12-1988

Early Events of Lethal Action by Tobramycin in *Pseudomonas aeruginosa*

Jane Elizabeth Raulston
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Jane Elizabeth Raulston entitled "Early Events of Lethal Action by Tobramycin in *Pseudomonas aeruginosa*." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Thomas C. Montie, Major Professor

We have read this dissertation and recommend its acceptance:

Leaf Huang, Gary Sayler, Robert Moore

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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EARLY EVENTS OF LETHAL ACTION BY TOBRAMYCIN IN PSEUDOMONAS AERUGINOSA

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Jane Elizabeth Raulston

December 1988

DEDICATION

This dissertation is dedicated in memory of my grandfather, James Wilbur Hunnicutt, who journeyed on to meet our Lord shortly after I began my studies at the University of Tennessee. Few people have had as tremendous an influence in my life as has "Daddy Jim." His courage and his stubborn determination to transcend any of life's obstacles, while maintaining hope that the best is yet to be, are just two of the many admirable legacies he left behind. But more important than these, his kindness, his patience, his devotion, and especially his love to his family was unsurpassable, and I am extremely fortunate to have witnessed his example.

"And now these three remain: faith, hope and love. But the greatest of these is love."

1 Corinthians 13:13

"I miss you...and I love you deeply, Daddy Jim."

ACKNOWLEDGEMENTS

I would like to acknowledge my committee members, Drs. Robert Moore, Leaf Huang, and Gary Sayler for their helpful criticisms and guidance. A special thanks to Dr. Moore for his help in my job search. As for my major professor, Thomas C. Montie, I feel that I could not have chosen a better person under whom to study. His time, his guidance, his patience, and his dedication, are deeply appreciated. I will miss and always value his friendship.

The people whom I worked with in this lab have helped to ease the burden along the way, and make this experience worthwhile. David Drake and Janice Allison deserve special credit for helping me get off to a good start, and I will always appreciate their friendship and much needed encouragement. Many cherished memories will never be forgotten from the time that I have spent with Tina Anderson and Kim Kelly. With them, each day was always far from being "uneventful" or "routine." Each will always have a very special place in my heart. Jerry Williams, Jr., Linda Summitt, and Robyn Walker deserve recognition for their wonderful assistance in this project. I would also like to give special thanks to Sherry Dougherty for her friendship.

Over the years, many wonderful weekends have been spent with the Fleming family; I can't thank them enough for their love and support. As for Teresa Fleming, it is best stated that few people are as fortunate in their lifetime to find as loving, caring, and as dedicated a friend as I have found in her.

I could elaborate for many pages about how much each member of my family has meant to me. The times spent together during our annual beach vacations, Thanksgiving, and Christmas holidays are particularly invaluable to me. I am indebted to each of my grandparents, James and Mabel Hunnicutt, and, Clarence and Helen Raulston, for their wonderful love, constant support, and their confidence in me. I am very proud of each of my brothers, Keith, Eric, and Jonathan. I only hope that I am able to give them as much joy, love, and self-assurance that they have so willingly given to me. Finally, my parents, William ("Bill") and Jane Raulston, in each their own way, are nothing short of angels from heaven. Without them, I couldn't possibly have experienced as rich or as wonderful a life. Because of their influence and their love, I have always known that there is nothing in this life I couldn't accomplish if I worked for it. For this priceless gift, I love them dearly.

ABSTRACT

The immediate activities of the aminoglycoside antibiotic, tobramycin, were investigated in Pseudomonas aeruginosa PAO1. The influence of carbon growth substrate and the antibiotic exposure environment in the magnitude of activity were examined. Lethality by 8 ug/ml tobramycin occurred rapidly (1 to 3 minutes). The release of specific cellular components into the supernatant was associated with lethality. This material was initially detected as an increase in UV-absorbance. Magnesium in the reaction mixture provided protection against lethality and leakage, but did not reverse lethal damage after a 3 minute tobramycin treatment. Also, uptake of ^{3}H-tobramycin was reduced in the presence of magnesium. Cells grown with glucose as a carbon source were more susceptible than organic acid grown cells as was the rapidity and amount of cell damage. Analyses of the leakage material revealed a 2-fold increase of protein in the supernatant after a 1-3 minute treatment which paralleled lethality. A prominant 29kDa protein was observed by SDS-PAGE in the released material, which has been identified as the periplasmic enzyme, β-lactamase. Tobramycin also elicited an increase of certain amino acids in the supernatant, particularly, basic amino acids. The immediate activities of tobramycin did not involve i) release of overall cell protein, ii) massive loss of total pool amino acids, iii) cell lysis, iv) inhibition of proline uptake, v)

release of lipopolysaccharide, or vi) leakage of ATP. Electron microscopy showed no apparent damage after a 3 minute exposure. Forty percent inhibition of protein synthesis had occurred by 3 minutes of exposure, while release of UV-absorbing material and lethality were detectable after only 1 minute. These data suggest that leakage occurs at least simultaneously, if not prior, to ribosomal interference. Resistant cystic fibrosis isolates of P. aeruginosa did not leak under the same experimental conditions, but one of two susceptible strains examined did show increased UV-absorbance following treatment.

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ATP	adenosine triphosphate
BHI	brain heart infusion
BSA	bovine serum albumin
CF	cystic fibrosis
CFU	colony forming unit
2-DAM	2-deoxystreptamine
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetate
FC	Folin Ciocalteau
HPLC	high pressure liquid chromatography
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
MH	Mueller Hinton
MIC	minimal inhibitory concentration
MSM	mineral salts medium
PMF	proton motive force
SBD	standard bacterial diluent
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
TLC	thin layer chromatography

CHAPTER I

INTRODUCTION

Pseudomonas Virulence and Antibiotic Treatment

P. aeruginosa is a pathogen in patients suffering from severe burn injuries, cystic fibrosis (CF), or immunosuppression. The pathogenicity is attributable to several properties of the organism. In addition to the most extensively studied virulence factor, exotoxin A, other virulence factors include exotoxin S, extracellular protease, adherence, and motility (OGaard et al.,1986). *Pseudomonas* also exhibits an exceptional degree of resistance towards antibiotics. Seven possible modes for antibiotic resistance in bacteria have been outlined in a recent review (Davies, 1986);

(i) the alteration or inactivation of the antibiotic itself,

(ii) alteration of the target site(s),

(iii) blocking the transport of the antibiotic,

(iv) by-passing the antibiotic sensitive step (by replacement),

(v) increasing the levels of the target enzyme (titration of the drug),

(vi) cell is spared the antibiotic-sensitive step by an endogenous or exogenous product, and

(vii) the production of a metabolite that antagonizes the action of the inhibitor.

An additional resistance mechanism involving active efflux of antibiotic has been described (McMurry et al., 1980). Resistant isolates exhibit one, or a combination of these properties, and regulation of these mechanisms is poorly understood (Davies, 1986).

The aminoglycoside antibiotics are currently among the most successful therapeutic agents used in the treatment of Pseudomonas (A.S.H.P., 1983; Price, 1986). They have several desirable properties in that their inhibitory activity is effective toward a large inoculum of bacteria, their action is usually rapid and completely bactericidal, the pharmacokinetics are predictable and dose-proportionate, and they appear to act synergistically with other antibiotics such as the β-lactams (Price, 1986; Scudamore and Goldner, 1982). One drawback is that aminoglycosides do exhibit some toxicity, such as ototoxicity and nephrotoxicity, in a small percentage of patients (A.S.H.P., 1983; Penketh et al., 1983; Price, 1986).

The current increase of aminoglycoside resistant P. aeruginosa in CF patients is alarming. Patients who become colonized are unable to clear the organism despite a potent immune response (Pier et al., 1986; Hoiby et al., 1975). Antimicrobial chemotherapy, in some cases, allows for considerable clinical improvement (Penketh et al., 1983).

Therefore, the benefits of aminoglycoside treatment outweigh the risks. Unfortunately, the drugs must be administered in high daily dosages, eliciting toxic side effects (Pederson et al., 1987).

Because of a substantial amount of successful treatments, researchers retain these antibiotics as ready components against gram negative infection. Recent emphasis has been placed on regulating dosage and development of safer analogs of this class. As the name implies, aminoglycoside antibiotics are composed of an aminocyclitol nucleus to which one or two amino sugars are glycosidically linked. The commonly known streptomycin differs from amikacin, gentamicin, kanamycin, neomycin, paramomycin, and tobramycin, in that it has a streptidine nucleus. The latter antibiotics possess a 2-deoxystreptamine (2-DAM) nucleus (A.S.H.P.,1983).

Effects of Aminoglycosides on Protein Synthesis

One of the most prominent original studies, regarding ribosomal activity, was published in 1965 (Davies et al.). In an *in vitro* translational assay utilizing 70S ribosomal fractions from *Escherichia coli*, Davies demonstrated that streptomycin, as well as the 2-DAM antibiotics neomycin, kanamycin, paromomycin, gentamicin, hygromycin B, and viomycin, induce misreading due to incorrect amino acid incorporation. Several studies since have supported these findings. However, a more recent observation suggests that the general mode of action for the 2-DAM

aminoglycosides is the inhibition of ribosomal translocation (Ahmad et al., 1980). Davis et al. (1986) attempt to resolve this issue by proposing an initial misreading function by smaller amounts of antibiotic, leading to incorporation of altered protein in the cell envelope, which ultimately results in a high amount of antibiotic influx and blockage of ribosomal translocation.

A particularly striking finding was made by Buckel et al. in 1977. It had been commonly accepted that aminoglycoside activity was restricted to the 30S ribosomal subunit. Buckel isolated gentamicin resistant mutants by exposure to ethylmethanesulfonate and obtained ribosomal profiles of these mutants. Results consistently showed that these isolates possess only one altered protein (L6), which belongs to the 50S ribosomal subunit. More recently, Tanaka et al. (1983) demonstrated that habekacin binds to both ribosomal subunits. Such studies indicate that there are multiple sites of action for aminoglycosides on the ribosome. Furthermore, there is evidence that the effects on protein synthesis are specific for a given aminoglycoside antibiotic.

Aminoglycoside Transport Kinetics

The transport kinetics of aminoglycosides have been studied by L. E. Bryan (1977). By assaying the uptake of radiolabelled streptomycin (and in some instances, gentamicin), under varying conditions known to alter membrane functions, Bryan has proposed

a three-phase mechanism. First, the interaction and uptake of aminoglycosides across the outer membrane is an energy-independent, physical process. Passage through the inner membrane is energy-dependent. Finally, ribosomal binding also appears to be an energy-requiring step. Hancock and collaborators (1981b) have shown that lysozyme enters the cell in the presence of gentamicin, irregardless of the presence of potassium cyanide. The uncouplers dinitrophenol and sodium azide also made no difference in permeability. In agreement with Bryan, Hancock concluded that outer membrane permeation does not require an energized membrane.

Role of the Outer Membrane, Cationic Charge, and Porin in Antibiotic Activity

The interactions of antibiotics with the cell surface of microorganisms have only recently been considered a relevant activity. Because of the rising number of "membrane resistant" strains, the past decade has experienced increased research on this particular aspect coinciding with an increase in the understanding of membrane structure. The availability of certain probes, such as the quantitation of β-lactamase by colorimetric methods, initiated membrane studies. One of the first studies, directed at membrane penetration of β-lactams in E. coli, showed that the location of β-lactamase is responsible for the protection of the bacterium (Zimmerman and Rosselet, 1977).

One study conducted with E.coli, Proteus mirabilis and Enterobacter cloacae, involved assessment of antibacterial activity by cephalosporin and penicillin (Sawai et al., 1982). Mutants were obtained possessing little to no porin (major transmembrane protein), and their resistance profiles were compared. The results revealed that mutants lacking porin are highly resistant to cephalosporins but not to penicillins. This indicated that porin protein is a major route of uptake. However, because there are differences in uptake of structurally related compounds, it is apparent that there are delicate differences among the functional properties of membrane proteins. In addition, it has been suggested that the nucleus of a particular antibiotic plays an important role in permeation. Sawai et al. (1982) also showed that there are some differences in the magnitude of antibiotic resistance between gram negative organisms. These differences are attributed to the fact that outer membrane protein, as well as secreted protein, differ amongst the genera. More recently, Godfrey and Bryan (1987) reported that porin is the limiting factor for penetration of β-lactam antibiotics. They additionally delineated differences between organisms by showing that E. coli and Salmonella typhimurium pores allow passage of neutral molecules up to 600 daltons, whereas P. aeruginosa porin take up molecules as large as 9,000 daltons. Overall, the literature suggests that in gram negative bacteria, in consideration of a wide variety of antibiotics, porin-mediated uptake appears to be a

major, and, perhaps, most commonly used route (Katsorchis et al., 1985; Jaffe et al., 1982; Werner et al., 1985; Yoshimura and Nikaido, 1985).

The idea that antibiotics diffuse through aqueous pores in the outer membrane, has been extended to the aminoglycosides. Some authors propose that this is the primary mode of entry. Nakae and Nakae (1982) utilized proteoliposomes and a liposome swelling technique to arrive at this conclusion. Mutant E. coli strains produced only three to four percent of porin in comparison with the wild type. Interestingly, the resistance profiles of these strains did not differ from that of the wild-type. Nakae and Nakae, thus, hypothesized that uptake is porin-mediated, and must be a highly efficient mechanism. Under their assay conditions, the diffusion of uncharged saccharides such as ribose, hexoses, dissaccharides, and raffinose, were observed using liposomes reconstituted with or without porin. The passage was size-dependent. The exclusion limits of the porin pores appearred to be much less than the molecular weights of the aminoglycosides. Their explanation for the uptake of these cationic compounds, is that, perhaps, the environment of the pore wall favors the passage of cations, or, conversely, that the porin opens and closes dependent upon the immediate environment.

Although studies of aminoglycosides have focused upon the role of porin-mediated uptake, apparently many other possible uptake mechanisms exist. Interactions with lipopolysaccharide

(LPS), diffusion through the phospholipid bilayer, crack formation in the membrane, passage through abnormal protein channels, and perhaps a yet unknown uptake pathway have also been proposed (Davis, 1986; Day and Day, 1982; Godfrey et al., 1984; Hancock et al., 1981b; Hancock and Wong, 1984; Katsorchis et al., 1985; Peterson et al., 1985). Because many membrane studies utilize E. coli as the model organism, it is important to note that the membrane of Pseudomonas differs significantly from that of the Enterobacteriaciae (Mizuno and Kageyama, 1979). Therefore, any proposed mechanism may or may not apply to all gram negative organisms. Likewise, evidence suggests that any mechanism proposed for a specific antibiotic may or may not be applicable to the complete class of related compounds (Davies, 1986).

Less is known about the outer membrane of P. aeruginosa compared to E. coli. The classification scheme of the outer membrane protein profile of P.aeruginosa is confusing, in part, because of differing isolation techniques and gel systems (Mizuno and Kageyama, 1978). Several authors have identified at least six major proteins: D (~50kDa), E (~45kDa), F (~33kDa), G and H (~21kDa), and I (~8kDa) (Brown et al., 1984; Mizuno and Kageyama, 1978, 1979). Protein I resembles Braun's lipoprotein in E. coli, and is the only protein considered analogous in both E. coli, and Pseudomonas (Hancock et al., 1981a; Mizuno and Kageyama, 1978, 1979).

Two other commonly encountered classification schemes for

P.aeruginosa exist in the literature. In many cases, only two proteins, A (~43kDa), and B (~16.5kDa) are acknowledged. Elsewhere, the following classification scheme has been reported: I (~56kDa), II (~53kDa), III (~38kDa), IV (~21kDa), and V (~16kDa). It is important to note that the outer membrane protein composition is strongly influenced by the growth media, and many of these proteins are modifiable by external elements such as heat (Hancock and Cary, 1979; Mizuno and Kageyama, 1978). Using various isolates, it is known that most outer membrane's possess a small number of specific proteins present in large amounts (Hancock and Nikaido, 1978).

Nicas and Hancock (1980, 1983a) have examined the outer membrane profile of P. aeruginosa grown in magnesium-deficient medium. A protein designated as H1 was overproduced in this environment. Interestingly, this isolate simultaneously acquired resistance towards the antibiotics polymyxin B, colistin, and gentamicin. Resistance to permeabilization by ethylenediaminetetraacetate (EDTA) was also observed. The role of magnesium (and other divalent cations such as calcium), has been proposed to be a key factor in the stabilization of the outer surface of gram negative bacteria (Gilliland et al., 1974; Nicas and Hancock, 1980; Vaara and Vaara, 1981). Growth in a magnesium-deficient medium results in structural and chemical differences in Pseudomonas (Nicas and Hancock, 1980). Such conditions reveal that cell envelope phosphorous levels decrease,

and the amount of carbohydrate and 2-keto-3-deoxyoctonate (KDO, a core component of the surface LPS) increases. Although the protein profiles alter (i.e. change in molecular weight), no quantitative differences exist.

The polycationic antibiotics have been observed to exhibit antagonism with magnesium (Nicas and Hancock, 1983a; Warren et al., 1985). It has been suggested that magnesium is readily displaced by gentamicin. A 1,000-fold molar excess of magnesium is required for protection against gentamicin, indicating that the affinity of the cell surface for gentamicin is much greater than that for magnesium. Measurements of magnesium levels in isolated outer membrane preparations suggest that as protein H1 increases, the amount of magnesium decreases, and this decrease in turn, limits possible binding sites for polycationic antibiotics. Nicas and Hancock (1983a) hypothesize that the outer membrane protein H1, when produced, displaces magnesium binding sites and protects the surface. Alternatively, H1 may itself be a magnesium binding protein which will also bind these antibiotics.

In a subsequent study, Nicas (1983b) isolated and used mutants which overproduce H1. The results seem to confirm that resistance to chelators and cationic antibiotics is closely correlated with the presence and amounts of protein H1. The absolute levels of divalent cations present in the outer membrane do not appear to be as significant.

The general effects of polycations on bacteria have been

studied elsewhere. Vaara and Vaara (1981, 1983a, 1983b) report that at least two different types of outer membrane properties exist in the presence of different cations. Hydrophobic probes, which are fluorescently labelled or radiolabelled have been commonly utilized to assess the permeability properties of phospholipid bilayers (Loh et al., 1984; Vaara and Vaara, 1983b). These probes, in addition to approaches such as sensitivity to sodium dodecyl sulfate, and electron microscopy, have been used by Vaara and Vaara (1981, 1983a, 1983b) to establish membrane effects by a $lysine_{20}$ peptide polymer and polymyxin B nonapeptide (deacylated polymyxin B). Each polycationic agent yielded a different type of activity. $Lysine_{20}$ peptide caused the release of 20-30% LPS from Salmonella typhimurium's outer membrane, sensitized it to anionic detergents, induced long, finger-like projections from the surface, but did not allow permeation of the hydrophobic probes. Conversely, polymyxin B nonapeptide allowed for the intercalation of probes, but did not cause release of LPS or allow for the lethal activity of SDS. In addition, polymyxin B nonapeptide caused sensitization of gram negative organisms to hydrophobic antibiotics. Electron microscopy revealed a wrinkled surface, rather than projections. In a more recent study involving P. aeruginosa, gentamicin was observed to induce a wrinkled outer membrane surface as well as produce holes through the membrane (Martin and Beveridge, 1986). These authors also quantitated the amount of LPS and reported it to be released following 30 minutes

exposure to gentamicin. The data taken together suggest that gentamicin expresses at least two different functional modes of activity which are common for both the lysine peptide and polymyxin B.

In addition to protein H1, studies by the Hancock group have been extended to other properties of the outer membrane proteins with respect to antibiotic activity. In a magnesium deficient medium, the major Pseudomonas porin (protein F), does not appear to be altered (Hancock et al., 1982, 1981b). In contrast with the antibiotics carbenicillin and tetracycline, which are thought to utilize protein F as the major uptake route, it has been tentatively stated that porin is probably not involved in aminoglycoside or polymyxin uptake (Hancock et al., 1981b). Another finding by this group is in direct contradiction in stating that porin number is important in polymyxin uptake (Hancock et al., 1979). Many of their studies have been directed at the general permeabilization effect of polycationic antibiotics (Hancock and Wong, 1984). For example, lysozyme is generally ineffective in the lysis of gram negative organisms because of the protection afforded by the outer membrane. However, the addition of polycationic antibiotics to P. aeruginosa allows lytic activity by lysozyme, as measured by a decrease in absorbance at 600nm.

