20 μg/ml, the increase of NPN fluorescence was rapid and plateaued within 20 sec after a

Figure 13. Excitation and emission spectra of NPN fluorescence in aqueous solution. A solution of 5 μM NPN in 5 mM Hepes was made. Emission and excitation maxima were observed at 460 (A) and 340 nm (B) respectively.

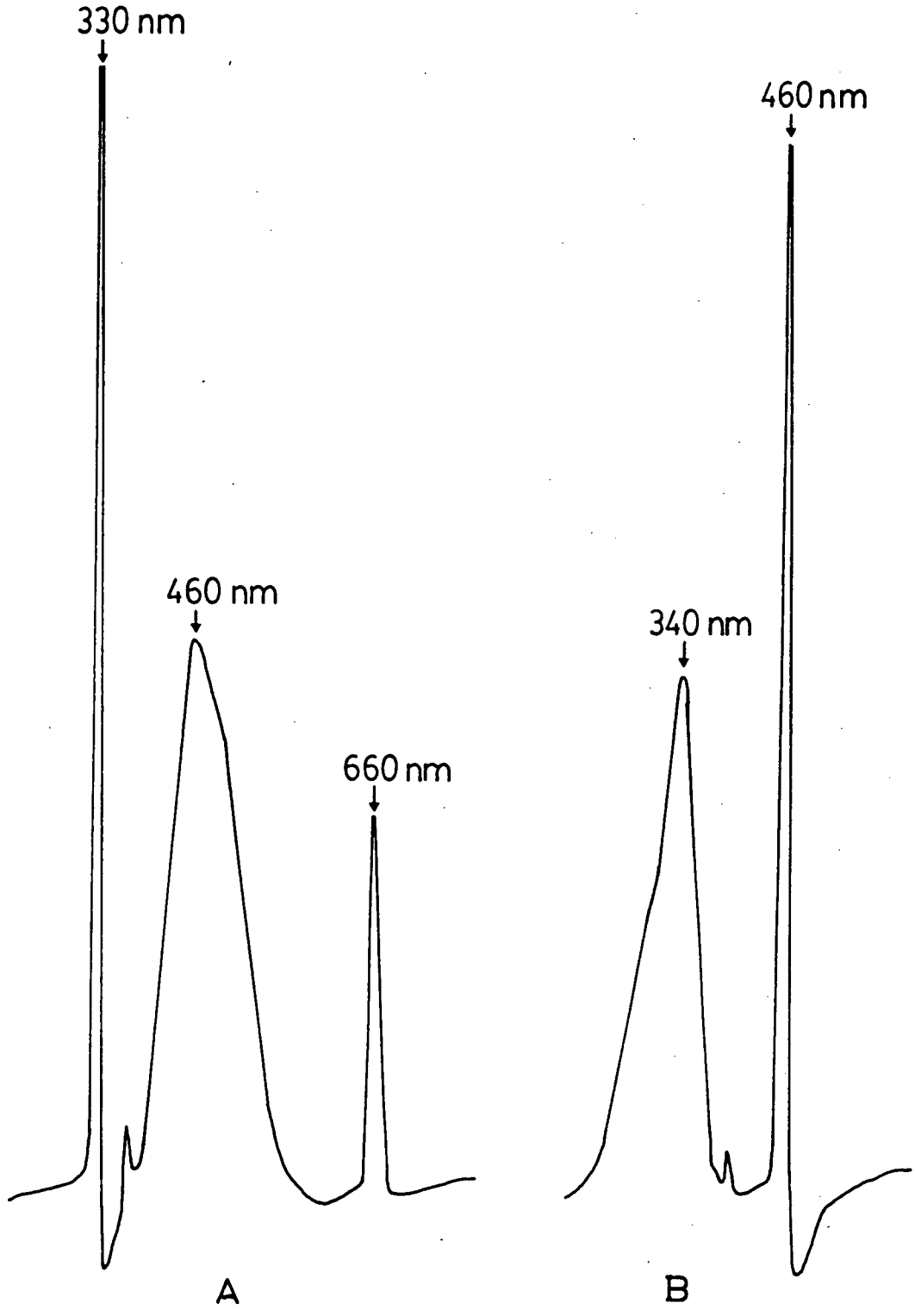
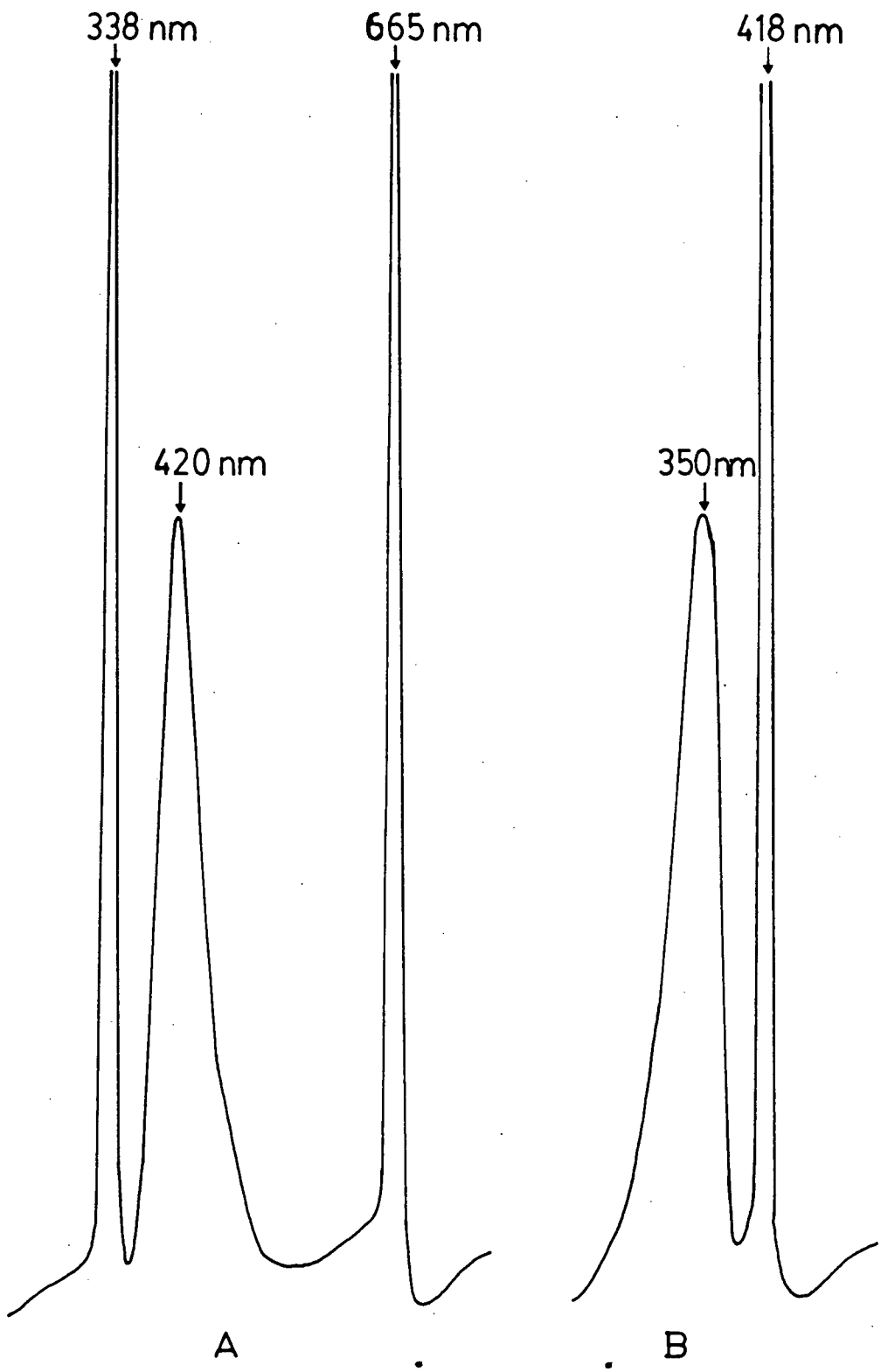

330 nm
460 nm
660 nm
A
460 nm
340 nm
B

Figure 14. Emission and excitation spectra of NPN fluorescence after addition of NPN to a suspension of P. aeruginosa H103 in the presence of gentamicin. H103 cells were resuspended in 5 mM Hepes buffer with 10 µM NPN and 20 µg/ml gentamicin. Emission and excitation maxima were observed at 420 nm (A) and 350 nm (B) respectively.