Hancock has proposed a theory described as "the self-promoted uptake pathway" for aminoglycosides and polymyxins (Hancock et al., 1981b; Nicas and Hancock, 1983b). The

basis for the theory is that if polycationic antibiotics are capable of promoting the transport of molecules such as lysozyme, they must also be capable of promoting their own transport through the outer membrane. Since Pseudomonas LPS is known to have a high phosphate content, it is suggested that the initial interaction is ionic and is with LPS. In a site-specific study of E. coli K12 with metallic ions, it has been shown that a high affinity for cations exists at the LPS, and that it can be attributed to phosphoryl substituents and not free carboxyl groups (Ferris and Beveridge, 1986).

Following some initial studies of the influence of biochemically altered LPS in mutants by Kropinski et al. (1982), it has been suggested that porin-mediated uptake is influenced by the interactions of porin protein with LPS. These may be ionic interactions of LPS phosphate with protein, or hydrophobic interactions of lipid A residues with acyl chains, or both. In turn, these interactions may cause an opening and closing mechanism of porin protein. Polysaccharide diffusion studies (Angus et al., 1982; Godfrey et al., 1984; Yoneyama and Nakae, 1986), in addition to studies with porins implanted into black lipid bilayer membranes (Hancock et al., 1982; T. C. Montie, personal communication), have resulted in the overall conclusion that P. aeruginosa has large transmembrane porin protein which remains in a closed state most of the time. Molecular seiving has also been suggested by Godfrey and Bryan (1987).

Studies utilizing the black lipid bilayer system have been under heavy criticism as a feasible model for comparisons with intact cells (Yoneyama and Nakae, 1986). This system consists of a Teflon chamber, separated into two chambers by a divider with a small hole in the center. Across this opening, a solution of oxidized cholesterol in *n*-decane is painted. Upon bilayer formation, the membrane turns black to incidental light. Protein can be added to the membrane and conductivity across the membrane (through porin) can be assessed (Hancock et al., 1982). Data indicate the exclusion limit of P. aeruginosa porin protein F, in relation to the diffusion of uncharged polysaccharides, to be approximately 6,000 daltons, and the cross-sectional area to be three times larger than that of E. coli porin (Hancock et al., 1982; Nicas and Hancock, 1983b). Also, studies of porin F and an additional porin P (induced under conditions of low phosphate) suggest that P. aeruginosa porins favor diffusion of cations two to four times over anions (Hancock et al., 1982).

The assumption that P. aeruginosa harbors large pores with exclusion limits of several thousand daltons, has been challenged by Yoneyama and Nakae (1986). They have examined the diffusion of pentoses, hexoses and several uncharged saccharides and disaccharides in plasmolyzed cells. The results show that pentoses and hexoses are able to diffuse, but uncharged saccharides having a molecular weight larger than 342 are impermeable. In conclusion, they state that the exclusion limit of

the major porin protein in P. aeruginosa is less than the size of hydrated sucrose. This is ten to twenty fold less than that reported from the black lipid bilayer studies, and is to their knowledge, the smallest limit yet reported among gram negative organisms. Electron microscopic observations support this interpretation.

The concept of opening and closing pores to explain permeability differences is not acceptable to some researchers. Yoneyama and Nakae (1986) state that an open-close mechanism has not been investigated in situ, and the black lipid bilayer results are an artifact....only a reflection of the system itself. It is a well-recognized observation that the low-molecular weight antibiotics such as penicillins, cephalosporins, chloramphenicols, and tetracyclines diffuse poorly through the outer membrane of P. aeruginosa. If large pores are present, it is not clear why these compounds don't permeate more rapidly. Hancock has stated (personal communication) that recent results indicate a small number of large pores and a large number of small pores exist.

Role of Lipopolysaccharide

The role of LPS in antibiotic interaction has been studied by several researchers (Bryan et al., 1984; Day and Day, 1982; Godfrey et al., 1984; Hancock et al., 1983, 1981b; Katsorchis et al., 1985). It's expression on the outer surface of P. aeruginosa appears to be complex; influenced by the environment as well as

the phase of growth. Day and Day (1982) mentioned that the LPS of P. aeruginosa differs significantly from the LPS of other gram negative organisms. The core is unusually rich in phosphorous, particularly because the inner region heptose residues are highly phosphorylated. Similar to observations obtained with other gram negative bacteria, P. aeruginosa LPS is released spontaneously into the environment under normal growth conditions (Cadieux et al., 1983).

In general, P. aeruginosa aminoglycoside resistant strains have altered surfaces due primarily to changes in LPS (Katsorchis et al., 1985). Bryan et al. (1984) state that "low level" impermeability-type aminoglycoside resistance in P. aeruginosa, results from conversion of smooth LPS to superficial or deeper rough LPS phenotypes. In summarizing low-level resistance, Bryan outlines that, i) P. aeruginosa clinical isolates do not have inactivating enzymes or ribosomal mutations to account for this resistance; ii) the resistance cannot be transferred by conjugation; iii) this property is not restricted to Pseudomonas, because Klebsiella pneumoniae is also shown to exhibit this type of resistance; and, iv) the resistance is stable and is maintained in the absence of antibiotics. Our observations with CF strains are consistent with Bryan's results. In contrast, Moore et al. (1984) demonstrated that low-level resistance toward polymyxins is reversible and cannot be maintained. Interestingly, Gilleland et al. (1988) reported that low level aminoglycoside resistance is

unstable in the absence of antibiotic, and that the type of resistance observed was either specific or cross-resistant, indicative of strain-specificity.

In Bryan's comparative studies using gentamicin (1984), he found that the expression of protein H1 is no different in resistant P. aeruginosa than in susceptible isolates. Recent studies by Gilleland et al. (1988) support Bryan's conclusions. These finding are in direct conflict with Nicas' (1980) studies mentioned earlier. Bryan believes that the differences in the amount or type of outer membrane protein could not be correlated with susceptibility or resistance.

To investigate the possible contributions by LPS in antibiotic susceptibility, Godfrey et al. (1984) approached the problem by using permeability mutants and correlating such mutations with changes in LPS structure. Results show that P. aeruginosa LPS structure and composition influences the activity of hydrophilic antibiotics, specifically the β-lactams. Peterson et al. (1985) examined the binding of antibiotics to P. aeruginosa LPS by displacement of a cationic spin probe. Their findings indicate that probe motility within the LPS head group decreases in the presence of cationic antibiotics. They believe a resultant rigidity disrupts the conformation of the outer membrane which leads to drug penetration. From another perspective, Moore et al. (1986) have studied the LPS binding kinetics of dansyl polymyxin, a fluorescent derivative of polymyxin B. The results reveal multiple

binding sites present on LPS. Aminoglycosides, magnesium, and other polycations are able to compete for some of the sites, but not all.

The vast majority of studies with the polycationic antibiotics have focused on either direct interactions or the penetration of probes through the outer surface. Only recently have researchers considered the effects of these drugs on secreted proteins or virulence factors (Clark et al., 1984; OGaard et al., 1986; Warren et al., 1985). Warren et al. (1985) showed that the presence of subinhibitory concentrations of gentamicin and tobramycin inhibited the secretion of P. aeruginosa protease. This is in support of the findings by Ogaard et al. (1986) who reported that gentamicin and tobramycin totally eliminate secretion of elastase. The more hydrophobic antibiotics, such as carbenicillin, elicited no such effects. A number of explanations for such observations exist, but selective inhibition of protein synthesis is considered least likely. Interference with protein secretion by reducing levels of cyclic nucleotides, or direct physical interference are considered the most likely possibilities.

Role of Phospholipid

The importance of phospholipid composition to antibiotic bactericidal activity, has for the most part been neglected. However, because of the toxicity elicited upon host tissues, some studies have been conducted (Au et al., 1986; Champlin et al.,

1983; Gilbertson et al., 1984). Low concentrations of linolenyl alcohol inhibit growth of the gram positive organism Streptococcus mutans (Crout et al., 1982). It has been proposed that the alcohol acts as a molecular spacer between hydrocarbon chains of the acyl-phosphatides, leading to structural alterations which increases the permeability of the plasma membrane.

Gilbertson et al. (1984) elaborated on this study by combining linolenyl alcohol with the permeabilizing antibiotic, gentamicin. Under conditions where gentamicin alone is ineffective, addition of linolenyl alcohol elicited a bacteriostatic effect. When tridecane-1-ol was substituted for linolenyl alcohol, gentamicin produced a bactericidal response. This indicated that, regardless of antibiotic binding, the state of the phospholipids cannot be ignored.

Another interesting study points out that polymyxin-resistant strains of P. aeruginosa exhibit a significant decrease in both phosphatidylethanolamine and phosphatidylglycerol with the concomitant appearance of an unidentified neutral lipid lacking phosphate or amino moities (Champlin et al., 1983; Moore et al., 1984). If polycationic antibiotics do interact with phospholipid head groups, this neutralization would surely limit their binding access. Champlin et al (1983) theorized that when a susceptible P. aeruginosa bacterium encounters polymyxin (or other cationic drugs), it

triggers a phospholipid degradation response possibly by an envelope-bound phospholipase.

Aminoglycosides and Active Transport

The effects of aminoglycosides on active transport have been investigated in studies where E. coli and P. aeruginosa have been grown in the presence of subinhibitory concentrations of aminoglycosides (Eagon et al., 1982). These bacteria were unable to transport proline effectively. The extrusion of protons appeared normal, but the maintenance of a normally efficient proton gradient was decreased.

The theory that aminoglycoside action on the outer membrane is energy-independent in all gram negative organisms does not appear to be altogether true. Martin and Beveridge (1986) suggested that this mechanism occurs in P. aeruginosa, but it appears to differ in E. coli (Iida and Koike, 1974). Both groups have performed numberous electron microscope analyses. They report observations of outer membrane blebbing, following antibiotic treatment of P. aeruginosa, irrespective of the presence of sodium azide or potassium cyanide. However, blebbing by E. coli K12 did not occur if these cells have been preincubated with chloramphenicol, tetracycline, or erythromycin, suggesting that active protein synthesis is a prerequisite for aminoglycoside effects on these cells. It is possible that the aminoglycosides interfere with cell energy levels following the inhibition of

ATPase. Such a phenomenon has been observed in a eucaryotic system (Dalhoff, 1987; Chahwala and Harpur, 1982).

Summary

To briefly summarize the observations of aminoglycoside activity, it has been suggested:

1. Aminoglycosides inhibit protein synthesis by a specific energy-dependent interaction at the ribosomal level.
2. Uptake of aminoglycosides across the inner membrane is energy-dependent, whereas uptake across the outer membrane is energy-independent.
3. Aminoglycosides probably utilize porin protein as a major route in P. aeruginosa.
4. Divalent cations may antagonize aminoglycoside interaction at the outer membrane.
5. Aminoglycosides bind to isolated LPS.
6. Phospholipid composition may influence the lethal activity of aminoglycosides, but this role is believed to be minor in comparison to that of protein and LPS.
7. Aminoglycosides may block the secretion of extracellular substances.

Specific Aims of This Study

As is evident from the background, the data available concerning the mechanism(s) of aminoglycoside activity is controversial and anomalities exist at all levels of investigation. It remains to be seen whether a number of these physiological events are all a part of a single sequence or cascade of events leading to cell death, or whether the antibiotic is simply working at many unrelated levels to introduce lethal damage. The antibiotics are often described as being "pleotropic" in their activities (Dalhoff, 1987). The translational aspects of aminoglycoside activity (Davies et al., 1965) were once generally accepted to be the only cause of cell death. This hypothesis remained unchallenged until more recent studies provided evidence for other possible lethal activities (see review, Schlessinger, 1988).

The overall objectives of this work were to i) establish a time frame for lethality of P. aeruginosa by tobramycin, and ii) investigate damage to the envelope associated with lethality. Time was considered the key parameter in these experiments because early observations of bacteriostatic and bacteriocidal effects indicated they occurred within minutes. Extended antibiotic exposure periods may reflect post-lethal effects (Miller et al., 1987). Lethality was experimentally defined here by standard plating methods, which reflect the cell's inability to divide and produce colonies. A low concentration of tobramycin

was used to magnify the most sensitive biochemical events of initial lethal activity, as well as avoid secondary effects.

The degree of lethality by aminoglycosides is often dictated by certain environmental components so that careful attention has been given in this study to define the protection provided by these compounds. These precautions were well taken because many medium components such as magnesium, phosphate, and carbon growth source influenced the extent of tobramycin activity.

Reports of membrane permeabilization by aminoglycosides (Hancock and Wong, 1984; Loh et al., 1984), and a critical role for LPS in the extent of activity (Bryan et al., 1984; Day and Day, 1982; Hancock et al., 1983), directed the attention of investigators to the effects of these antibiotics at the cell envelope. In addition to lethality studies, we established that cells were releasing UV-absorbing material into the supernatant following tobramycin exposure. It was therefore necessary to assess the nature of this released material. Supernatants from tobramycin treated cells were assayed for general protein, LPS, as well as amino acids and selected enzymes.

The major concept behind the approach desribed here has emphasized the rapid physiological responses occurring concomitant with lethality. Irreversible "permeabilization," as indicated by abnormal release of certain cell components, was consistently tied to lethality within minutes. To separate these events from non-relevant events, and to underline the elements of

specificity of tobramycin effects noted, we examined some more general cell parameters. For example, the condition of the cell with respect to protein synthesis, active transport, and structural integrity was evaluated. Finally, the general observations of cellular leakage, as well as broad antibiotic susceptibility profiles, were initiated for P. aeruginosa CF strains in this study. It is thought that future studies, conducted in parallel with the PAO1 results obtained here, may eventually reveal a role for a specific envelope component(s) in these "low level" resistant isolates.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains, Storage, and Growth Conditions

Pseudomonas aeruginosa PAO1, a standard wild type, was utilized in the majority of these studies. This strain was obtained from A. Kropinski, Queen's University, Kingston, Canada. *P. aeruginosa* cystic fibrosis (CF) strains were isolated from the sputum of CF patients by M. J. Thomassen, Case Western Reserve School of Medicine, Cleveland, Ohio. A protease export mutant, *P. aeruginosa* 902 x 503^{-}, and it's parent strain 902 x 503^{+}, were gifts from Bengt Wretlind, Karolinska Hospital, Stockholm, Sweden. *P. aeruginosa* 34mut, an LPS leaky isolate, and it's parent strain 9027, were obtained from Day and Day (1982), and the American Type Culture Collection, respectively. Finally, *P. aeruginosa* PJ108-0331, a multiflagellated mutant, was a gift by Tetsuo Iino, University of Tokyo, Japan.

Storage of strains was at -70°C. Thirty to fifty milliliter (ml) cultures were grown 12-15 hours in brain heart infusion (BHI) broth (Gibco, Madison, WI) at 37°C in a gyratory water bath shaker at 175rpm. Cell pellets were obtained in sterile tubes by centrifugation at 6,000rpm for 15 minutes (25°C). Cells were washed twice by resuspension in equivalent volumes of sterile BHI broth and centrifugation. Aliquots (0.5ml) of the final suspension

were placed into sterile Cryovials (Sarstedt, Princeton, New Jersey), along with 0.5ml aliquots of 50% sterile glycerol. Vials were immediately frozen and placed in a -70°C Revco ultralow freezer. Daily stocks were maintained at 4°C in BHI broth or slants. Every four to six months, stocks were replaced with fresh isolates from the -70°C storage.

For the majority of these studies, bacteria were grown in a mineral salts medium (MSM) which consists of 4.0×10^{-2}M K_2HPO_4, 2.2×10^{-2}M KH_2PO_4, 7.5×10^{-3}M $(NH_4)_2SO_4$, 2.0×10^{-4}M $MgSO_4 \cdot 7H_2O$, 9.3×10^{-6}M $FeCl_3 \cdot 6H_2O$, and 0.5% glucose, citrate or succinate as a carbon source. For some studies, bacteria were grown in BHI broth or Mueller Hinton (MH) broth (BBL Microbiology Systems, Cockeysville, MD). In all experiments, 30-50ml primary cultures were obtained by overnight growth at 37°C, in a gyratory water bath shaker at 175rpm. Secondary cultures for experimental use were begun by inoculation of the primary culture into fresh medium, and incubation under the same standard conditions. Growth was monitored by following the absorbance at 590 nanometers (nm) with a Bausch and Lomb Spectronic 20. Growth curves were established by plotting absorbance versus time, either with both axes on a standard arithmetic scale, or with the absorbance readings plotted on a semi-logarithmic scale.

Standard curves for quantitating cell numbers of P. aeruginosa PAO1 were established for growth with each carbon source, and in the different complex media. This was accomplished

by washing cells twice in equivalent volumes of standard bacterial diluent (SBD). SBD consists of 25mM potassium phosphate buffer (pH=7.0), 0.9% NaCl, 0.2mM $MgSO_4 \cdot 7H_2O$, and 0.01% gelatin. The final suspension was then diluted by four, two-fold dilutions, followed by one, one hundred-fold dilution, and four ten-fold dilutions. 0.1ml from each of the last five dilution tubes was added to 5mls of 0.8% BHI soft agar (48°C) and plated in duplicate on BHI agar plates. The optical density at 590nm was recorded for the first four tubes, and after obtaining plate counts, the corresponding colony forming units per milliliter (CFU/ml) was established for each absorbance reading. By utilization of simple linear regression, the best fitting straight line of optical density versus CFU/ml was plotted for each experimental growth condition. Extrapolation of this line following an optical density reading (for cultures grown under the same conditions) then yielded the approximate CFU/ml.

The effect of tobramycin on growth was assessed by inoculation of a series of flasks containing 50mls MSM, the selected carbon source, and 8, 4, 2, 1, 0.5 or 0 micrograms per milliliter (ug/ml) tobramycin. The absorbance at 590nm was monitored over a period of six hours.

Antibiotic Susceptibility

The antibiotic profiles of 26 *P. aeruginosa* CF isolates against seven different antibiotics were obtained by the

Kirby-Bauer (KB) zone assay method. Each strain was grown in MH broth overnight at 37°C, 175rpm, in a gyratory water bath shaker. Strains were not passed more than twice for these analyses. Cells were washed twice with 0.85% NaCl and diluted until the density matched that of a barium chloride turbidity standard (0.048M $BaCl_2$ in 0.36N H_2SO_4). Following dilution, suspensions were thoroughly applied to the entire surface of MH agar plates. With a dispensing apparatus, antibiotic discs were applied to the inoculated surface. Plates were incubated for 16-18 hours at 37°C, and the clear zone around each disc was interpreted for antibiotic reactivity according to NCCLS standard zone sizes (mm) provided by Difco Laboratories, Detroit, Michigan, 1981.

Tube dilution assays were done for several P. aeruginosa strains. With tobramycin as a model aminoglycoside, several two-fold dilutions of this antibiotic were prepared in sterile distilled water. The various tobramycin concentrations were then added to 9.0mls of MH broth. Approximately 10^5 CFU/ml of each organism were inoculated into a series of MH tubes containing the various concentrations of tobramycin. Tubes were incubated overnight at 37°C, and examined the following morning for turbidity. Clear tubes having the lowest concentrations of tobramycin were recorded as the minimal inhibitory concentration (MIC) for each strain.

Lethality Studies

P. aeruginosa PAO1 was grown in MSM with glucose or citrate as the carbon source. Cells were pelleted by centrifugation at 6,000rpm for 15 minutes, and resuspended in fresh MSM (without carbon source), 0.01M potassium phosphate buffer (pH=7.0), 2 x 10^{-2}M or 2.0 x 10^{-4}M $MgSO_4 \cdot 7H_2O$, 20% sucrose, or sterile distilled water at 37°C. Tenth ml aliquots were removed upon resuspension for dilutions and plating at zero time. Suspensions were incubated at 37°C as untreated controls, or following exposure to 8 ug/ml tobramycin, and 0.1ml aliquots were removed after 1, 3, 5, or 10 minutes for dilutions. The dilution scheme consisted of one, one hundred-fold dilution, followed by four, ten-fold dilutions in 0.85% NaCl. In some experiments, to assess the effect of temperature in tobramycin killing, saline dilution tubes were pre-warmed to 37°C and 48°C. Samples were plated in duplicate by either a soft agar (0.8%) overlay technique (or "pour plate"), or by the spread plate method.

Experimental efforts to restore viability, following brief tobramycin treatment, was attempted. MSM/glucose grown cells were suspended in 37°C, 0.01M potassium phosphate buffer or sterile, distilled water. Cells were exposed to 8 ug/ml tobramycin for three minutes, and aliquots were removed at both zero and three minutes for dilutions and plating. Immediately after the three minute exposure, positively charged compounds were added, and incubation was continued for seven minutes, after which an

aliquot was removed for dilutions and plating. The charged compounds investigated include 2.0 x 10^{-2}M $MgSO_4 \cdot 6H_2O$, 1.7 x 10^{-3}M L-lysine, or a mixture of L-lysine, L-arginine, L-histidine, spermine, spermidine, and putrescine at a concentration of 1.7 x 10^{-3}M.

Electron Microscopy

Negative staining and thin-sectioning of P. aeruginosa PAO1, grown in glucose, suspended in water and exposed to tobramycin, was conducted as described by Martin and Beveridge (1986). Briefly, for negative staining, cells were placed on copper grids coated with carbon and Formvar, and stained by an ammonium molybdate and uranyl acetate method. For thin sections, cells were fixed with 5% glutaraldehyde and 1% osmium tetroxide, and dehydrated in an ethanol series (25, 50, 80, 95, and 100%). Following sectioning, samples were poststained with uranyl acetate and lead citrate. Samples were examined with a Hitachi H-600 transmission electron microscope operating at 100kV, and equiped with a liquid nitrogen cold trap.

Transport Assay

The transport of L-proline in the presence of absence of tobramycin was determined by modification of the procedure of Montie and Montie (1979). Cells were grown in MSM with glucose, citrate, or succinate, were washed twice, and suspended in 0.05M

potassium phosphate buffer (pH=7.0), 0.005M magnesium chloride and 17mM carbon source. 200 ug/ml chloramphenicol was added to the suspension for 15 minutes, at 37°C, prior to uptake. The experiment was then begun by addition of 0.1ml ^{3}H-L-proline (290 mCi/mmol, final concentration=1 x 10^{-5}M), with or without 8 ug/ml tobramycin, to the incubating cell suspension. At one minute time intervals, samples were filtered through 0.45u Schleicher & Schuell (Keene, NH) nitrocellulose filters on an Amicon (Danvers, MA) filtering manifold. Each sample was immediately chased with 10mls of phosphate-magnesium buffer. Background radioactivity was determined by filtration of ^{3}H-L-proline without cells. Filters were dried and immersed in 5mls of Bray's scintillation cocktail (Research Products, Inc.). All samples were then placed on a Beckman LS 7000 (Irvine, CA) scintillation counter.

Protein Synthesis

The activity of tobramycin on inhibition of protein synthesis was accomplished by following the incorporation of ^{3}H-L-proline into protein. Approximately 5 x 10^{8} CFU/ml, grown in MSM/glucose, were centrifuged at 6,000rpm for 15 minutes, suspended in 0.01M potassium phosphate buffer at 37°C, and divided into two flasks. The experiment was begun by addition of 0.1ml ^{3}H-L-proline (1uCi/umol, 2 x 10^{-5}M) to both cell suspensions at zero time. Tobramycin (8 ug/ml) was added to one flask 2 minutes after proline addition. At one minute time

intervals, 0.5ml samples were added to 0.5ml of ice-cold 10% trichloroacetic acid (TCA), and allowed to remain on ice for 15 minutes. Samples were then filtered on Whatman #3 (England) paper over the Amicon filtering manifold. Filters were washed with two-1ml volumes of 5% ice-cold TCA, two-1ml volumes of 95% ethanol, and 1ml of ether. After drying, each filter was placed into 5mls of Bray's solution for counting.

Quantitation of ATP

Analysis of ATP levels in supernatant preparations was conducted according to the method described by Colowick et al. (1978). This assay is based on the quantitation of light following addition of luciferase-luciferin. In the presence of ATP, these components form a luciferyl adenylate complex which is immediately oxidized to generate light. The amount of light generated is proportionate to the amount of ATP present in the sample. Light is measured with a bioluminescent photometer (Dupont, Inc., Wilmington, DE).

P. aeruginosa PAO1, was grown to log phase with either glucose or citrate as the carbon substrate. Cells were centrifuged (6,000rpm for 15 minutes), and suspended in sterile, distilled water. Cells were divided in half, and one portion was exposed to 8 ug/ml tobramycin. 1ml samples were removed at 0, 3, and 12 minutes following addition of tobramycin, and immediately centrifuged for 1.5 minutes at 13,000 x g. 10ul of supernatant was

withdrawn with a syringe for addition to the reaction mixture. The photometer was calibrated with an ATP standard (Sigma, St. Louis, MO) suspended in 25mM glycylglycine buffer (pH=7.4). The luciferase-luciferin enzyme was supplied by Sigma as a concentrated solution in glycine buffer with magnesium sulfate and EDTA. Following the recommended dilution, 10ul of each sample was added by injection into a cuvette in the dark chamber of the photometer. Counts from unknowns were compared against a standard curve of known ATP concentrations.

Radiolabelled Tobramycin Uptake

To assess the extent of tobramycin uptake, a radiolabelled assay was conducted. Glucose grown P. aeruginosa PAO1 was suspended in 0.01M potassium phosphate buffer (pH=7.0) plus 2 x 10^{-2}M magnesium sulfate, phosphate buffer alone, magnesium sulfate alone, or water. ^{3}H-tobramycin (8 ug/ml, 1.62 uCi/ml) was added to the suspensions at zero time, and incubated at 37°C. Samples were removed at three minute intervals, and washed once with phosphate buffer. Filters were then dried, immersed in Bray's scintillation cocktail, and counted.

Preparation of Supernatant Samples and UV Scans

Primary cultures of P. aeruginosa PAO1 were grown overnight in MSM plus glucose, citrate, or succinate at 37°C in a gyratory water bath shaker at 175rpm. Secondary cultures of

100-1000mls were inoculated the next morning and the optical density at 590nm was monitored. Upon reaching the exponential growth phase ($O.D._{590nm}$=0.1 to 0.8), cells were centrifuged at 6,000rpm for 15 minutes. Supernatants were discarded, and cells were gently resuspended in either 0.01M potassium phosphate buffer (pH=7.0) or sterile water at 37°C. Suspensions were divided into control and treatment flasks, and at time zero, tobramycin (8 ug/ml) was added to the treatment flask. After three minutes, suspensions were centrifuged at 10,000 x g for 5 minutes (4°C), and the supernatants were filtered through 0.45u Nalge or Millipore filters.

Supernatants were then placed in lyophilization flasks and shell-frozen in an acetone dry-ice bath. Concentrated samples from overnight lyophilization were resuspended in small equivalent volumes of water or buffer, and ultraviolet (UV) scans of released material were made with a dual beam UV-visible recording spectrophotometer (Shimadzu Corp., Kyoto, Japan). Data were obtained as "difference spectra." Samples were then stored at -70°C until further analyses could be made. Some supernatant samples from phosphate-suspended cells were dialyzed against water (24-48 hours, 4°C) before storage at -70°C.

Analysis of Supernatants for Protein and Lipopolysaccharide by Colorimetric Methods

Two separate assays were routinely employed to assess the amount of overall protein present in samples. A modified Lowry method (Markwell et al., 1978) was utilized which is based on the reaction of phosphomolybdic and phosphotungstic acids with tyrosine and tryptophan residues (Abs. max.=600nm or 660nm). Another assay for protein quantitation was the microBiorad (Biorad Laboratories, Richmond, CA), which involves the interaction of Coomassie blue dye with protein (Abs. max.=595nm). Bovine serum albumin (BSA) served as the protein standard for both assays.

For the Lowry assay, a stock solution (A) of 0.19M Na_2CO_3, 0.10M NaOH, and 5.7mM KNa tartrate, as well as a stock solution (B) of 0.16M $CuSO_4 \cdot 5H_2O$ was prepared. Before each experiment, 50 volumes of solution A was added to one volume of solution B to make solution C. A diluted phenol reagent was prepared by adding 1ml distilled water per 1.4mls of Folin Ciocalteau (FC) reagent. Supernatant samples were removed from -70°C, and resuspended by equivalent dry weight amounts or obtained directly following tobramycin treatment. Samples and BSA standards were added to 5mls of solution C and allowed to incubate at room temperature for 10 minutes. After this period, 0.5ml of the FC reagent was added to samples and mixed on a Vortex mixer. Samples were incubated for 30 minutes, then absorbance readings were made at

600nm. For the microBiorad method, samples and BSA standards were diluted to a volume of 0.8ml each. 0.2ml of the concentrated Biorad reagent was added, and samples were mixed on a Vortex mixer. After five minutes, absorbance readings at 595nm were obtained. A standard curve of the BSA standards was obtained by simple linear regression, and unknown samples were quantitated by extrapolation from this curve.

The quantitation of lipopolysaccaride (LPS) in supernatant samples was determined by the amount of 2-keto-3-deoxyoctonate (KDO), an LPS core component present (Karkhanis et al., 1978). High purity KDO (Sigma, St. Louis, MO) was utilized as the standard. Both samples and standards were hydrolyzed by suspension in 1ml 0.2N H_2SO_4 and boiling for 30 minutes at 100°C. Samples were then centrifuged at 13,000 x g for one minute to remove particulates. 0.5ml of each sample was then added to 0.25ml of 0.04M periodic acid in 0.125N H_2SO_4, mixed on a Vortex mixer, and incubated at room temperature for 20 minutes. Next, 0.25ml of 2.6% sodium arsenate ($NaAsO_2$) in 0.5N Hydrochloric acid (HCl) was added to samples, mixed on a Vortex mixer, and allowed to stand until the brown color disappeared. Following this, 0.5ml of 0.6% thiobarbituric acid (TBA) was added, samples were mixed on a Vortex mixer, and heated at 100°C for 15 minutes. One ml of dimethyl sulfoxide (DMSO) was then added to hot samples. After cooling, the absorbance at 548nm for each standard was made

against a blank (minus KDO), and the amount of LPS in unknowns was quantitated following absorbance readings and extrapolation of a standard curve of the known LPS values.

Polyacrylamide Gel Electrophoresis

Lyophilized supernatant samples and molecular weight markers were suspended in 0.5M Tris-Cl buffer (pH=6.8) with 2.5% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue. Samples, but not molecular weight markers, were heated at 100°C for ten minutes. Polyacrylamide gel electrophoresis (PAGE) was conducted by utilization of either a 10% acrylamide gel or with a 10-15% gradient gel.

The 10% acrylamide separation gel was poured in a thin (0.75mm) vertical slab unit (Hoeffer Scientific, San Francisco, CA). It was composed of 10% acrylamide / NN' - methylene - bis - acrylamide (30:0.8), and 0.1% SDS in 0.375M Tris-Cl (pH=8.8). 5% acrylamide, 0.1% SDS in 0.125M Tris-Cl (pH=6.8) was employed as a stacking gel. Following deaereation, gels were polymerized with 10% ammonium persulfate and tetramethylethylenediamine (TEMED). 10-50ul of samples were applied to gels with a Hamilton 50ul syringe and electrophoresis was carried out at a constant current of 15mA per slab. The running buffer was 0.025M Tris base, 0.19 M glycine and 3.5mM SDS.

The PhastSystem Phastgel (Pharmacia, Piscataway, NJ) was

composed of a continuous 10 to 15% acrylamide gradient in 0.112M acetate and 0.112M Tris (pH=6.4). Each gel was approximately 0.45mm thick, with a 32mm separation zone. Phastgel SDS buffer strips were composed of 0.2M tricine, 0.2M Tris, and 0.55% SDS (pH=7.5) with 2% agarose. 1ul samples and molecular weight markers were added with a PhastSystem applicator, and electrophoresis was conducted at 10mA per gel.

Following electrophoresis, gels were visualized for protein (Wray et al., 1981) or LPS (Tsai and Frasch, 1982), by silver-staining. The protein method involved overnight fixation in 50% methanol. This was followed by staining in a 0.8% (w/v) silver nitrate, 0.08% sodium hydroxide, and 0.14% (v/v) ammonium hydroxide solution. The gel was then developed in a solution of 0.005% (w/v) citric acid and 0.019% (v/v) formaldehyde. 50% methanol served as the stop solution. The PhastSystem protein stain procedure includes several initial wash steps in 50% ethanol/10% acetic acid and 10% ethanol/5% acetic acid. Gels were then sensitized with 8.3% glutardialdehyde, and washed with water. Gels were stained in 0.25% (w/v) silver nitrate and developed in 2.5% sodium carbonate with 400ul formaldehyde per liter of solution. Development was stopped with 5% acetic acid.

For LPS-silver staining, the gels were fixed overnight in 40% (v/v) ethanol and 5% (v/v) acetic acid. The next morning, gels were oxidized for 10 minutes in the same solution plus 30mM periodic acid. Three, 20 minute wash steps were done with one

liter volumes of distilled water. Gels were then stained in 0.019N NaOH, 0.67% (w/v) silver nitrate, and 0.1% (w/v) ammonium hydroxide. Three wash steps, 10 minutes and 200mls each, were done, and gels were developed in 0.5% (w/v) citric acid and 0.019% (v/v) formaldehyde. After staining, the vertical slab gels were heat dried under vacuum.

Thin-Layer Chromatography

An equivalent amount of each supernatant sample, was dissolved in 0.3N hydrochloric acid (HCl). 20mM solutions of L-lysine, L-aspartate, L-threonine, L-proline, L-tryptophan, L-phenylalanine, and L-leucine as standards, were also prepared in 0.3N HCl. 3 ul of standards and samples were spotted onto cellulose preformed filter paper (Eastman Kodak Co., Rochester, NY; Whatman Ltd., England) and placed in a development chamber. The running solvent consisted of 60 parts *n*-butanol, 30 parts glacial acetic acid, and 10 parts water. After 2 hours and 20 minutes, papers were removed and allowed to air dry completely. Amino acids were detected using a 0.2% ninhydrin (Sigma, St. Louis, MO) + collidine reagent, and compared with the mobilities of the standards.

Amino Acid Analysis of Supernatant Samples by High Pressure Liquid Chromatography (HPLC)

Approximately 1 x 10^8 CFU/ml of MSM/glucose grown P. aeruginosa PAO1 were either suspended in distilled water, or washed three times with 0.01M potassium phosphate buffer (pH=7.0) and resuspended in distilled water. 100ml portions of washed and unwashed cells either served as controls or were treated for 3 minutes with 8 ug/ml tobramycin at 37°C. Aliquots were then centrifuged, and supernatants were filtered and concentrated by lyophilization.

For hydrolysis, aliquots of each sample were placed into hydrolysis vials, 200ul of 6N HCl was added, and samples were sealed under vacuum. Following a 24 hour exposure to 105°C, the vials were opened and lyophilized. For both hydrolyzed and nonhydrolyzed samples, a "redry" step was done for the removal of ammonia and other extraneous peaks. A solution of water, methanol, triethylamine, and phenylisothiocyanate (7:1:1:1) was added for a 30 minute incubation at 25°C. Samples were lyophilized. Finally, each sample was diluted in 400ul of a sodium phosphate buffer (pH=7.4) and 4ul was removed for separation over a Novapak reversed-phase C18 column. Results were compared with a standard containing 20 amino acids (PICO TAG, Amino acid analysis Operator's manual, Waters Associates Inc., Manual Number 88140, Dec. 1984, Revision A).

Amino Acid Extraction

To assess the amount of pool amino acids in glucose growing, log phase P. aeruginosa PAO1, extractions were conducted according to that outlined by Montie and Montie (1975). Cells were collected by centrifugation and either washed three times with 0.01M potassium phosphate buffer (pH=7.0) or not washed. Pellets were then suspended in an equivalent volume of 70% ethanol, and allowed to incubate for 30 minutes at 4°C. The suspension was then centrifuged, filtered, and concentrated. The resultant material was then subjected to analysis by HPLC.

Protein Isolation and Molecular Weight Determination

The relative molecular weight (M_r) of a specific tobramycin-released protein was initially determined by it's electrophoretic mobility, in a 10% polyacrylamide gel, against the mobilities of Biorad low molecular weight markers. These include phosphorylase B (M_r=92.5kDa), bovine serum albumin (M_r=66.2kDa), ovalbumin (M_r=45kDa), carbonic anhydrase (M_r=31kDa), soybean trypsin inhibitor (M_r=21.5kDa), and lysozyme (M_r=14.4kDa).

Further characterization of the tobramycin-released protein profile was accomplished by gel filtration over a Sephacryl S-200 (Pharmacia, Piscataway, NJ) column. Concentrated and dialyzed supernatant samples from one liter of phosphate-suspended, 8 ug/ml tobramycin treated (3 minutes) cells were placed over a

1.5cm x 50cm Pharmacia column. The running buffer was 0.01M potassium phosphate (pH=7.5) + 0.02% sodium azide. 1ml samples were collected overnight (at 4°C) with a Pharmacia fraction collector and the absorbance at 280nm and 260nm was recorded spectrophotometrically. Peak fractions were pooled, lyophilized, and examined by SDS-PAGE. The elution characteristics of the tobramycin-released protein were compared against the elution profiles of bovine albumin (M_r=66kDa), β-lactoglobulin (M_r=36.8kDa), and cytochrome C (M_r=13kDa). A standard curve of fraction number versus $\log_{10}$ molecular weight was utilized to obtain the relative molecular weight of the unknown protein. A void volume of 20mls was determined using Blue Dextran (Sigma, St. Louis, MO) as a tracking dye.

β-Lactamase Assay

Detection of the periplasmic enzyme, β-lactamase, was accomplished by a modification of the procedure outlined by O'callaghan et al. (1972). Nitrocephin is a chromogenic β-lactam which undergoes a color change from yellow to red as the β-lactam ring is hydrolyzed by a β-lactamase. The enzyme reaction was conducted at 37°C in 0.05M potassium phosphate buffer (pH=7.0) with 10^{-4}M nitrocefin. At this concentration, the change in optical density at 482nm upon complete hydrolysis of nitrocefin totalled 1.59 units. Therefore, enzyme units were quantitated as follows:

$$\text{E. U.} = \frac{\Delta \text{O.D.482 / minute}}{1.59} \times \text{micromoles of nitrocefin} \times v$$

where *v* is the factor adjusting the volume to 1ml. The specific activity was then obtained as enzyme units per milligram of protein (microBiorad).

The samples investigated for the presence of β-lactamase include i) glucose grown, P. aeruginosa PAO1 supernatants, either exposed to 8ug/ml tobramycin for 3 minutes or untreated, ii) pooled, concentrated fractions from S-200 gel filtration of tobramycin treated cell supernatants, and iii) cell sonicates. Sonication of cells was conducted by suspension of cells in 0.01M potassium phosphate buffer (pH=7.0), placement in ice, and three exposures to 4 amperes at 30 second intervals. A probe sonifier (Branson Instruments, Inc., Stamford, CN) was utilized. The sonicate was then centrifuged, and the supernatant analyzed for β-lactamase.

Osmotic Shock

An osmotic shock procedure used for obtaining transport binding proteins from P. aeruginosa (Hoshino, 1979; Hoshino and Kageyama, 1980), was conducted with glucose grown PAO1. Exponential phase cells were suspended in 50mM Tris-HCl (pH=7.3) containing 0.2M magnesium chloride at a concentration of 20mls per gram of wet weight cells. The suspension was incubated for 10

minutes at 30°C, then rapidly chilled to 4°C for 15 minutes. Cells were exposed a second time to 30°C for 10 minutes and chilled again for 15 minutes. A pellet was obtained by centrifugation (10 minutes, 7,000rpm, at 4°C), rapidly dispersed in distilled water at 25°C, and allowed to stand for 20 minutes before centrifugation. The supernatants from both the magnesium chloride extract and water extract were combined and centrifuged at 20,000 x g for 10 minutes at 4°C. This supernatant was then concentrated by lyophilization, and dialyzed against water before analysis by SDS-PAGE.