338 nm
665 nm
420 nm
418 nm
350nm
A
B

4 fold increase in fluorescence emission (Fig. 8, curve A). The fluorescence increase in the presence of 100 µg/ml gentamicin 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 (see Fig. 8, curves A and B).

The addition of the divalent cations, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+} and Sr^{2+} at low levels (eg. 50 µM Mg^{2+}, Fig. 8, curve C; Fig. 15) inhibited the enhancement, by gentamicin, of NPN fluorescence. This effect was observed in both cyanide and Na azide-pretreated cells. Increasing concentrations of 0 - 50 µM Mg^{2+} or Ca^{2+} produced corresponding decreases in the initial rate of NPN fluorescence enhancement (Fig. 16A). 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. 16B). As mentioned above, Ca^{2+} and Mg^{2+} or any of the other cations used, did not themselves cause enhancement of NPN fluorescence in whole cells or phospholipid vesicles.

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 I) suggested that a combination of these two effects was responsible for the observed fluorescence increase. The amount of NPN

Figure 15. Inhibition of the gentamicin-promoted enhancement of NPN fluorescence in intact cells of P. aeruginosa H103 by divalent cations. At the arrow labelled 1, H103 cells were resuspended in 5 mM Hepes buffer with 1 mM KCN. At arrow 2, Mg^{2+} was added to 5 mM. At arrow 3, NPN was added to 10 µM and at arrow 4, gentamicin was added to a final concentration of 20 µg/ml. Similar results were obtained with addition of Mn^{2+}, Ca^{2+}, Ba^{2+} or Sr^{2+} to 5 mM.

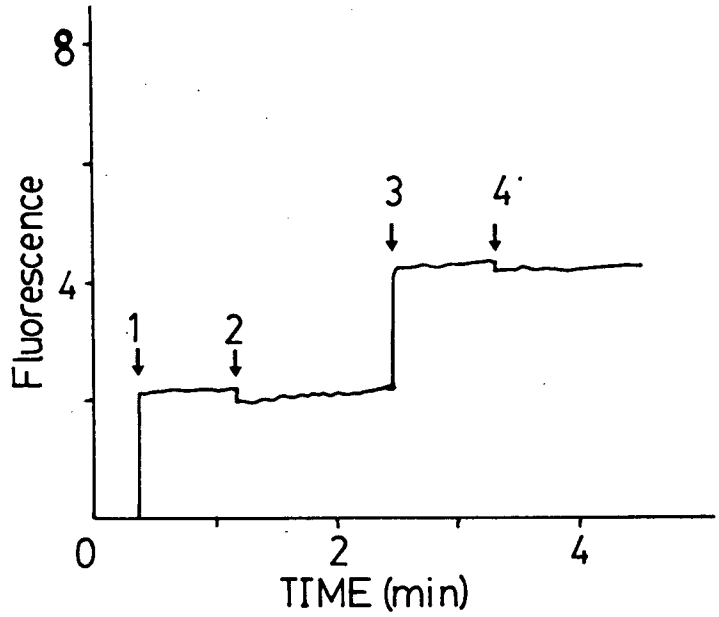

8
4
Fluorescence
1
2
3
4
0
2
4
TIME (min)

Figure 16. 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 Methods. Immediately afterwards 20 µg/ml gentamicin was added to each cuvette and the kinetics of fluorescence increase followed over time. In panel B either 50 µM Mg^{2+} (▲), 40 µM Ca^{2+} (●) or no cation (O) were added prior to the addition of varying amounts of gentamicin to the final concentration given on the X axis. All experiments were performed on cyanide-treated cells.