Chemicals

The carbon growth substrates, citrate and succinate, were purchased as sodium salts from Sigma Chemical Co. (St. Louis, MO). α-D(+)-glucose was also obtained from Sigma with less than 5% of the β-anomer present. Amino acids, ATP, KDO, tobramycin, chloramphenicol, and blue dextran were purchased from Sigma at the highest purity available. Also from Sigma were buffer salts, glycerol, sucrose, and TLC reagents.

Enriched growth media were obtained from both Gibco (Madison, WI), and BBL Microbiology Systems (Cockeysville, MD). Acrylamide, methylene bisacrylamide, TEMED, SDS, ammonium hydroxide, and low molecular weight markers for electrophoresis were purchased in a highly purified form from Biorad Laboratories (Richmond, CA). Sephacryl S-200 was obtained from Pharmacia

(Piscataway, NJ). The chromogenic β-lactam, nitrocefin, was obtained from BBL Microbiology Systems. The radiolabelled compounds, L-(2, 3, 4, 5 -^{3}H) proline, and (6" <n> - ^{3}H) tobramycin, were both purchased from Amersham, Arlington Heights, IL.

CHAPTER III

RESULTS

Influence of Growth Substrate and Environmental Conditions on the Extent of Activity by Tobramycin Toward Pseudomonas aeruginosa PAO1

Inhibition of Growth. It was of initial interest to examine and establish inhibitory levels of tobramycin in a defined medium. Growth profiles were obtained for bacteria grown in a mineral salts medium (MSM) with glucose, citrate, or succinate as the carbon substrate. The tobramycin concentrations present in the medium ranged from 0.5 to 8.0 ug/ml, which is just above and below the minimal inhibitory concentration (MIC) of 1.0 ug/ml for P. aeruginosa PAO1 in Mueller Hinton (MH) broth.

It was found that the degree of inhibition by tobramycin is related to the particular carbon source utilized for growth. As shown in Figure 1, cells growing with glucose (A) were more susceptible to tobramycin than were cells growing with citrate (B), or succinate (C), as a carbon source. For example, after six hours of incubation in the presence of 1.0 ug/ml tobramycin, the glucose growing cells were inhibited by approximately 86% as compared to the control, whereas citrate growing cells and succinate growing cells were inhibited by 6% and 38%,

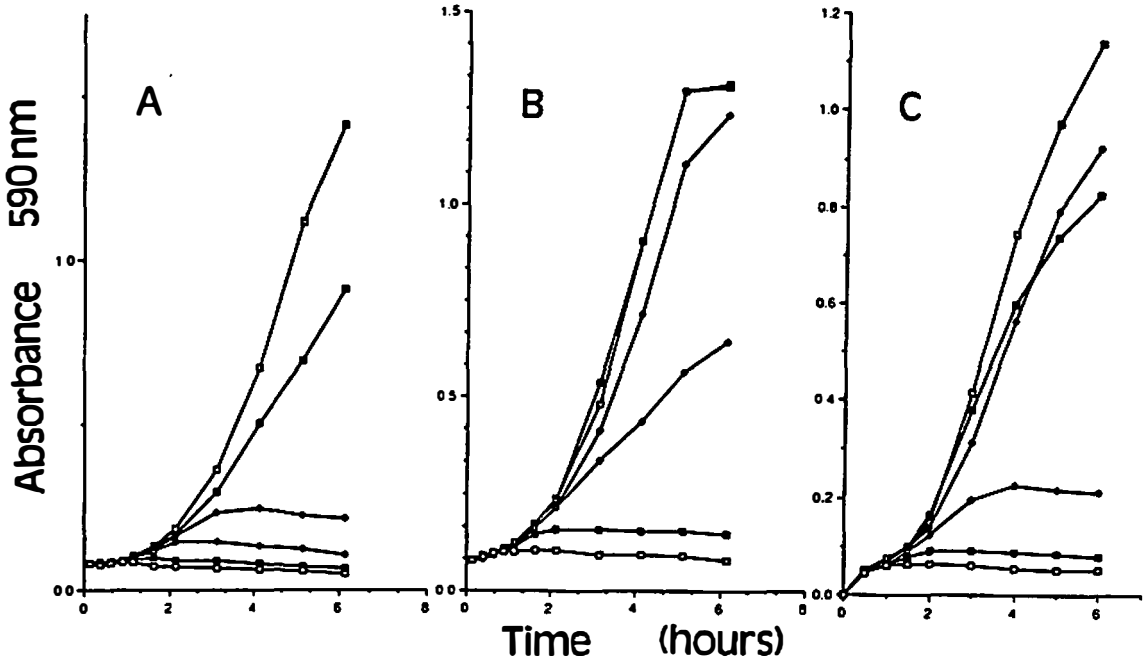

Figure 1. Inhibition of growth of Pseudomonas aeruginosa PAO1 by various concentrations of tobramycin in MSM with glucose (A), citrate (B), or succinate (C), as a carbon source. Symbols: ⊡ , no tobramycin; ◘ , 0.5 ug/ml; ● , 1.0 ug/ml; ◆ , 2.0 ug/ml; ■ , 4.0 ug/ml; □ , 8.0 ug/ml of tobramycin. Results represent the average of duplicate experiments.

respectively. Interestingly, inhibition by 1.0 ug/ml of tobramycin in glucose growing cells is very comparable to the MIC value assessed in MH broth.

Lethality and Environmental Components. In these studies, the extent of lethality following a brief exposure to tobramycin was assessed under various environmental conditions. Evidence has indicated that magnesium and other divalent cations compete with aminoglycosides presumably at their outer membrane target site(s) (Abdel-Sayed et al., 1982; Eagon, 1984; Hancock et al.,1981; Loh et al., 1984; Moore et al., 1986; Nicas and Hancock, 1983; Peterson et al., 1985). The presence of phosphate in the environment may also interfere with aminoglycoside activity (Hancock et al., 1981).

It is well established that growth with different carbon substrates alters the expression of certain envelope components, such as protein (Hancock, 1980) and lipid compostion (Conrad, 1979). Different substrates are also known to influence cellular energy levels (Montie and Montie, 1979). Therefore, it was deemed necessary to conduct studies with each carbon source considered as an individual "system."

To investigate the protection by environmental components, cells were incubated into early exponential growth in MSM, and then harvested. They were suspended in either fresh MSM (which contained magnesium and phosphate), phosphate buffer, sucrose, or

water, and treated with 8 ug/ml tobramycin for only three minutes (37°C). Table 1 shows that the degree of lethality by tobramycin was twice as great in phosphate buffer-suspended cells (66%) in comparison to the MSM-suspended cells (29%). Cells suspended in water, to eliminate any protective components, were killed to the greatest extent (96%). These studies indicate that both magnesium and phosphate provided some protection, consistent with previous suggestions.

Lethality studies also show that glucose grown cells (Table 1) were found to be more susceptible to tobramycin than were citrate grown cells (Table 2) under all environmental conditions studied. For example, following antibiotic treatment, for glucose grown cells suspended in phosphate buffer, colony forming units (CFU's) were reduced nine-fold compared to citrate grown cells. Such results suggest a possible physiologically-specific role for growth substrates in aminoglycoside studies.

Growth Phase and Experimental Plating Conditions. Since the specific salt conditions modify response to tobramycin, we decided to investigate the possibility that the degree of lethality may be related to the phase of growth. Previous studies (Tables 1 and 2), were conducted with cells harvested at the onset of exponential growth. In this study, glucose growing cells were allowed to reach the late logarithmic growth phase before centrifugation (Table 3). Cells were then suspended in either fresh

Table 1. Immediate bactericidal action of 8ug/ml tobramycin toward glucose-grown Pseudomonas aeruginosa PAO1 under various environmental conditions.

Environment	Exposure Time (minutes)	CFU/ml[a]	% Lethality[b]
Mineral Salts Medium (MSM)[c]	0	2.1×10^8	-
	3-Control	2.1×10^8	0
	3-Treated	1.5×10^8	29
0.01M Potassium Phosphate Buffer (pH=7.0)	0	8.7×10^7	-
	3-Control	8.8×10^7	0
	3-Treated	3.0×10^7	66
20% Sucrose	0	3.7×10^9	-
	3-Control	2.6×10^9	30
	3-Treated	9.1×10^8	75
dH_2O	0	1.5×10^8	-
	3-Control	1.2×10^8	20
	3-Treated	6.5×10^6	96

[a]A soft agar overlay technique was utilized for plate counts.
[b]% lethality was measured as the percent decrease of CFU/ml as compared to the zero minute values. Results represent the average of duplicate samples, with no individual value exceeding 4%.
[c]Carbon substrate was not included.

Table 2. Immediate bactericidal action of 8ug/ml tobramycin toward citrate-grown Pseudomonas aeruginosa PAO1 under various environmental conditions.

Environment	Exposure Time (minutes)	CFU/ml[a]	% Lethality[b]
Mineral Salts Medium (MSM)[c]	0	2.7×10^8	-
	3-Control	3.3×10^8	0
	3-Treated	3.4×10^8	0
0.01M Potassium Phosphate Buffer (pH=7.0)	0	8.4×10^7	-
	3-Control	8.4×10^7	0
	3-Treated	7.8×10^7	7
dH_2O	0	1.6×10^8	-
	3-Control	1.4×10^8	12
	3-Treated	1.2×10^7	92

[a] A soft agar overlay technique was utilized for plate counts.
[b] %lethality was measured as the percent decrease of CFU/ml as compared to the zero minute values. Results represent the average of duplicate samples, with no individual value exceeding 3%.
[c] Carbon substrate was not included.

Table 3. Comparative lethality by 8ug/ml tobramycin in glucose-grown Pseudomonas aeruginosa PAO1 from different growth phases.

Environment & Growth Phase	Time (minutes)	CFU/ml[a]	%Lethality[b]
Mineral Salts	0	2.1×10^8	-
Medium (MSM)[c],	3-Control	2.1×10^8	0
Early Log	3-Treated	1.5×10^8	29
MSM[c],	0	8.5×10^8	-
Late Log	3-Control	8.2×10^8	3
	3-Treated	5.5×10^8	35
dH_2O,	0	1.5×10^8	-
Early Log	3-Control	1.2×10^8	20
	3-Treated	6.5×10^6	96
dH_2O,	0	5.0×10^8	-
Late Log	3-Control	4.4×10^8	12
	3-Treated	3.2×10^7	94

[a]A soft agar overlay technique was utilized for plate counts.
[b]%lethality was measured as the percent decrease of CFU/ml as compared to the zero minute values. Results represent the average of duplicate samples, with no individual value exceeding 3%.
[c]Carbon substrate was not included.

MSM or water, and treated with tobramycin as before. Results indicated that growth phase had no influence on lethality by tobramycin in P. aeruginosa PAO1.

A soft agar overlay (or pour plate) technique is often employed for studies with Pseudomonas because of this organism's motility. The method allows for clean separation of colonies. However, it involves placing the bacterial suspension into agar which has been warmed to 48°C. The amount of killing observed in one accidental experiment (soft agar temperature=>52°C) differed as much as 8%. Although brief exposure to i) a temperature of 48°C, and ii) reduced aeration in soft agar has a negligible effect on normal, intact P. aeruginosa PAO1, it was of interest whether or not lethality in an aminoglycoside-damaged cell may be enhanced by these factors.

Such questions were examined in a series of experiments whereby glucose grown cells were suspended in sucrose or water, treated with 8 ug/ml tobramycin for three minutes, and plated by both the spread-plate (S) and pour-plate (P) procedure. Saline dilution tubes utilized in the spread-plate method were adjusted to three separate temperatures (25°C, 37°C, and 48°C) to examine the influence of temperature alone. It must be emphasized that cells were exposed to these temperatures for less than 10 seconds, which represents the time evolved during mixing and diluting. Results in Figure 2 show that the decline in CFU/ml in untreated, control (C) cells was the same by either method, as

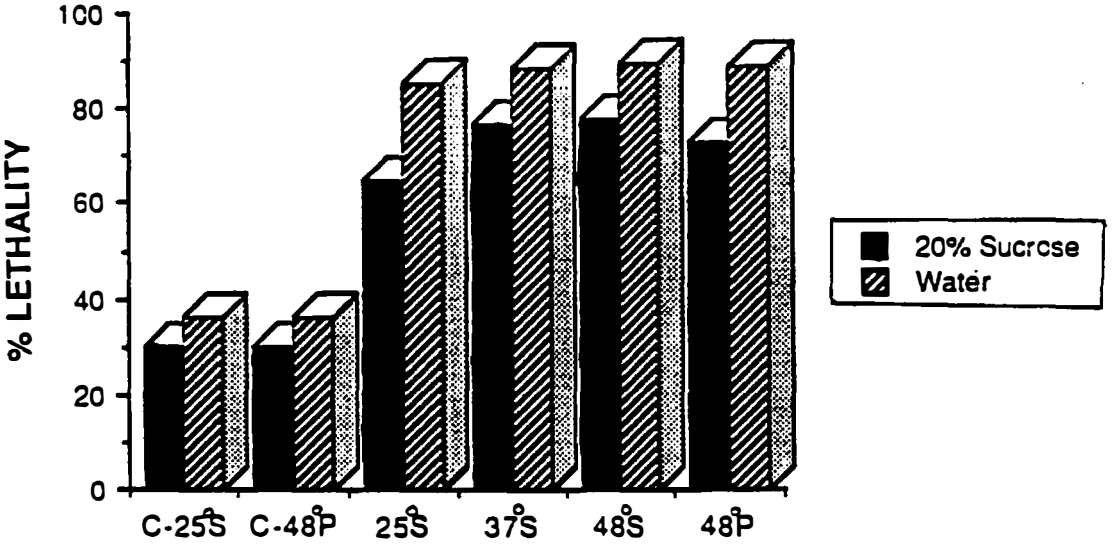

Figure 2. Lethality by 8 ug/ml tobramycin, in glucose grown Pseudomonas aeruginosa PAO1, suspended in 20% (w/v) sucrose or water, and plated by different methods and temperatures. C, control; S, spread-plate; P, pour-plate. Results represent the average of duplicate experiments.

expected. Lethality in tobramycin treated cells was likewise similar by either method. Although a few differences (up to 10%) were observed as the temperatures were increased in tobramycin treated cells, these were not considered large enough to pursue further.

Magnesium as a Protectant. The results in Tables 1, 2, and 3 (pages 50, 51, and 52, respectively) clearly show that cells suspended in MSM were best protected from tobramycin lethality. This was presumably due to the presence of magnesium ions. To confirm the protective role, glucose grown cells were suspended in different concentrations of magnesium sulfate. Protection by phosphate buffer was excluded. Cells were treated with tobramycin and plated as before. The results in Table 4 demonstrate that a magnesium sulfate concentration of 2.0×10^{-4}M (which is the concentration present in MSM) allowed for only 31% lethality. The value of 29% lethality in Table 1, page 50, is in excellent agreement with these results. It is interesting to note that Nicas and Hancock (1983a) have stated that a 1,000-fold excess of magnesium is necessary for protection of P.aeruginosa against gentamicin. A 2.0×10^{-4}M concentration of magnesium sulfate utilized here is only 12-fold greater than 8 ug/ml tobramycin (1.7×10^{-5}M), and provides protection to the cells.

Table 4. Comparative magnesium protection of Pseudomonas aeruginosa PAO1[a] from lethality by 8 ug/ml tobramycin.

Molarity of $MgSO_4 \cdot 7H_2O$[b]	Exposure Time (minutes)	CFU/ml[c]	% Lethality[d]
2.0×10^{-1}M	0	2.4×10^8	-
	3 - Control	2.5×10^8	0
	3 - Treated	2.0×10^8	19
2.0×10^{-2}M	0	3.7×10^8	-
	3 - Control	3.8×10^8	0
	3 - Treated	3.2×10^8	14
2.0×10^{-4}M	0	3.8×10^8	-
	3 - Control	3.4×10^8	11
	3 - Treated	2.6×10^8	31

[a]Cells were grown in MSM + glucose, and suspended in different concentrations of magnesium sulfate at 37°C.
[b]In control experiments, >90% lethality was consistently observed for water-suspended cells treated for 3 minutes.
[c]A soft agar overlay technique was utilized for plate counts.
[d]%lethality was measured as the percent decrease of CFU/ml as compared to the zero minute value. Results represent the average of duplicate samples, with no individual value exceeding 6%.

However, a magnesium concentration of 2.0 x 10^{-2}M (over 1,000-fold excess compared to tobramycin) did provide the best protection (Table 4).

The brief tobramycin exposure period of 3 minutes suggested that the lethal damage might be concentrated at the outer surface or at least accessible. It was thought that these effects may be easily reversible. Cells were treated with tobramycin as usual, then supplied with 2.0 x 10^{-2}M magnesium sulfate immediately following the 3 minute exposure period. As evident in Table 5, it was impossible to restore viability. Therefore, the lethal damage by tobramycin occurs rapidly and is irreversible within even the short time frame of 3 minutes.

Uptake of ^{3}H-Tobramycin. As additional support to the lethality data, and to clearly show that magnesium antagonizes the initial interaction of tobramycin, glucose grown P.aeruginosa PAO1 was exposed to ^{3}H-tobramycin. In Figure 3, cells were suspended in magnesium sulfate (A), magnesium sulfate in phosphate buffer (B), phosphate buffer (C), or water (D), and exposed to 8 ug/ml of ^{3}H-tobramycin for 3 minutes. Following filtration and one-10ml phosphate buffer wash step, the amount of radioactive tobramycin adhering to or taken up by cells (on the filter) was determined.

In these experiments, it was not possible to distinguish between transported drug and that which was tightly bound to the

Table 5. Inability of magnesium sulfate to reverse lethality of Pseudomonas aeruginosa[a] by 8 ug/ml tobramycin.

Sample	Time (minutes)	CFU/ml[b]	% Lethality[c]
Control[d]	0	2.8×10^8	-
	3	2.7×10^8	3
	10	2.8×10^8	0
Tobramycin[e]	0	3.3×10^8	-
	3	6.0×10^5	>99
	10	8.0×10^4	>99
Tobramycin and Magnesium[f]	0	3.4×10^8	-
	3	3.5×10^5	>99
	10	6.6×10^5	>99

[a]Cells grown in MSM + glucose.
[b]A soft agar overlay technique was utilized for plate counts.
[c]%lethality was measured as the percent decrease of CFU/ml as compared to the zero minute value.
[d]Cells were plated upon suspension in 37°C distilled water, as well as following 3 and 10 minutes of incubation.
[e]Cells were exposed upon suspension, and plated over 10 minutes of incubation.
[f]A 2.0×10^{-2}M concentration of magnesium sulfate was added immediately following 3 minutes of tobramycin exposure and incubated 7 additional minutes.

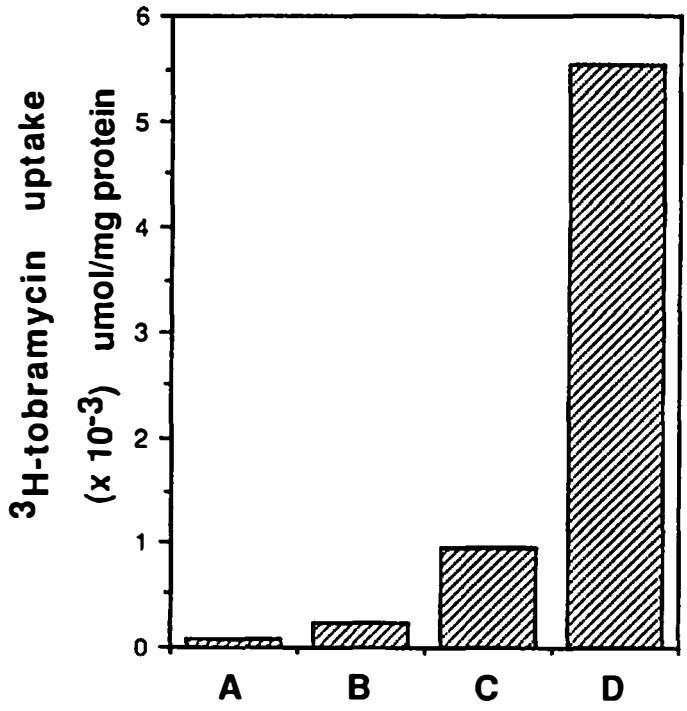

Figure 3. Uptake of labelled tobramycin under different salt conditions. Pseudomonas aeruginosa PAO1 was suspended in 2.0 x 10^{-2}M magnesium sulfate (A), 2 x 10^{-2}M magnesium sulfate in 0.01M potassium phosphate buffer (pH=7.0) (B), potassium phosphate buffer (C), or distilled water (D). Results represent the average of duplicate experiments.

surface. However, the distinctive environmental roles served by magnesium and phosphate were evident. Cells suspended in magnesium revealed the lowest degree of tobramycin uptake, whereas that for phosphate-suspended cells was 12 times greater. Furthermore, in comparison to magnesium-suspended cells, water-suspended cells revealed an increased uptake of 69-fold. Although not as substantial a difference, it was found that tobramycin associates 2.9-fold greater with cells in magnesium-phosphate buffer than in magnesium alone. This probably represents some interaction of magnesium and phosphate ions in solution. Under all conditions except phosphate, tobramycin was taken up within three minutes, because even after 12 minutes of exposure, no more than 22% additional drug was recorded. Phosphate buffer suspended cells contained exactly twice as much tobramycin after 12 minutes of exposure.

Effect of Tobramycin on Protein Synthesis and Amino Acid Transport in Pseudomonas aeruginosa PAO1 with Respect to Time

Amino Acid Transport. Transport of the amino acid, proline, was investigated by methods previously described (Montie and Montie, 1979). It was thought that this system would provide a most sensitive approach for detecting immediate tobramycin-induced alterations to the envelope, and also to indirectly assess the energy level of these cells. Glucose (A),

citrate (B), and succinate (C) grown cells (Figure 4) were washed and suspended in transport buffer with the respective carbon substrate. Before proline addition, cells were preincubated with chloramphenicol to eliminate proline incorporation into protein. ^{3}H-L-proline was added with or without tobramycin, and uptake was observed over a five minute period. The presence of tobramycin did not alter the efficiency of proline transport within this short time frame.

Protein Synthesis Inhibition. As outlined in Chapter I, the precise activities of aminoglycosides at the ribosomal level is a controversial issue (Ahmad et al., 1980; Davis et al., 1986). However, it is clear that these drugs do interact and lead to the eventual inhibition of protein synthesis. It was of interest to establish the degree of protein synthesis inhibition, within a short time frame, under conditions utilized for lethality and leakage experiments. Glucose grown P. aeruginosa PAO1 was suspended in phosphate buffer, and the amount of ^{3}H-L-proline incorporated into protein was determined at one minute time intervals (Figure 5). Tobramycin (arrow) was added after two minutes of proline uptake. Results show no difference after a one minute exposure, and just below 40% inhibition following a three minute exposure. Therefore, although the role of ribosomal inhibition cannot be totally excluded in lethality experiments, leakage of cellular material occurs well prior to three minutes (see below).

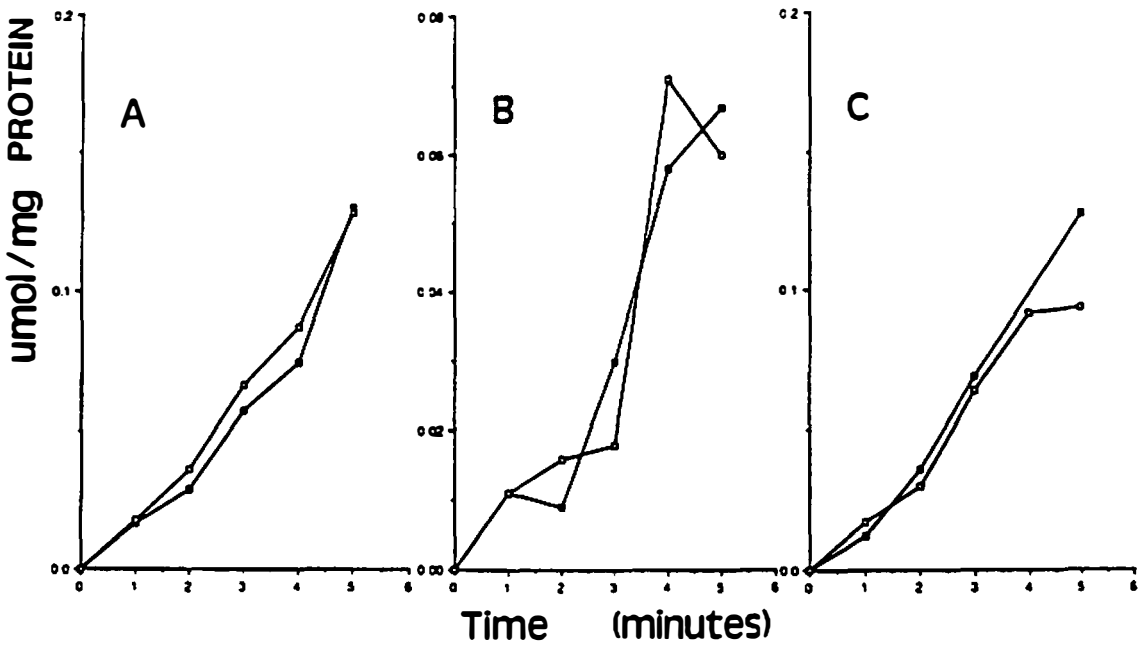

Figure 4. Proline transport in glucose (A), citrate (B), and succinate (C) grown Pseudomonas aeruginosa PAO1 in the presence (■) or absence (□) of 8 ug/ml tobramycin. Results represent the average of duplicate experiments.

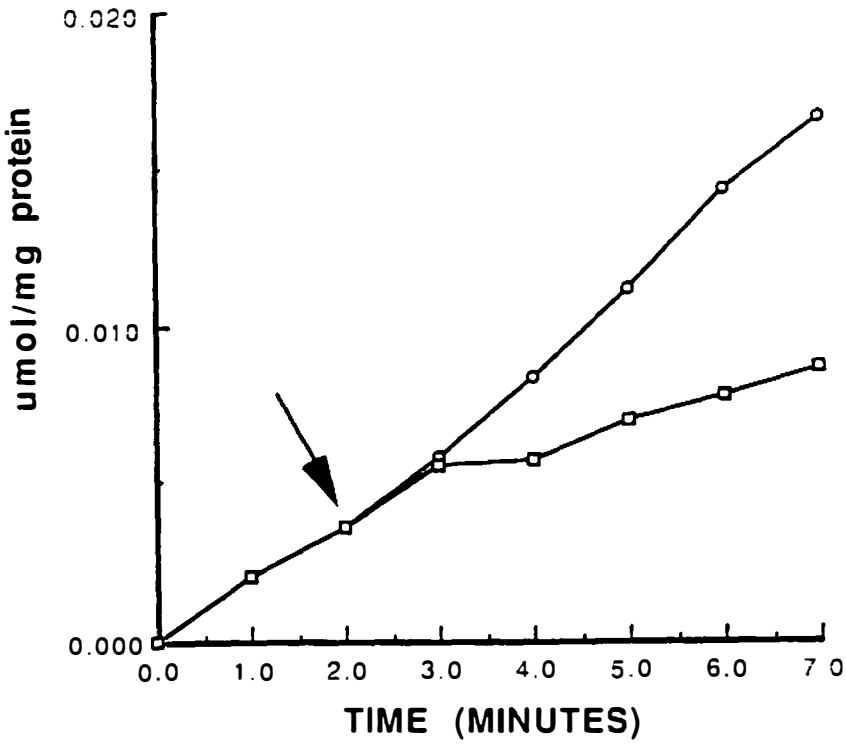

Figure 5. Protein synthesis inhibition by 8 ug/ml tobramycin in glucose grown Pseudomonas aeruginosa PAO1. ^{3}H-L-proline was added at zero time in both control (o) and treatment (□) suspensions, and tobramycin was added (arrow) two minutes after proline uptake. Results represent the average of duplicate experiments.

Leakage of Cellular Components from Pseudomonas aeruginosa PAO1 Following Short Term Exposure to Tobramycin

For many years, aminoglycoside studies focussed primarily on the mechanism of activity at the ribosomal level. There were a few early reports of small molecules leaking from the cell following treatment, but these were soon ignored. Only recently is aminoglycoside-induced damage at the cell envelope recognized as relating to potential lethal activity.

The majority of studies conducted to meaure damage or "permeabilization" at the envelope, have employed the uptake of certain probes, such as a specific enzyme substrate, or a fluorescant or radiolabelled substance, as an indicator (Hancock et al., 1981b; Hancock and Wong, 1984; Loh et al., 1984; Moore et al., 1986). Here, the cell surface activity of tobramycin initially was assessed by spectrophotometrically examining cell supernatants for material released as a result of envelope damage.

General Composition of Leakage Material. The same conditions used for studying lethality were employed to examine tobramycin-induced leakage by spectrophotometric analysis of released material following brief exposure to the antibiotic. Following the treatment of glucose grown, phosphate buffer-suspended P. aeruginosa PAO1 with 8 ug/ml tobramycin, supernatants were concentrated by lyophilization, and resuspended

in ice-cold water at equivalent concentrations by weight for spectrophotometric examination in the ultraviolet (UV) region. "Difference spectra" between treated and untreated cell supernatants were used to highlight released compounds resulting from tobramycin effects. Figure 6 is a scan from cells exposed to tobramycin for only one minute. It was found that the tobramycin treated cell supernatant contained a larger amount of UV-absorbing material than did the untreated cell supernatant. This release, as mentioned earlier, occurred prior to any apparent inhibition of protein synthesis.

Comparative UV scans of i) cells grown with citrate, succinate, or glucose as the carbon substrate, ii) cells suspended in buffer or water, iii) cells suspended in 2.0×10^{-2}M magnesium sulfate, and iv) cells treated with 8 ug/ml tobramycin for three minutes or longer were obtained. Regardless of carbon growth substrate, all cells were observed to release UV-absorbing material upon exposure to tobramycin. A broad peak at 260-280 nm was consistently found, yet the magnitude of the peak at 210 nm (Figure 6) was much greater in cells grown in glucose, than in cells grown in an organic acid. The relative amount of released material was not only in greatest amounts from glucose grown cells, but also occurred most rapidly within one to three minutes. In the case of citrate and succinate grown cells, the release was more progressive over a 12 minute period. Release was more rapid and extensive in water-suspended cells compared to

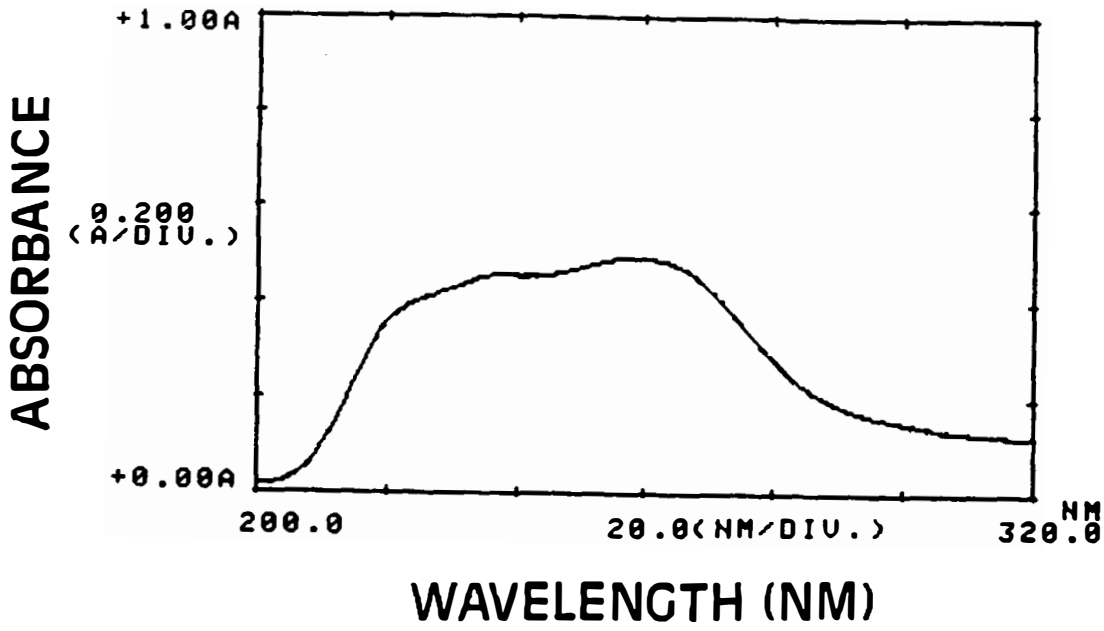

Figure 6. Release of UV-absorbing material from Pseudomonas aeruginosa PAO1 following exposure to 8 ug/ml tobramycin for one minute. Results are expressed as a "difference spectrum" between untreated and tobramycin treated resultant supernatant fractions.

buffer-suspended cells. However, the peak profiles were very similar (data not shown). No leakage was observed for cells suspended in magnesium sulfate. In one experiment, phosphate-suspended cell supernatants were dialyzed overnight against water to reduce the amount of buffer salts. Dialysis resulted in a loss of some of the UV-absorbing material, which indicated that non-dialyzed supernatants contained small components, possibly amino acids and small polypeptides.

It has been reported that gentamicin potentiates the release of LPS from P. aeruginosa (Martin and Beveridge, 1986). Therefore, it was of interest to assay supernatants for this component and for the general release of protein. It should be noted that LPS is spontaneously released from P. aeruginosa during normal growth (Cadieux, 1983). Two methods were utilized for detection of LPS. The first employed the 2-keto-3-deoxyoctonate (KDO) microassay (Karkhanis et al., 1978), which quantitates the amount of KDO, an LPS core component, in a given sample. The second approach involved a silver-staining technique for LPS following SDS-PAGE (Tsai and Frasch, 1982). Similarly, two separate colorimetric methods were used to quantitate the amount of protein present in the supernatant.

Initial analyses on crude, unconcentrated supernatant samples are shown in Figure 7. For this experiment, cells were grown in MSM plus glucose, suspended in water, and treated with tobramycin for three minutes. Based on the lethality data, these

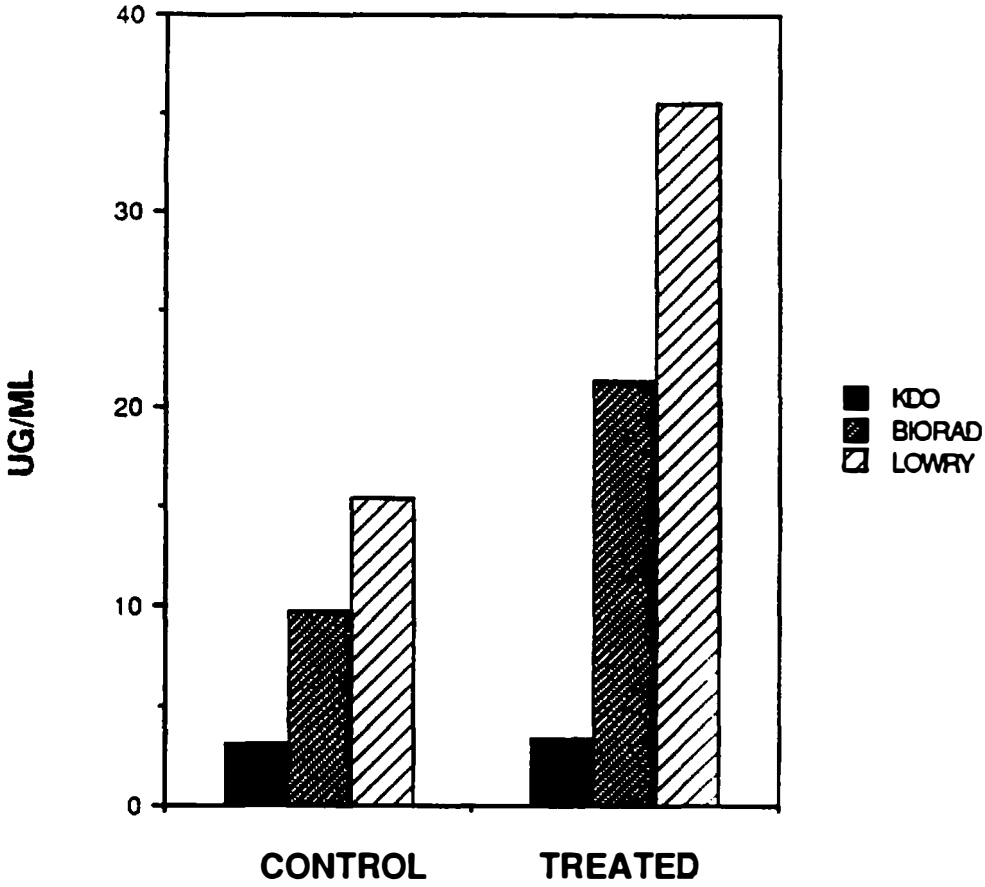

Figure 7. Direct comparative analysis of Pseudomonas aeruginosa PAO1 supernatants for released protein and lipopolysaccharide. Cells were suspended in water (37°C) and either treated with 8 ug/ml tobramycin for 3 minutes or left untreated. Unconcentrated supernatants were analyzed by the microBiorad and Lowry protein assays as well as the KDO microassay for LPS quantitation. Results represent the average of duplicate experiments.

were the most severe conditions which would allow for easy detection of these cellular components. It was found that there was a more than two-fold increase of protein in the supernatant. The specific amount of protein was greater by the Lowry method than that observed by the Biorad analysis. This observation, in addition to the fact that dialysis of supernatants results in a loss of UV-absorbing material, strengthened the suggestion that free amino acids or small peptides may be released.

Also shown in Figure 7 was the failure to detect an additional amount of KDO in the tobramycin treated cell supernatant. Results from silver-stained gels for LPS support these findings. However, the release of LPS was detected following a 12 minute exposure with cells grown under all conditions (glucose, citrate, and succinate).

It has previously been shown that glucose grown cells have a significantly lower ATP level in comparison to citrate grown cells (Montie and Montie, 1979). In P.aeruginosa RM46, a methionine auxotroph, the levels of ATP in glucose growing cells were only 28-46% of the total present in citrate growing cells. It may be that aminoglycosides inhibit ATPase, although there are no known previous reports of such an occurrence in bacteria. Alternatively, ATP may leak out through the damaged outer membrane. The loss of ATP, or it's reduced presence in glucose grown cells for repair may explain the enhanced susceptibility of these cells to tobramycin.

Detection of ATP in the supernatants of both glucose and citrate grown cells was performed using a firefly luciferase method. Cells were suspended in water and treated with tobramycin for a 12 minute period. Supernatants were not concentrated before analysis, but were instead analyzed immediately following exposure. The results shown in Table 6 suggest that leakage of ATP into the supernatant does not occur.

Protein Release and Lethality. Because protein is a major component released into the tobramycin treated cell supernatant, it was of interest to correlate this event with lethality in a short time frame. Cells were suspended in phosphate buffer, instead of water, to eliminate any osmotic effects and to allow for a more progressive lethal event over time. Figure 8 clearly illustrates that protein release parallels the rapid lethal activity by tobramycin. After only one minute of exposure, viable colony forming units were reduced by 39% and nearly twice as much protein was found in the supernatant (a 1.7-fold increase).

Effect on Cell Morphology. Tobramycin has been found to elicit the greatest extent of damage to cells suspended in a water environment. Therefore, water-suspended cells were observed by electron microscopy for any changes in cell structure as a result of short term tobramycin exposure. As shown in Figure 9, there appeared to be no immediate morphological alterations, such as

Table 6. Amount of ATP found in Pseudomonas aeruginosa PAO1[a] supernatants following treatment with 8ug/ml tobramycin.

Growth Source	Time (minutes)	pmol[b]
Glucose	0	0.76 (±0.33)
	3-Treated	0.70 (±0.24)
	12-Treated	0.68 (±0.12)
	12-Control	0.73 (±0.20)
Citrate	0	0.64 (±0.17)
	3-Treated	0.64 (±0.10)
	12-Treated	0.64 (±0.08)
	12-Control	0.64 (±0.10)

[a]Log phase cells were centrifuged and suspended in sterile, distilled water (37°C) before tobramycin treatment.
[b]picomoles of ATP were determined by comparison against standard concentrations of ATP in a luciferase-luciferin assay. Standard deviations from four separate experiments are shown in parenthesis.