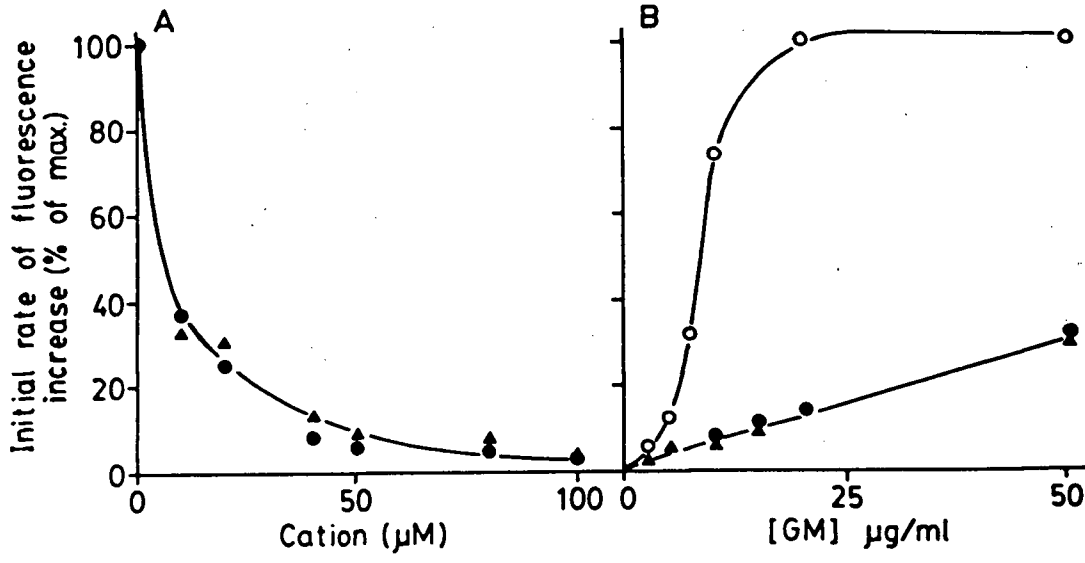

A
B
Initial rate of fluorescence increase (% of max.)
100
80
60
40
20
0
0
50
100
Cation (μM)
0
25
50
[GM] μg/ml

Table I. Influence of gentamicin concentration on the total uptake of NPN and enhancement of NPN fluorescence in P. aeruginosa cells.

Gentamicin Concentration (µg/ml)	Cell bound NPN in Arbitrary Units (µmol 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 µmol NPN taken up by reference to a standard curve constructed by addition of 3% Triton X-100 to different amounts of NPN.