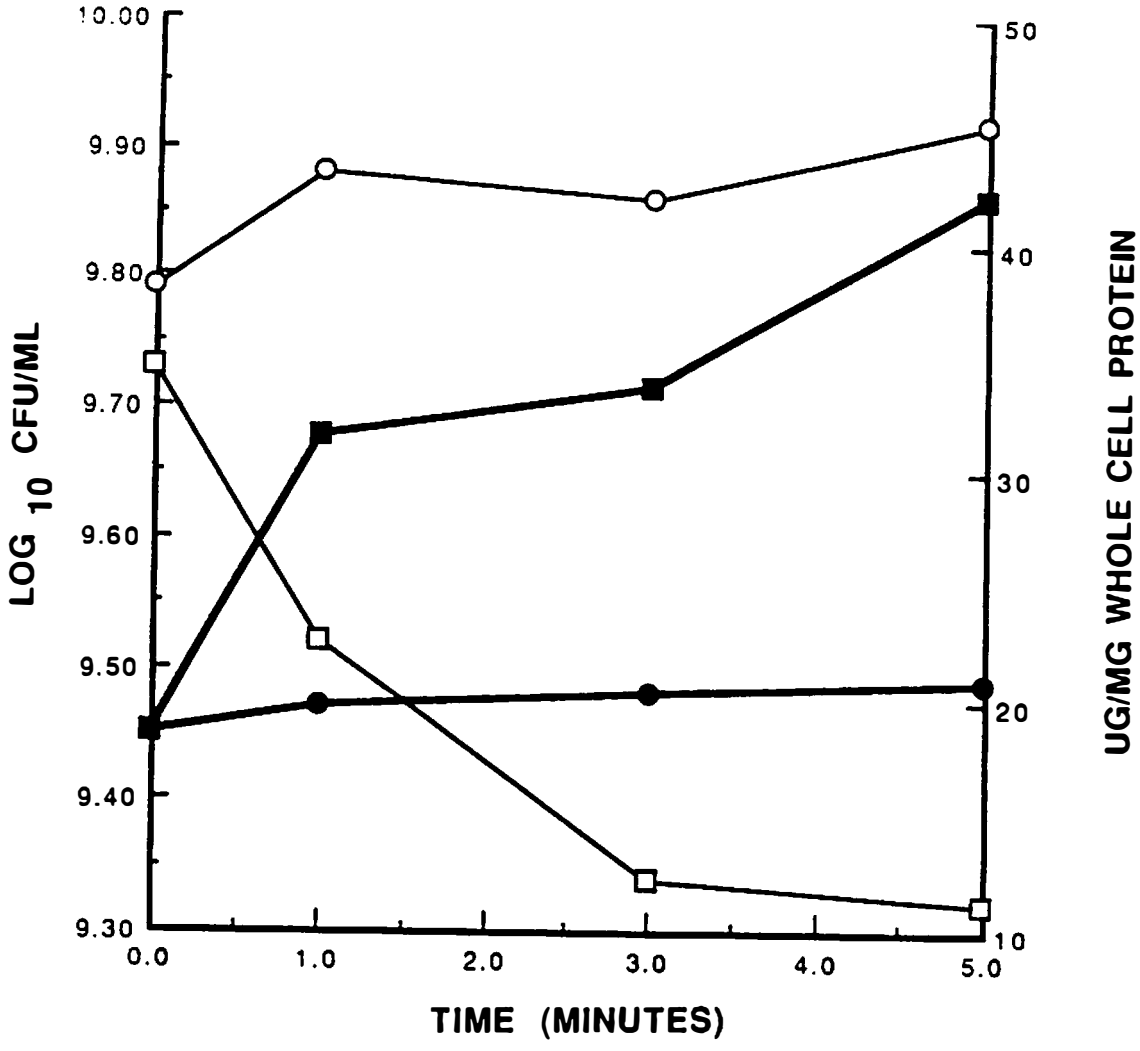

Figure 8. Release of protein from Pseudomonas aeruginosa PAO1 associated with lethality by 8 ug/ml tobramycin. Cells were suspended in 0.01M potassium phosphate buffer (pH=7.0) at 37°C. Lethality over time for control (○) and treated (□) cells was determined by plate counts. Protein release over time for control (●) and treated (■) cells was quantitated by Lowry analysis of the concentrated supernatants. Results represent the average of duplicate experiments.

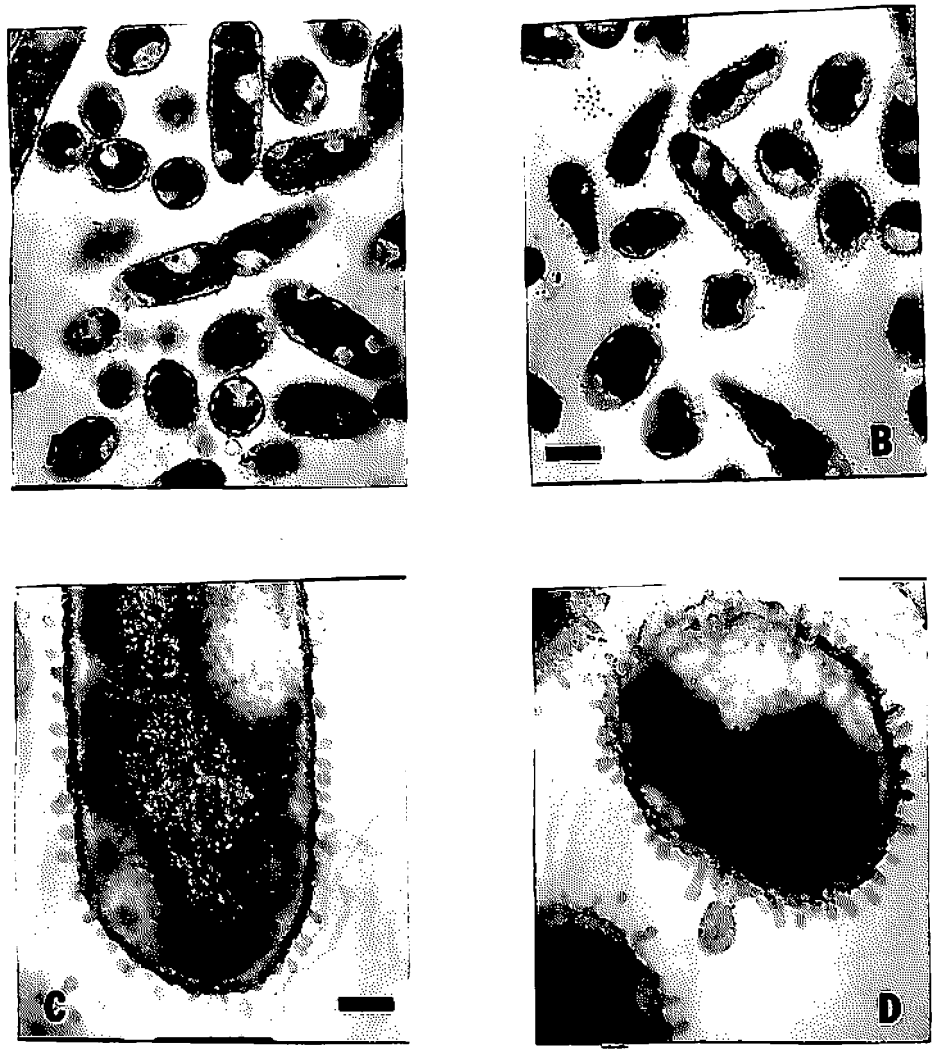

Figure 9. Structure of Pseudomonas aeruginosa PAO1 before and after tobramycin treatment. Morphology was examined by thin-section electron microscopy. The cells shown above were grown on MSM with glucose and suspended in water. (A) Control cells at a magnification of 20,000; (B) cells treated with 8 ug/ml tobramycin for 3 minutes at a magnification of 20,000 (▬ =0.5uM); (C) same as (A), except at a magnification of 100,000 (▬ =0.1uM); and (D) same as (B), except at a magnification of 100,000.

excessive outer membrane blebbing (Tanaka et al., 1983), or the appearance of holes (Martin and Beveridge, 1986) through the surface as has been observed previously. However, the surface features of treated cells in Figure 9D did not appear to be as confluent as the control cells in Figure 9C. It was reported that an association between increased LPS release and bacterial surface blebbing exists following treatment with 25 ug/ml tobramycin for 15 minutes (Martin and Beveridge, 1986). From the electron micrographs in Figure 9, a significant ($P>0.05$ by student's *t* test) increase in the number of blebs per mm between the control and treated cells did not exist. These observations are in good agreement with the data in Figure 7 (page 68) showing no detectable amount of KDO released as a result of short term tobramycin exposure.

Release of Amino Acids from Pseudomonas aeruginosa PAO1 Following Short-Term Exposure to Tobramycin

Initial Detection of Amino Acids. The loss of small cellular components such as potassium (Dubin and Davis, 1961) and nucleosides (Roth et al., 1960) from gram negative bacteria following exposure to aminoglycosides has been previously described. Quantitative differences obtained from protein assays (Figure 7, page 68) of tobramycin treated cell supernatants indicated a possible release of amino acids and/or small peptides.

Supportively, overnight dialysis of supernatant samples caused a subsequent loss of released material as observed by spectrophotometric scans. Therefore, nondialyzed supernatant samples from control and tobramycin treated log phase cells, grown in MSM plus glucose, citrate or succinate, were subjected to thin layer chromatography (TLC). Amino acids were detected with a ninhydrin/collidine reagent, and the mobilities were compared against representative standards of various classes of amino acids (i.e., acidic, basic, aromatic).

As outlined in Table 7, an increased amount of ninhydrin-reactive material was present in tobramycin treated cell supernatants. Strongly reactive component(s) (Rf=0.03) emerged from the origin of the chromatograph in all treated samples. This slowly migrating material probably represents small peptides and polypeptides. An additional, highly mobile component (Rf=0.97) was detected in the glucose grown, tobramycin treated cell supernatants alone, indicative of more extensive damage in these cells.

Amino Acid Analysis. For a more specific analysis of the amino acids released, concentrated supernatants from glucose grown cultures were analyzed by reverse-phase high pressure liquid chromatography (HPLC). The results, as presented in Table 8, revealed that basic amino acids were predominantly released. Compared to the control, a 4.9-fold increase of lysine, a 9.5-fold

Table 7. Resolution of Pseudomonas aeruginosa PAO1 released components[a] by thin layer chromatography.

Standards	Rf	Samples & Growth Source	Rf
Lysine	0.16	Glucose Control	0.54
		Glucose Treated[b]	0.03
Aspartate	0.30		0.40
			0.54
Threonine	0.48		0.97
		Citrate Control	0.54
Proline	0.61	Citrate Treated[b]	0.03
			0.40
Tryptophan	0.66		0.54
		Succinate Control	0.54
Phenylalanine	0.79	Succinate Treated[b]	0.03
			0.40
Leucine	0.86		0.54

[a]Mobile components detected as ninhydrin-collidine positive components.

[b]Approximately 10^8 CFU/ml were treated with 8ug/ml tobramycin for 3 minutes.

Table 8. Release of amino acids from Pseudomonas aeruginosa PAO1[a] following a 3 minute exposure to 8ug/ml tobramycin.

Amino Acid	Control (pmol/mg)	Treated (pmol/mg)	Fold Increase
Basic			
Lysine	1.0	4.9	4.9
Arginine	12.3	116.4	9.5
Histidine	1.0	12.8	12.8
Acidic			
Aspartate	112.8	103.4	-
Glutamate	1175.4	1292.3	1.1
Aromatic			
Tyrosine	0	0	-
Phenylalanine	1.0	1.2	1.2
Tryptophan	0	0	-
Sulfur-containing			
Methionine	0	0	-
Aliphatic			
Glycine	86.9	64.4	-
Alanine	34.7	81.1	2.3
Valine	14.5	33.1	2.3
Leucine	5.7	5.9	1.0
Isoleucine	1.0	2.1	2.1
Imino			
Proline	45.8	45.0	-
Aliphatic Hydroxyl			
Serine	1.0	37.4	37.4
Threonine	14.3	21.3	1.5

[a]Concentrated supernatants were analyzed by reverse-phase HPLC. Results were compared against a 20 amino acid standard and expressed as picomoles per milligram of whole cell protein.

increase of arginine, and a 12.8-fold increase of histidine was observed. If cells were subjected to wash steps with phosphate buffer, prior to tobramycin treatment, the overall amounts of certain amino acids were reduced (Table 9). The curious release of serine, shown in Table 8, was reduced to zero after wash steps, suggesting that this amino acid is loosely associated with the surface. The basic amino acid lysine consistently remained to be a major component released (3.5-fold) even following washing. Additionally, the specific amounts (pmol/mg) of lysine were similar under both preparation conditions.

For comparison, pool amino acids were extracted from log phase, glucose growing cells with ethanol, as outlined in Chapter II. The concentrated extract from both washed and unwashed cells was analyzed by HPLC and compared against standard amino acids. As expected (Table 10), cells subjected to wash steps contain much lower amounts of free amino acids. On the average, there was 5.4 times as many amino acids associated with unwashed cells compared to washed cells. Consistent with the serine release observed in Table 8, washing reduced the amount of cell-associated serine by 6.6-fold. Of particular interest, reduction of the amounts of lysine and arginine, by either prepared extract, was less than 2-fold. The observation that cells retain lysine and arginine better than other amino acids, strengthens the possible mechanism of controlled specific release by tobramycin.

Table 9. Release of amino acids from washed cells of P.aeruginosa PAO1[a] following a 3 minute exposure to 8ug/ml tobramycin.

Amino Acid	Control (pmol/mg)	Treated (pmol/mg)	Fold Increase
Basic			
Lysine	1.0	3.5	3.5
Arginine	24.2	38.5	1.6
Histidine	0	0	-
Acidic			
Aspartate	9.3	11.4	1.2
Glutamate	55.4	55.2	-
Aromatic			
Tyrosine	6.1	7.9	1.3
Phenylalanine	1.0	1.6	1.6
Tryptophan	0	0	-
Sulfur-containing			
Methionine	0	0	-
Aliphatic			
Glycine	1.0	17.9	17.9
Alanine	13.3	39.6	3.0
Valine	12.6	17.1	1.4
Leucine	11.9	20.9	1.8
Isoleucine	5.0	7.4	1.5
Imino			
Proline	16.3	15.9	-
Aliphatic Hydroxyl			
Serine	0	0	-
Threonine	12.3	15.2	1.2

[a]Concentrated supernatants were analyzed by reverse-phase HPLC. Results were compared against a 20 amino acid standard and expressed as picomoles per milligram of whole cell protein.

Table 10. Pool amino acid extraction from exponentially growing Pseudomonas aeruginosa PAO1[a].

Amino Acid	Unwashed Cells[b](pmol/mg)	Washed Cells[c](pmol/mg)
Basic		
Lysine	760.7	419.0
Arginine	2034.0	1294.6
Histidine	ND*	1279.1
Acidic		
Aspartate	7192.3	546.5
Glutamate	50,760.8	3458.1
Aromatic		
Tyrosine	14.8	57.2
Phenylalanine	185.6	44.2
Tryptophan	ND*	ND*
Sulfer-containing		
Methionine	0	0
Aliphatic		
Glycine	2183.0	746.3
Alanine	2203.4	706.1
Valine	976.8	219.4
Leucine	439.5	232.7
Isoleucine	ND*	70.1
Imino		
Proline	1605.6	233.4
Aliphatic Hydroxyl		
Serine	2004.6	304.2
Threonine	1466.3	431.7

[a]Results compared against a 20 amino acid standard.
[b]MSM/glucose grown cells were directly suspended in ethanol.
[c]MSM/glucose grown cells were washed 3 times with 0.01M potassium phosphate buffer (pH=7.0), before ethanol extraction.
*Not determined.

Environmental Supplementation of Basic Compounds. The release of basic amino acids, particularly lysine, suggested that tobramycin may serve a specific lethal role by depletion of the cell's supply of positively charged compounds necessary for growth. Similar to the magnesium experiments described earlier (Table 5, page 63), an attempt was made to restore viability, following a brief exposure to tobramycin, with an exogenous supply of L-lysine at a concentration 100 times that of tobramycin. As revealed in Table 11, this measure had no effect.

Reversal of lethality was then attempted in the more favorable phosphate buffer environment, and cells were supplied with a mixture of basic compounds at 100 times the concentration of tobramycin. In addition to the basic amino acids (lysine, arginine, and histidine), cells were also provided with the polyamine compounds spermine, spermidine, and putrescine. As shown in Table 12, this effort yielded no restored viability. It is apparent that the complex nature of the lethal mechanism of tobramycin can not be explained by a simple depletion of the cell's necessary supply of basic compounds.

Table 11. Inability of lysine to reverse lethality of Pseudomonas aeruginosa PAO1[a] by 8 ug/ml tobramycin.

Sample	Time (minutes)	CFU/ml[b]	% Lethality[c]
Control[d]	0	3.8×10^8	-
	3	3.7×10^8	3
	10	4.9×10^8	0
Tobramycin[e]	0	3.8×10^8	-
	3	1.1×10^7	97
	10	1.5×10^6	>99
Tobramycin and Lysine[f]	0	3.8×10^8	-
	3	1.1×10^7	97
	10	1.3×10^6	>99

[a]Cells were grown in MSM + glucose.
[b]A soft agar overlay was utilized for plate counts.
[c]%lethality was measured as the percent decrease of CFU/ml as compared to the zero minute values.
[d]Cells were plated upon suspension in 37°C distilled water, as well as following 3 and 10 minutes of incubation.
[e]Cells were exposed upon suspension, and plated over 10 minutes of incubation.
[f]A 1.7×10^{-3}M concentration of lysine was added immediately following 3 minutes of tobramycin exposure, and incubated 7 additional minutes.

Table 12. Inability of basic compounds to reverse lethality of Pseudomonas aeruginosa PAO1[a] by 8 ug/ml tobramycin.

Sample	Time (minutes)	CFU/ml[b]	% Lethality[c]
Control[d]	0	1.5×10^9	-
	3	1.5×10^9	0
	10	1.4×10^9	7
Tobramycin[e]	0	1.5×10^9	-
	3	4.1×10^8	73
	10	2.8×10^7	98
Tobramycin and Basic Mixture[f]	0	1.5×10^9	-
	3	4.1×10^8	73
	10	3.6×10^7	98

[a]Cells were grown in MSM + glucose.
[b]A soft agar overlay technique was utilized for plate counts.
[c]%lethality was measured as the percent decrease of CFU/ml as compared to the zero minute values.
[d]Cells were plated upon suspension in 37°C potassium phosphate buffer (0.01M, pH=7.0), as well as following 3 and 10 minutes of incubation.
[e]Cells were exposed upon suspension, and plated over 10 minutes of incubation.
[f]A 1.7×10^{-3}M concentration of L-lysine, L-histidine, L-arginine, spermine, spermidine, and putrescine was added immediately following 3 minutes of tobramycin exposure, and incubated 7 additional minutes.

Release of β-Lactamase from Pseudomonas aeruginosa PAO1 Following Short-Term Exposure to Tobramycin

Release of a 29kDa Protein. In addition to the release of small components such as basic amino acids, it was evident in preliminary studies (Figure 7 and 8, pages 68 and 72, respectively) that tobramycin is highly effective in provoking the release of cellular protein. It was necessary to determine whether or not this event constituted a loss of general protein, or, similar to the amino acids, showed some selectivity. Morphological examinations (Figure 9, page 73) are indicative of a more specific process because general lysis of cells had not occurred. Concentrated supernatants from P. aeruginosa PAO1 i) grown with glucose, citrate, or succinate as a carbon source; ii) suspended in water or phosphate buffer; and iii) exposed to 8 ug/ml tobramycin for three to twelve minutes were analyzed for their specific protein content by SDS-PAGE. Silver-staining for protein (Wray et al., 1981) was chosen over Coomassie Blue as an indicator because of the greatly enhanced sensitivity of this method. As shown in the representative gel (Figure 10), a specific protein (arrow, lane B, M_r=29kDa), in addition to smaller molecular weight material, was released as a consequence of tobramycin exposure. It should be noted that the dark area below the dye front (lane B) includes tobramycin itself.