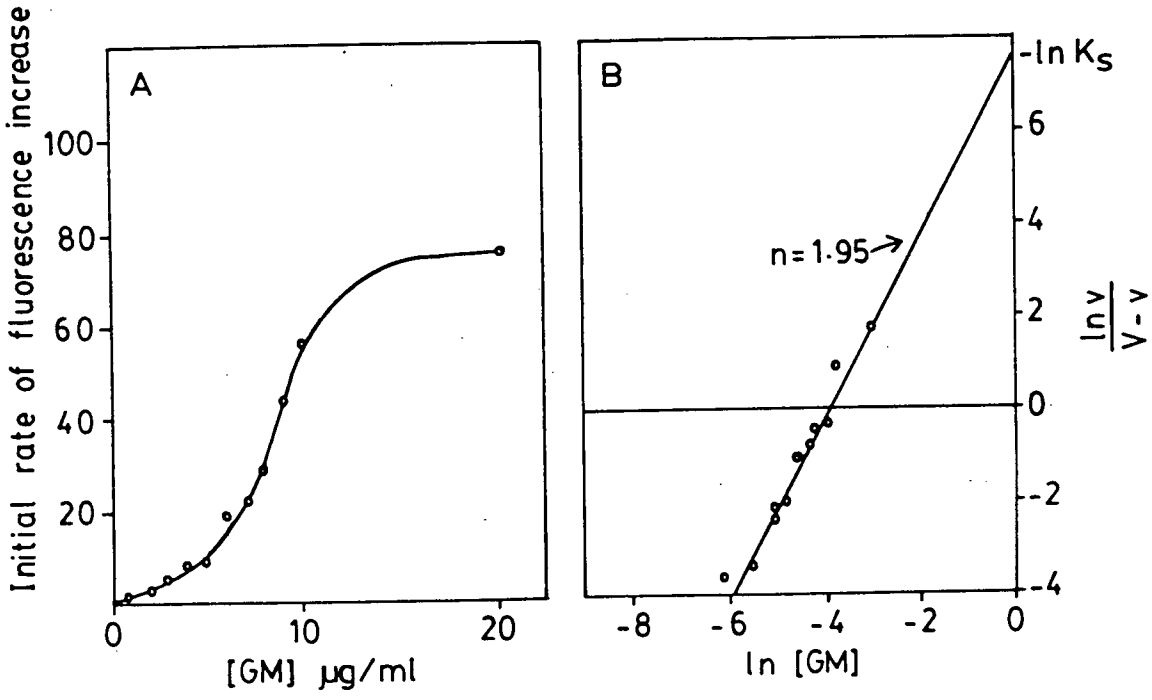
[b]Total fluorescence was the actual increase in fluorescence measured in the 10 min after addition of gentamicin to cells and is the fluorescence intensity of the cell sample after gentamicin addition minus the fluorescence intensity prior to gentamicin addition. [Prior to gentamicin addition, 4 fluorescent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

[c]Calculated as the ratio of total fluorescence (column 3) to cell bound NPN (column 2). This is the increase in the fluorescence of cell bound NPN over and above the expected fluorescence in 3% Triton X-100.

bound to cells before and after gentamicin treatment was determined by a centrifugation technique using Triton X-100. Triton X-100 was previously demonstrated to enhance only the fluorescence of cell-free NPN but had no effect on cell bound NPN (16). In the absence of added gentamicin, approximately 4 fluorescent units of NPN were associated with cells. After addition of gentamicin and sufficient time to allow fluorescence to reach a steady state level, cell-associated fluorescence had increased four to seven fold. 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 (compare columns 2 and 3 of Table I). 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 (see column 4, Table I; N.B. 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 (9). 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, using gentamicin concentrations between 1 and 20 μg/ml, were performed to evaluate how the rate of NPN uptake was affected by the gentamicin

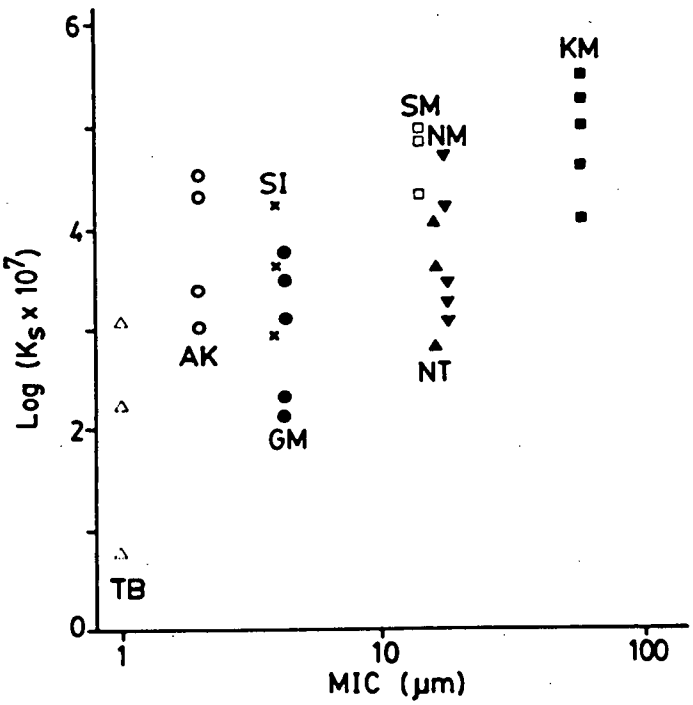
Figure 17. Kinetics of gentamicin promoted increase in NPN fluorescence of intact cells. In panel A the initial rate of fluorescence increase (V expressed in arbitrary fluorescence units per min per mg cell dry weight), taken from a series of experiments like those depicted in curves A and B of Fig. 8, was plotted against the concentration of gentamicin (GM) in μg/ml. All experiments were performed with cyanide-treated cells. This data was reanalyzed according to a Hill plot (panel 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 (n = 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.


A
Initial rate of fluorescence increase
100
80
60
40
20
0
10
20
[GM] µg/ml
B
-ln Ks
6
4
2
0
-2
-4
n=1·95
ln v / V - v
-8
-6
-4
-2
0
ln [GM]

concentration present. We obtained a family of sigmoidal curves of fluorescence increase over time after gentamicin addition (eg. Fig. 8, curves A and B). Due to the complexity of 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. 17A). From this, it appeared that a cooperative interaction had occurred between gentamicin and cells giving rise to the uptake of NPN, ie. the interaction of one molecule of gentamicin with the cell surface enhanced subsequent interactions. The data were replotted as a Hill plot (Fig. 17B) 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 5 experiments) indicating a cooperative interaction with a minimum of 2 - 3 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 (pseudo-association constant) for the interaction of gentamicin with cells, since the Y-axis intercept is -ln Ks. The calculated K_s values were used for relative comparisons of the different aminoglycosides since they varied over 4 orders of

Figure 18. Relationship between the minimal inhibitory concentrations (MIC) of the different aminoglycoside antibiotics and the $\log_{10}$ of the pseudo-association constants K_s, extrapolated from Hill plots such as those shown in Fig. 17. The abbreviations for the aminoglycoside antibiotics and their symbols were: tobramycin = TM (Δ); amikacin = AK (O); sisomycin = SI (x); gentamicin = GM (●); streptomycin = SM (□); netilymycin = NT (▲); neomycin = NM (▼); kanamycin = KM (■).


6
4
2
0
Log (K_S x 10^7)
KM
SM
NM
SI
AK
NT
GM
TB
1
10
100
MIC (μm)

magnitude. A plot of log K_s against the logarithm of the minimal inhibitory concentrations (MIC) for the different aminoglycosides is shown in Figure 18. There were significant variations for each antibiotic in individual determinations of the K_s despite the fact 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 number) 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. 8, curve C; Fig. 15). 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 interaction 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 MIC for different aminoglycosides.

Polarization Studies. Addition of the neutral probe DPH to cells followed by gentamicin addition caused similar kinetics of fluorescence increase as those shown in Fig. 8 for NPN (Fig. 19). The enhancement of NPN (Table I) and DPH fluorescence after gentamicin-promoted uptake suggested that these probes were partitioning into a more fluid environment. To confirm this, we performed fluorescence polarization studies with DPH, which has

frequently been used for such studies. DPH in acetone gave a low polarization value (Table II) since the probe is highly mobile in this solvent (Freifelder, 1982). Decreasing mobility or binding of the probe results in increased polarization (Freifelder, 1982) as demonstrated in Table II for DPH in water and cell-associated DPH in the absence of added antibiotic. In the presence of gentamicin, or another polycationic antibiotic polymyxin B, a decrease in polarization was observed. This suggested that these antibiotics caused DPH to move into a more mobile environment within the cells.

Figure 19. The effect of gentamicin addition on the polarized fluorescence intensity of DPH added to intact cells of P. aeruginosa. Strain H103 cells were resuspended in 2 ml of 5 mM Hepes buffer with 2 µM DPH.

a - fluorescence intensity with the input polarizer set at 90° and the output polarizer set at 0°.

b - fluorescence intensity with th input polarizer set at 90° and the output polarizer set at 90°.

c - fluorescence intensity with the input polarizer set at 0° and the output polarizer set at 90°.

d - fluorescence intensity with the input polarizer set at 0° and the output polarizer set at 0°.

At the arrow, 25 µg/ml gentamicin was added with the polarizers set as for d and the fluorescence increase followed over 12 min.