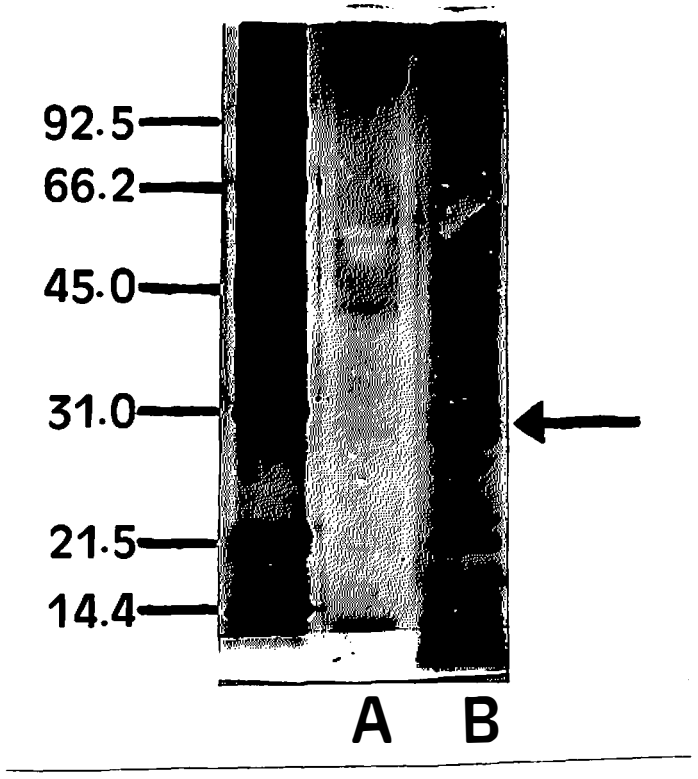

Figure 10. SDS-PAGE of glucose grown Pseudomonas aeruginosa PAO1 concentrated cell supernatants. (A) Water suspended, untreated, glucose grown cell supernatant; and (B) supernatant from glucose grown cells, suspended in water, and treated with 8 ug/ml tobramycin for 3 minutes.

The 29kDa protein was consistently released regardless of environmental conditions or carbon growth source. In agreement with UV-scan observations mentioned earlier, the organic acid grown, tobramycin exposed cells, appeared to release the protein progressively (i.e. the intensity of the band increased over time). However, the overall SDS-PAGE protein profile remained remarkably similar between carbon substrates. In spite of the considerable differences in growth inhibition and lethality between glucose and citrate grown cells, the data accumulated concerning the composition of the tobramycin released material, suggests a minimal role for carbon source in leakage, if any. Therefore, carbon substrate comparisons were discontinued, and glucose grown cells were employed in following experiments as the "most susceptible" system in order to accentuate tobramycin activity.

Isolation and Identification. Experiments were conducted to isolate and identify the 29kDa protein in an effort to trace it's origin. Based on previous studies of aminoglycoside membrane permeabilization (Hancock et al., 1981b; Hancock and Wong, 1984; Loh et al., 1984; Moore et al., 1986) and proteolytic effects (OGaard et al., 1986; Warren et al., 1985), two separate possibilities were particularly appealing. First, if the export of protease is blocked by aminoglycosides, the 29kDa protein may be a degradation product of enhanced proteolytic activity at the outer

membrane. Two assays, i) general hydrolysis by protease in skim-milk-peptone protease agar plates, and ii) the quantitative, colorimetric azocasein method (Jagger et al., 1983), were employed to investigate enhanced protease activity. No activity was detected by either technique in both tobramycin treated and untreated cells. It has been suggested that MSM/glucose growing cells do not produce a sufficient quantity of protease to be detected (Bengt Wretlind, personal communication).

The possibility that the 29kDa protein originated from the periplasmic space was investigated next. Two enzyme assays were investigated as indicative "markers" of periplasmic leakage. Supernatants were scanned for the presence of i) alkaline phosphatase, and ii) asparaginase, which are both located in the periplasmic space. The method of Thomassen and Lutenberg (1980) was employed for detection of alkaline phosphatase. It was found that this enzyme is not induced under the growth conditions in these experiments. Asparaginase, as examined by a modification of the procedure by Yellin-Wriston (1966) coupled to the Berthelot ammonia reaction (Chaney and Marbach, 1962), was present in cell sonicates but not in supernatant samples.

To isolate and further study the 29kDa protein, liter volumes of tobramycin treated cell supernatants were obtained, and concentrated and dialyzed extensively against water (4°C). The lyophilized material was then passed through a Sephacryl S-200 gel filtration column. The elution profile is shown in Figure 11.

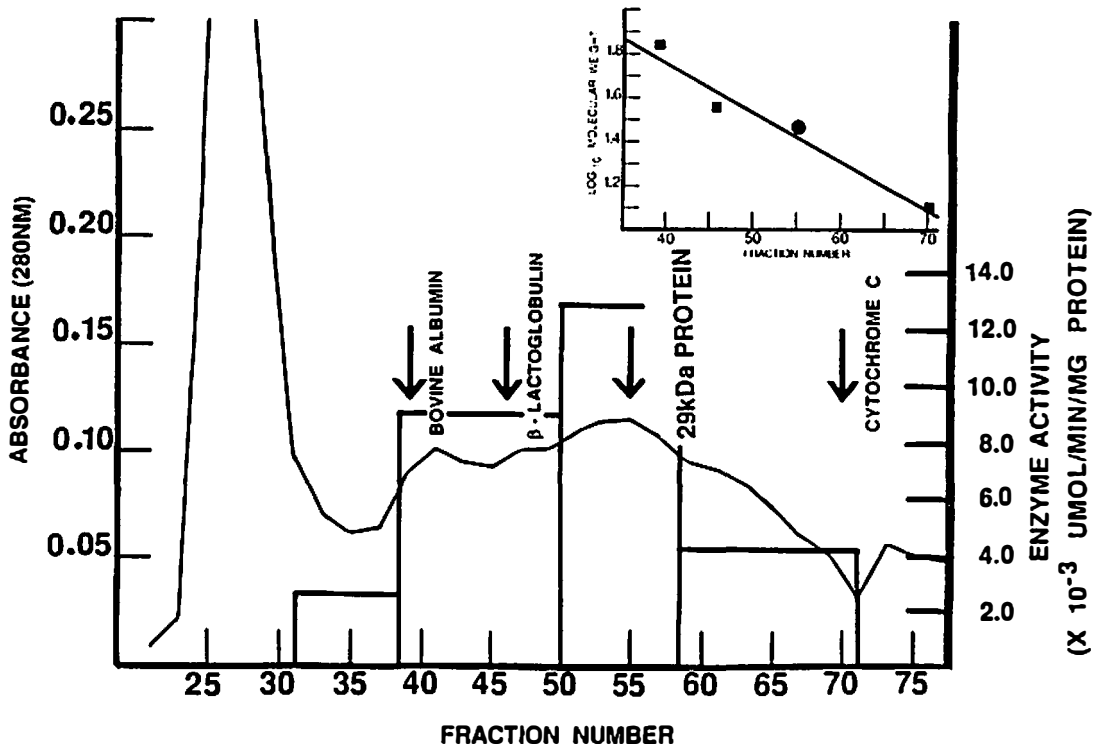

Figure 11. Elution profile, molecular weight determination of protein over Sephacryl S-200, and β-lactamase activity of selected pooled fractions. The concentrated supernatant from tobramycin treated Pseudomonas aeruginosa PAO1 was placed over a 1.5cm x 50cm column. The 29kDa protein was the predominant species in fractions 50-58, and the relative molecular weight was estimated by comparison with standard proteins (see inset, correlation coefficient=0.99 by simple linear regression).

Fraction numbers 50 through 58 contained predominantly the tobramycin released low molecular weight protein as observed by SDS-PAGE (Figure 12, lane B). A small amount of this particular protein appeared in fraction numbers 38-49 (Figure 12, lane A), however, high molecular weight material was also present. Fraction numbers 59-71 contained very little protein (Figure 12, lane C), as did all fractions below 37. The results shown in Figure 12 are from an automated PhastSystem 10-15% gradient polyacrylamide gel, which was used to expedite analysis. Although the profiles were generally comparable, in comparison to the standard 10% gel (Figure 10, page 85), certain discrepancies exist in the rate of migration of both the tobramycin released protein, as well as the standard molecular weight markers. Also, some of the lower molecular weight proteins appearred as "doublets" in the gradient gel for which we have no explanation.

To obtain a more accurate estimate for the molecular weight of the tobramycin-released protein, three standard proteins were applied over the S-200 column. By extrapolation from a standard curve (see inset, Figure 11) of the standard protein elution profiles, the native molecular weight of the tobramycin released protein was determined as 28kDa, which is very close to that initially observed by SDS-PAGE (Figure 10, page 84).

Since P. aeruginosa is known to produce a low basal level of a 29kDa β-lactamase (Nordstrom and Sykes, 1974), pooled

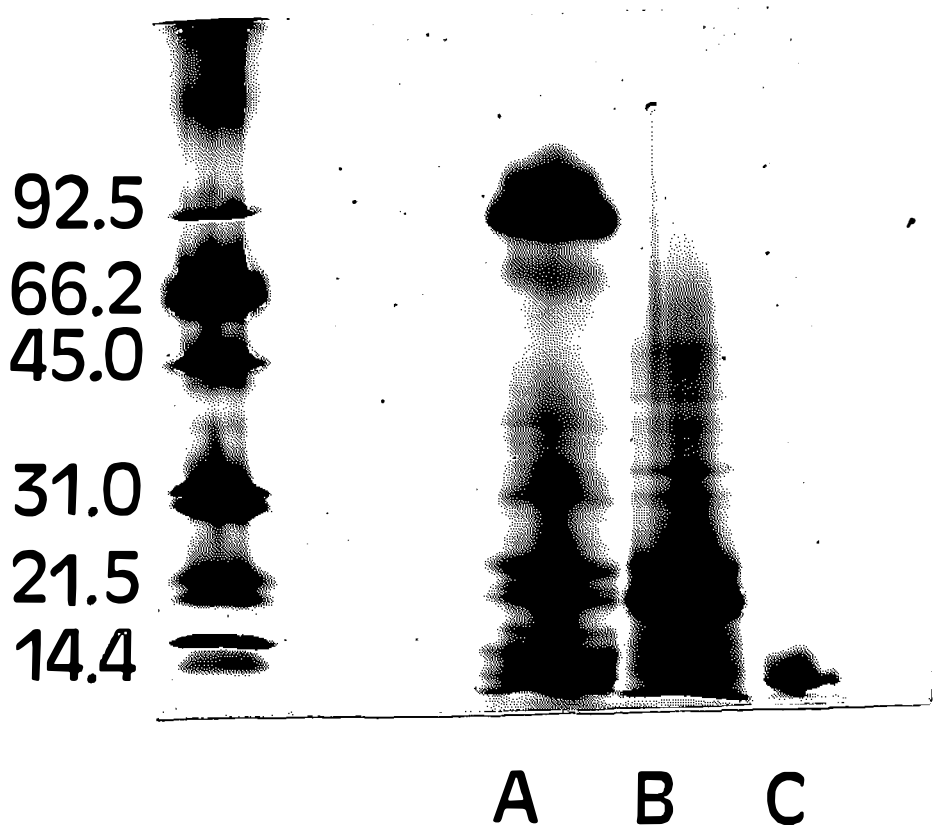

Figure 12. Protein profiles by SDS-PAGE and silver-staining of Pseudomonas aeruginosa PAO1 tobramycin treated cell supernatants following separation over Sephacryl S-200. (A) fraction numbers 38-49; (B) fraction numbers 50-58; and (C) fraction numbers 59-71.

column fractions were investigated for the presence of this enzyme by nitrocefin hydrolysis. It was found that enzyme activity was associated with fraction numbers 50 through 58, which contained the 29kDa protein seen in gel electrophoresis profiles. These data provided strong evidence that the released protein is indeed β-lactamase, a periplasmically-located enzyme. Attempts were made to induce the enzyme for easier detection, by growth in the presence of subinhibitory concentraions of benzylpenicillin. Unfortunately, the amount necessary for induction resulted in critical morphological alterations such as increased adherence and elongation of individual cells, as observed by microscopy. The effort was discontinued due to these undesirable side effects.

Because isolation procedures may inactivate enzyme activity, concentrated supernatants were examined for β-lactamase. The results in Table 13 show that concentrated supernatants from tobramycin exposed cells exhibited 7.3 times as much specific enzyme activity than in untreated supernatants. By comparison with the extracts of sonicated cells, 42% of this enzyme activity in exponentially growing MSM/glucose cells (sonicate) was present in tobramycin treated cell supernatants. Only 6% of the activity was found in untreated, control cell supernatants.

Although the periplasmic marker, asparaginase, was not detected in cell supernatants, it was desirable to confirm that a short-term exposure to tobramycin does not potentiate a flux of

Table 13. Release of β-lactamase from Pseudomonas aeruginosa PAO1 by exposure to 8 ug/ml tobramycin for 3 minutes.

Sample	Enzyme Units (umol/min/ml)[a]	Specific Activity (umol/min/mg)[b]	% activity
Sonicate		3.39×10^{-2}	100
Supernatant from untreated cells	1.3×10^{-3}	1.95×10^{-3}	6
Supernatant from tobramycin-treated cells	1.8×10^{-2}	1.42×10^{-2}	42

[a]Micromoles of substrate destroyed per minute per milliliter of sample at 37°C, pH=7.0.
[b]Micromoles of substrate destroyed per minute per milligram of protein at 37°C, pH=7.0.

general periplasmic contents. Therefore, an osmotic shock procedure was conducted with exponentially growing cells. As shown in Figure 13, SDS-PAGE (gradient gel) analysis of the osmotically released material revealed the presence of several low molecular weight proteins in addition to the 29kDa protein.

Aminoglycoside Activity Toward Pseudomonas aeruginosa Cystic Fibrosis Isolates

Resistance of P. aeruginosa toward aminoglycoside antibiotics is gradually increasing, particularly in cystic fibrosis (CF) patients. The strains isolated rarely possess resistance plasmids (MeNeill et al., 1984), but instead exhibit unusual cell surface properties. These altered characteristics are thought to be the primary reason for resistance. Within this section, studies have been initiated for the eventual assessment of aminoglycoside activities at the cell surface of CF strains.

Comparative Evaluation of CF and Other Clinical Isolates. To assess the antibiotic response(s) of CF strains, the Kirby-Bauer (KB) disc diffusion assay was conducted with 26, nonmucoid, sputum isolates obtained from patients at various stages of infection. The results in Table 14 show that aminoglycosides as a group (kanamycin, gentamicin, tobramycin, and amikacin) reflect the suspected resistance patterns currently emerging.

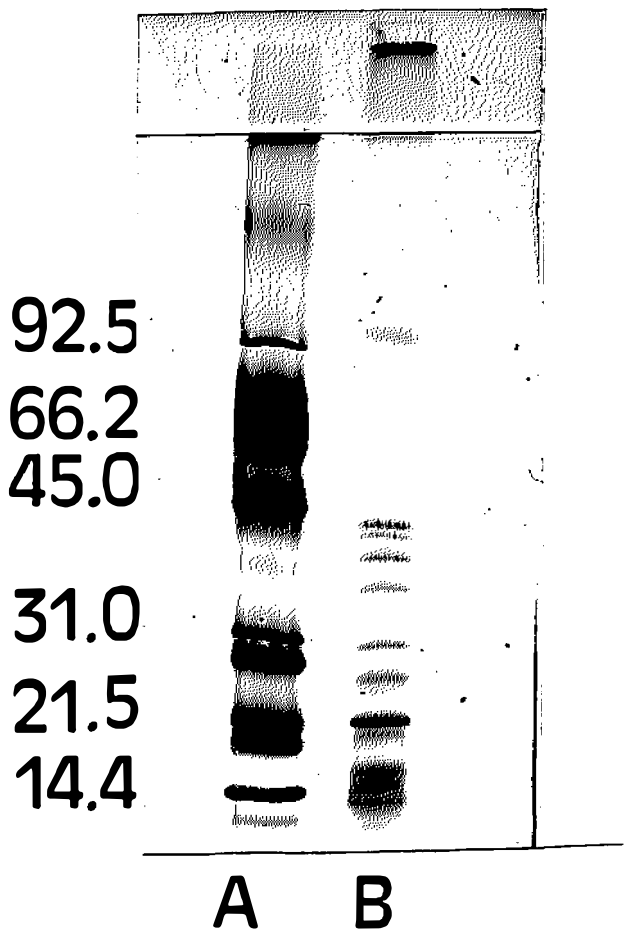

Figure 13. Analysis of periplasmic protein content by SDS-PAGE of glucose grown, Pseudomonas aeruginosa PAO1, following osmotic shock. (A) Molecular weight markers, and (B) osmotically released protein.

Table 14. Antibiotic responses of a series of cystic fibrosis strains of Pseudomonas aeruginosa[a].

Antibiotic	Resistant		Intermediate		Susceptible	
	#/total	%	#/total	%	#/total	%
Kanamycin (30 ug)	26/26	100	0/26	0	0/26	0
Tobramycin (10 ug)	5/26	19	7/26	27	14/26	54
Gentamicin (10 ug)	17/26	65	7/26	27	2/26	8
Amikacin (30 ug)	9/26	35	9/26	35	8/26	31
Piperacillin (100 ug)	3/26	12	2/26	8	21/26	81
Carbenicillin (100 ug)	7/26	27	2/26	8	17/26	65
Polymyxin B (30 ug)	0/26	0	0/26	0	26/26	100
Colistin (Polymyxin E) (10 ug)	0/26	0	0/26	0	26/26	100

[a]Kirby-Bauer assay of 26 isolates.

An interesting finding from these data is that the organisms are 100% susceptible to polymyxins. This is unusual because of presumptions made about aminoglycosides and polymyxins sharing the same mechanism of activity in permeabilization of the outer surface (Dalhoff, 1987; Hancock and Wong, 1984; Rivera et al., 1988). No correlations could be made with respect to resistance or susceptibility between rough or smooth isolates, or between CF patients in poor or good clinical condition.

For comparison, the aminoglycoside response properties of both CF and 145 random clinical isolates of P. aeruginosa were examined. The source of isolation is outlined in Table 15. Antibiograms (or disc diffusion tests) were conducted, and the results are presented in Table 16. As shown, the susceptibility percentages of CF to clinical strains reitterate the unique aminoglycoside resistance properties belonging to CF strains. For example, 98% of general clinical isolates are susceptible to gentamicin, compared to only 8% of CF strains.

In order to strengthen the KB results obtained, a minimal inhibitory concentration (MIC) tube dilution assay in MH medium was conducted with selected strains. Tobramycin served as the model aminoglycoside. In addition to the P. aeruginosa wild type strains PAO1 and ATCC-9027, three mutant strains, and five CF strains were examined (Table 17). As expected, wild-type organisms were highly susceptible to tobramycin because there are no known surface mutations or plasmids. The multiflagellated

Table 15. Source of isolation of Pseudomonas aeruginosa for antibiograms.

Isolates	Urine	Wound	Resp.	Blood	Genit.	Stool	Fluid
Clinical (145 samples)	39%	6%	40%	8%	1%	1%	1%
Cystic Fibrosis (26 samples)			100%				

Table 16. General antibiograms of clinical vs. cystic fibrosis isolates of Pseudomonas aeruginosa.

Type	Gentamicin		Tobramycin		Amikacin		Kanamycin	
	Clin.	CF	Clin.	CF	Clin.	CF	Clin.	CF
Susceptible	91%	8%	93%	54%	98%	31%	-	-
Intermediate	6%	27%	3%	27%	1%	35%	13%	-
Resistant	3%	65%	3%	19%	1%	35%	86%	100%

Table 17. Comparative response of Pseudomonas aeruginosa strains to tobramycin.

Strain	MIC (ug/ml)[a]
PAO1 (wild type)	1.0
ATCC-9027	0.5
PJ108-0331 (multiflagellated mutant)	1.0
902 x 503^{+} (secretion mutant)	2.0
902 x 503^{-} (secretion mutant)	1.0
35c (CF)	1.0
572b (CF)	1.0
572d (CF)	0.5
351g (CF)	4.0
503cc (CF)	8.0

[a]Minimal inhibitory concentrations (MIC's) determined in Mueller-Hinton media with an inocula of 10^5 CFU/ml.

mutant and protease secretion mutants were also susceptible to tobramycin.