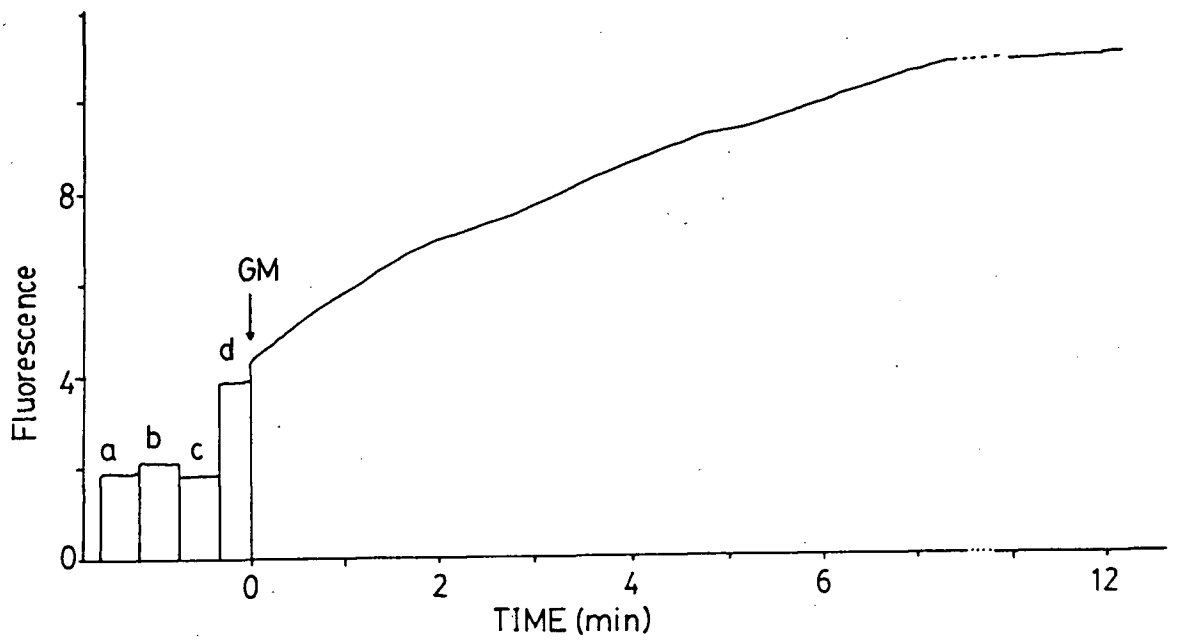

Fluorescence
8
4
0
a
b
c
d
GM
0
2
4
6
12
TIME (min)

Table II: Fluorescence polarization of the DPH taken up after the interaction of gentamicin and polymyxin B with *P. aeruginosa* cells.

Experimental Conditions	Polarization
DPH in water	0.16
DPH in acetone	0.05
DPH cells	0.36
DPH + cells + gentamicin (25 µg/ml)	0.27
DPH + cells + polymyxin B (20 µg/ml)	0.28

DISCUSSION

In this thesis, the effectiveness of fluorescent probes in studying the interactions of AG's with P. aeruginosa cells has been demonstrated. Since experiments with the anionic probe ANS produced rather complex kinetics, such that the probe did not specifically indicate what was happening at the outer surface of P. aeruginosa cells, all subsequent experiments were carried out with NPN. The uptake of NPN that was measured probably reflects outer membrane permeabilization to NPN by amino glycosides. As evidence for this, enhancement of NPN uptake by gentamicin could only be demonstrated in intact bacterial cells. We found that gentamicin did not stimulate uptake of NPN into bacterial outer membrane vesicles, inner membrane vesicles or phospholipid liposomes prepared synthetically. In other words, the increase in NPN fluorescence upon addition of GM was independent of gentamicin concentration. These vesicles became immediately saturated with NPN, which had spectral properties similar to the NPN taken up by gentamicin-treated whole 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 was disorganized by the French Press treatment used to make outer membrane vesicles, and synthetic phospholipid vesicles are simple lipid bilayer liposomes.

In further agreement with this concept, the rate of NPN fluorescence increase was related to the amount of gentamicin added (Figs. 8, 17), although the final amount of NPN associated with cells was relatively

constant. This suggests that NPN enters the bacterial cell via a limited number of access areas, but that NPN uptake, once initiated, proceeds until the sites with which NPN associates are saturated. Presumably the number of access sites for NPN is determined by the concentration of gentamicin in the system.

It was previously proposed in this lab, that the interaction sites for aminoglycosides on the outer membrane of P. aeruginosa are those sites where cations noncovalently crossbridge adjacent LPS molecules (14,15). 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 gentamycin-promoted uptake of NPN (Fig. 8,16). This result 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 published 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.

In further agreement with this both polymyxin B (12) and EDTA (9) cause an increase in the fluorescent intensity of hydrophobic fluorescent probes added to cells. Helgerson and Cramer (9) postulated that EDTA treatment removed the permeability barrier of the outer membrane of E. coli to NPN. This would allow the NPN molecules greater access to binding sites on the cell surface. More recently, it has been shown that polymyxin B interacts with a divalent cation binding site on LPS (17). Schindler and Osborn (17) used fluorescence analysis of dansylated

derivatives of LPS to show that LPS contains one and possibly two types of binding sites for Mg^{++} and Ca^{++}, in the KDO and phosphate groups. Furthermore, it has been shown that polymyxin B and gentamicin each compete with a cationic spin label probe, CAT_{12}, for a divalent cation binding site on P. aeruginosa LPS (AA Paterson, REW Hancock, EJ McGroarty, 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 initial rates of fluorescence increase as a function of gentamicin concentrations gave a sigmoidal plot suggesting positive cooperativity. To confirm this, we replotted the data as a Hill plot. The advantage of this 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], has no units and thus is independent of the arbitrary fluorescent units. Thus, assuming that the rate of NPN uptake directly reflects the interaction of gentamicin with outer membranes, which seems likely, the Hill plot provides kinetic data which reflect only this interaction. Similar arguments are valid for each of the eight aminoglycosides subjected to kinetic analysis by this method. In Hill plot analyses, of all eight aminiglycosides incorporating 30 separate sets of data, the Hill number (the slope of the Hill plot) was 2.26 ± 0.26. Since this number provides an estimation of the minimum number of interaction sites involved in the cooperative permaeabilization of outer membranes to NPN, we can make the assumption that at least three or more sites are involved.

While the Hill numbers were remarkably similar for each of the 8 aminoglycosides, it was interesting that the pseudoassociation constant Ks varied substantially. The highly significant ($p < 0.001$) linear relationship between the Ks 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 and/or rate of killing. In agreement with this, Mg^{++} and Ca^{2+} which strongly antagonize the transport of and killing by aminoglycosides (1) also significantly inhibited the enhancement of NPN uptake by aminoglycosides (Fig. 8, curve C, 16). 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 following the treatment of cells with aminoglycosides strongly mimicked the time course of aminoglycoside uptake (1,7).

Our experiments also suggested that the increase in NPN fluorescence after gentamicin addition to cells was due to a combination of NPN being taken up into the cells and (subsequently) an alteration in the environment of NPN molecules which enhanced its fluorescence yield. Furthermore, polarization studies with DPH, another neutral fluorescence probe, confirmed that the presence of gentamicin in a cell suspension caused the probe molecules to partition into a more highly mobile or fluid environment within the cells, as shown by a decrease in polarization value. Alternatively this decrease in polarization might be explained by

an alteration in fluorescence lifetime although we could not measure this on our apparatus.

Although this does not formally demonstrate that aminoglycosides interact with outer membranes to promote their own uptake, we have described the enhancement after gentamicin treatment of the uptake of hydrophobic probes NPN and DPH, and previously of the protein lysozyme and ß-lactam antibiotic nitrocefin (8). This data is thus consistent with the self-promoted uptake hypothesis (7,15). To further test and prove this hypothesis, it will be necessary to learn more about the events following the initial interaction. Attempts to fluorescently tag the aminoglycoside antibiotics directly are currently underway for this reason.

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