The CF isolates, 572b and 572d, were determined to be aminoglycoside susceptible by the KB method. In agreement, these strains exhibit a low MIC. P. aeruginosa 35c was designated as having an intermediate response to aminoglycosides. However, the MIC of 35c was comparable with susceptible strains. Finally, aminoglycoside resistant CF isolates (by KB) did possess a low-level of resistance to tobramycin. Resistance to low concentrations of antibiotics is indicative of outer surface alterations, whereas high-concentration resistance is characteristic of a plasmid-oriented enzyme modification response.

Release of Material in CF Strains. Leakage studies were initiated to examine the similarities or differences, in response to tobramycin of CF isolates compared to wild-type PAO1. Supernatants were obtained as described previously for PAO1, of the representative CF strains from Table 17. Difference spectra of supernatants were compared (Figure 14). The resistant strains, 503cc (A) and 351g (B), demonstrated no enhanced leakage from tobramycin exposure. KB-intermediate strain 35c (C) also showed no release. Strain 572b (D), a KB-susceptible isolate, did reveal increased absorbance, whereas 572d (E) (also susceptible) did not. The UV profile of 572b (D) varied slightly in comparison with the

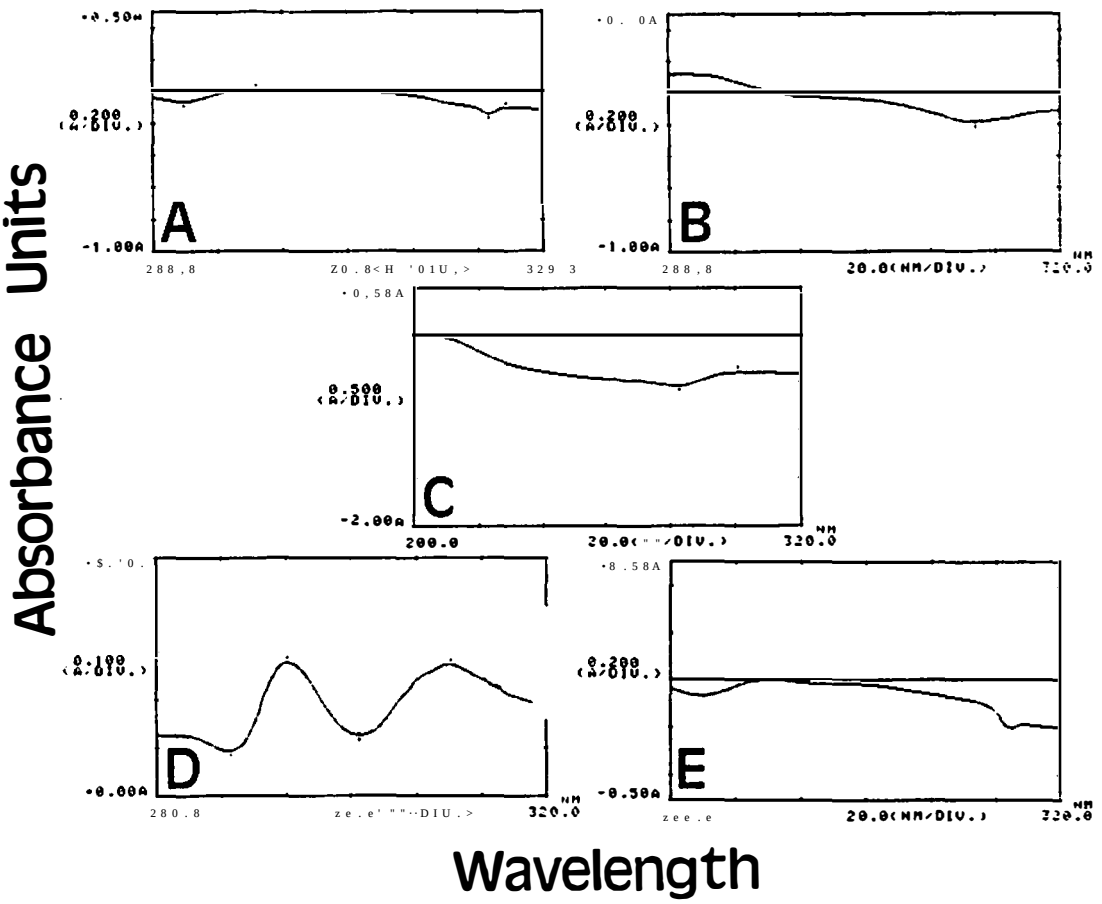

Figure 14. Release of UV-absorbing material from Pseudomonas aeruginosa CF isolates following exposure to 8 ug/ml tobramycin. Strains examined include 503cc (A), 351g (B), 35c (C), 572b (D), and 572d (E).

PAO1 profile, indicative of some differences in the specific composition of the material. Also, the amount of material released was not as great as that from PAO1. These results indicate that the approach used with P. aeruginosa PAO1, as a model susceptible strain, can be applied to the analysis of susceptibility and resistance mechanisms in selected clinical isolates.

CHAPTER IV

DISCUSSION

Research with aminoglycoside antibiotics has been conducted for more than 25 years, yet considerable controversy still exists concerning their lethal activities and cellular resistance mechanisms. To amend some of the confusion, Taber et al. (1987) suggest that each antibiotic be considered individually in reference to a mechanism of action or to the energetics of uptake. They also expressed the "need to explore the possibility that a given aminoglycoside may enter cells of different species by somewhat different routes." From the results in Chapter III, (Figure 1, and Tables 1 and 2, pages 47, 50, and 51, respectively), it is evident that both the growth conditions and the drug exposure environment influence the magnitude of activity by aminoglycosides. Many inconsistencies found in the literature can be attributed to the various experimental conditions utilized. Additional confusion is encountered due to extrapolations made between similar groups of antibiotics or bacteria. These problems are then compounded by the many reported aminoglycoside observations which may or may not relate to the initial lethal event. In the literature, it is often difficult to distinguish between what constitutes a lethal activity and what is the result of lethality. In this study, great care has been taken to maintain

consistent, well-defined growth conditions, and to outline the influence of various environmental components during antibiotic exposure. Additionally, these studies attempt to differentiate between the primary and secondary effects elicited by tobramycin with respect to time following exposure. Simplification of the reaction mixture minimizes variables which can confuse proper interpretation of results.

Summation of Results

The cellular response to aminoglycoside exposure is described as pleotropic (Dalhoff, 1987). An effort was made in this study to sort out some of these activities by focussing on rapidly occurring physiological events associated with lethality. For this reason, a somewhat broader approach to this problem was initially taken in an effort not to exclude or overlook alternative mechanisms. An initial observation that cell leakage was occurring rapidly remained the central observation and basis for further investigation. A brief summary of the results may help to clarify and condense this study for the purposes of discussion. Therefore:

1. Lethality by tobramycin is immediate (1-3 minutes), appears to be irreversible, and does not seem to necessitate additional influx of antibiotic for lethality, following the initial exposure.

2. Tobramycin-potentiated release of β-lactamase, as well as certain amino acids, occurs concomitantly, if not prior, to

inhibition of protein synthesis. Also, leakage appears directly associated with lethality.

3. The extent of tobramycin uptake, leakage, and lethality is influenced by the amount of magnesium in the environment.

4. Comparative carbon growth source studies suggest specific metabolic roles may exist for the magnitude of lethal damage by tobramycin.

5. The inhibition of amino acid uptake (as exemplified by proline), the release of LPS, the general loss of protein or ATP, the depletion of general pool amino acids or polyamines, the occurrence of drastic morphological alterations, as well as overall cell lysis, are not involved in the primary stages of lethality by tobramycin.

6. Resistant CF strains do not release material under the same tobramycin-exposure conditions as P. aeruginosa PAO1. However, one of two susceptible CF isolates does release material.

Lethality, Influence of Environmental Components, and Role of Carbon Growth Substrate

As shown in Tables 1 and 2, (pages 50 and 51), lethality (defined as the inability to form colonies) occurred immediately upon exposure to tobramycin (1-3 minutes). This particular observation is of considerable importance because many previous studies have been conducted with antibiotic exposure periods of ten or more minutes. Additionally, the drug concentrations utilized

by others were often much higher than the 8 ug/ml employed here. This low concentration was chosen to prolong the initial activities (50-80% lethality) and reduce post-lethal events. Also, 8 ug/ml tobramycin is ineffective in growth inhibition of some resistant CF isolates so that comparative analyses may be pursued in future studies. It has been cited elsewhere (Miller et al., 1987) that "abbreviated time-kill studies may be preferable to conventional 24 hour data....." and that "standard 24 hour studies are unnecessary and possibly misleading." In agreement, we question the validity of such studies, relative to killing, when as revealed here, greater than 50% lethality occurred after only three minutes of exposure to 8 ug/ml tobramycin in a buffered environment.

From another perspective, one could argue that a separate means for defining lethality is needed. The results presented here are actually a reflection of the lost function of cell division. For example, it was found that azlocillin produces striking ultrastructural changes by deposition of regular arrays of electron-dense material within P. aeruginosa (Elliott and Greenwood, 1983). The authors speculate that this material may be excess, abberant formation of peptidoglycan due to the absence of septation. If true, the cells are still actively metabolizing peptidoglycan, but are unable to divide which eventually leads to death. However, as shown in Figure 9 (page 73), no such morphological changes are found in these studies. The activities of aminoglycosides at the ribosomal level (Figure 5, page 63),

eliminate protein synthesis as an appropriate measure for lethality. Perhaps the use of vital dyes or stains will provide a valid, additional means for defining lethality. It is not understood why only a few investigators have attempted to measure and correlate lethality with their aminoglycoside observations.

The protective role of tobramycin competitive or inhibitory substances in the environment, such as magnesium and phosphate, had to be ascertained in these studies. Other studies indicated that the initial interaction of aminoglycosides on the cell surface of P. aeruginosa involves an ionic displacement of magnesium where this cation serves as a cross bridge for adjacent molecules of LPS (Eagon, 1984; Hancock and Wong, 1984; Loh et al., 1984; Nicas and Hancock, 1983; Peterson et al., 1985; Rivera et al., 1988). Additionally, the presence of magnesium in the surroundings is found to increase the minimal inhibitory concentrations (MIC's) of aminoglycosides (Abdel-Sayed et al., 1982). It should be noted that the protection of P. aeruginosa by magnesium or calcium is not due to a modification of the antibiotics (aminoglycosides, polymyxins) by these ions (Zimelis and Jackson, 1973).

Consistent with previous suggestions, it was found in these studies that decreasing or eliminating magnesium in the surroundings resulted in an enhanced susceptibility to tobramycin (Table 4, page 56). Furthermore, it was directly established that the increased susceptibility was associated with decreased uptake of tobramycin at the cell surface (Figure 3, page 59). In support,

Thompson and Eagon (1985) have shown that uptake of dihydrostreptomycin in P. putida membrane vesicles is not inhibited by magnesium (or spermidine), therefore protection must exist at an external site in the gram negative envelope. The much smaller degree of protection provided to cells by phosphate, compared to water, is thought to be attributable not only to some interaction of tobramycin with phosphate ions in solution, but also the more osmotically favorable environment. In consideration of the protective role of magnesium, previous reports which employ lengthy antibiotic exposure periods in an enriched medium, may be partially justified. However, the higher drug concentrations utilized may, in turn, compensate for the presence of these cations. Although the effect of magnesium on the MIC was not determined here, it is of interest to note that the inhibition of MSM/glucose growing cells (Figure 1, page 47) is very comparable to the MIC determined in the enriched MH medium (Table 17, page 98, magnesium content unknown). This suggests that the extent of tobramycin interaction with the cell surface in MSM versus enriched MH medium does not differ.

As mentioned earlier, Nicas and Hancock (1983a) imply that gentamicin has a much higher affinity for the cell surface of P. aeruginosa because 1,000-fold excess of magnesium (compared to gentamicin) is absolute for protection. However, data reported here show that only a 100-fold excess of magnesium provides a substantial degree of protection to cells (Table 4, page 56). It may

be that the affinity of tobramycin for P. aeruginosa is not as strong as that of gentamicin. In the case of divalent cations, for instance, calcium is observed to exhibit tighter binding than magnesium to P. aeruginosa LPS complexes (Rivera et al., 1988). On the other hand, a higher affinity by gentamicin, compared to tobramycin, would not account for the higher tobramycin susceptibility observed in CF strains (Table 16, page 97). This is, of course, assuming that the interaction site is exposed in these isolates. It may be of future interest to conduct antagonistic studies between different cationic antibiotics, which may reveal the unique properties of activity of each compound.

These data show that the degree of susceptibility to tobramycin is related to growth on a particular carbon substrate. By growing PAO1 in a defined medium with glucose as the carbon source, the inhibition of growth (Figure 1, page 47), as well as the degree of killing (Tables 1 and 2, pages 50 and 51), is greater than that observed with cells grown in the same medium with citrate or succinate as the carbon source. It is interesting that citrate grown cells, which may possess depleted magnesium due to chelation, were most resistant. Also, Conrad et al. (1979) report adaptive resistance of P. aeruginosa toward polymyxin when grown on glucose or glutamate. The possibility that the phase of growth may account for differences in these studies was examined (Table 3, page 52) and excluded. All data taken together suggest that a specific physiological function related to carbon metabolism may

be involved in the degree of susceptibility or resistance. Also, differences in resistance mechanisms may exist between similar cationic compounds.

It is known that certain proteins of the glucose and gluconate transport systems, and the Entner-Doudoroff enzymes, are induced during growth on glucose (Eagon, 1984). It may be that one of these inducible proteins serves to enhance the interaction with, or permeabilization of the outer membrane by, tobramycin. Another possibility for the increased susceptibility of glucose grown cells may be a lack of intracellular energy levels necessary for cell surface repair mechanisms following damage. It is known that glucose grown P. aeruginosa has a significantly lower level of ATP than when an organic acid serves as the substrate (Montie and Montie, 1979). Finally, since porin is known to serve as a major route of uptake for many antibiotics (Nakae and Nakae, 1982), it is possible that a glucose-inducible porin (Hancock and Carey, 1980) may serve to enhance the uptake of tobramycin into the cell.

Leakage of Cellular Material

Following an assessment of the contributions by carbon growth substrate and environmental components in lethality, continued cell surface studies were conducted with a suitable system which would outline and accentuate the precise and primary effects elicited by tobramycin. Both phosphate buffer and water environments were chosen for examination. Also, more

detailed studies were conducted with glucose grown cells, as a most sensitive model system.

As revealed in Chapter III (Figures 6, 7, and 8 pages 66, 68, and 72), tobramycin potentiates the release of certain cellular components. Leakage was first denoted by an increase of UV-absorbing material (Figure 6) in tobramycin treated cell supernatants. The material, as discussed in more detail later, contained specific protein and amino acids. Early investigations of the mechanism of streptomycin activity in E. coli, revealed an efflux of potassium ions following aminoglycoside exposure (Dubin and Davis, 1961). The release of nucleotides has also been reported (Roth et al., 1960). Although nucleotides may partially account for UV absorbance, it is stated that release of these compounds is not associated with lethality (Tzagoloff and Umbreit, 1962). Schlessinger (1988) stated in a recent review that the data concerning the efflux of potassium ions, suggest that this is a post-lethal event. Based on these reports, the presence of potassium ions and nucleotides in the supernatant was not investigated here. Additional substances may also be present, but the importance of identifying minor constituents is difficult to assess.

There was little difference in the extent of general protein released between phosphate and water suspended cells. In each case, an approximate 2-fold release occurred by three minutes. Absorbance profiles were very similar as well. Surprisingly, the

release of protein was qualitatively similar among cells grown with various carbon substrates. The only difference was that protein release in citrate and succinate grown cells was more gradual over time. Apparently the role of carbon source is minimal in tobramycin-potentiated leakage. With regard to the importance of energy levels for cellular repair, it was thought that glucose grown cells may show enhanced leakage of ATP. However, no such effect was observed (Table 6, page 71). It is known that the sodium-potassium ATPase is inhibited by gentamicin in erythrocyte membranes (Chahwala and Harpur, 1982), but a very high concentration of antibiotic (10^{-2}M) is necessary for >50% interference. From these combined data, it is doubtful that ATP plays a prominent role in carbon source antibiotic response differences.

In excellent agreement with lethality studies, cells suspended in magnesium sulfate (2.0×10^{-2}M) do not show tobramycin-elicited leakage as detected by UV absorbance profiles. However, cells suspended in 0.85% NaCl reveal enhanced leakage. Such findings strengthen the opinion that one major interaction site(s) for aminoglycosides resides where divalent cations stabilize the macromolecular arrangement of the cell surface by cross-bridging. Monovalent cations may occupy these sites in a more unsteady conformation, and the antibiotic is not only successful in penetrating the surface and potentiating leakage, but the effects are enhanced.

Sequence of Events Following Tobramycin Exposure

One recent proposal concerning the sequence of events by aminoglycosides leading to death of P. aeruginosa, is outlined by Martin and Beveridge (1986). They reported a loss of 34% total cell protein, 30% LPS, and predominant morphological abberations. They hypothesize that gentamicin displaces cations in the outer membrane, which leads to the extraction of outer membrane components such as protein and LPS. Blebbing of membranous material occurs initially, is followed by the appearance of holes through the envelope, which eventually leads to cell lysis and death. However, the earliest time point examined following exposure was 15 minutes, with a gentamicin concentration of 25 ug/ml utilizing buffer suspended cells.

In a more referenced paper, Davis et al. (1986) have proposed that aminoglycoside activity begins with slight initial entry of the drug into the cell. This initial amount then i) interacts with the ribosomes, causing misread protein to be incorporated in the envelope, which ii) leads to increased uptake of additional antibiotic through abnormal channels, and iii) results in death. Again, cells were exposed to 20 ug/ml streptomycin for 20 minutes or longer.

From the data presented in this text, not only does lethality occur within 1-3 minutes by only 8 ug/ml tobramycin, but this initial amount of drug is apparently sufficient for lethality. If Davis' proposal that eventual increased influx of antibiotic was

necessary for death, it would seem logical that the later addition of magnesium (Table 5, page 57) would prevent increased uptake of tobramycin, and cells would survive the initial dose. This opinion is supported in the review by Schlessinger (1988) which outlines that attempts to dilute aminoglycosides to a sub-lethal concentration, following initial exposure, also did not restore viability.

Within the scope of this text, a substantial degree of interference with protein synthesis by tobramycin had occurred by 3 minutes (Figure 5, page 63), but the UV-leakage in Figure 6 (page 66) was obtained from the supernatant of cells exposed to tobramycin for only one minute (environmental growth conditions were the same for both experiments). Less than 1% protein synthesis inhibition had occurred by one minute (Figure 5, page 63). These data, in addition to i) the lack of abnormal envelope morphology in a highly sensitive system (Figure 9, page 73), and ii) the lack of increased LPS in the supernatant following tobramycin exposure (Figure 7, page 68) suggest a separate series of activitie(s) than those outlined by Martin and Beveridge (1986) or Davis et al. (1986).

These data taken together suggest that immediately following cell surface contact, tobramycin first potentiates the release of specific cellular components, which is associated with lethality by initiation of irreversible damage at the envelope level. Interference with protein synthesis rapidly follows this damage.

However, the release of LPS (Martin and Beveridge, 1986), morphological abnormalities (Martin and Beveridge, 1986; Tanaka et al., 1983), and cessation of transport (Eagon et al, 1982; Figure 4, page 62) are probably events which follow lethality.

Loss of Certain Amino Acids

An investigation of the tobramycin-leakage material revealed that basic amino acids are preferentially released (Table 8, page 77). Also, lethality did not involve a simple cellular depletion of basic compounds by tobramycin (Tables 11 and 12, pages 82 and 83). This amino acid release may be an indication of osmotic stress brought about by tobramycin. It is suggested that in Vibrio cholerae, amino acids probably play a direct role in osmoregulation (Miller and Mekalanos, 1988). In fact, it is known that enteric bacteria control osmotic stress, partially, by alteration of intracellular pool sizes of certain amino acids such as proline or glutamate (LeRudulier et al., 1984). The complex nature of osmotic stress signaling is exemplified in a recent study by Csonka (1988). He found that there is an increase of intracellular proline with increased osmotic stress in Salmonella typhimurium. This is thought to be due to osmotically stimulated proline transport systems (ProP and ProU). In sharp contrast, this investigator was unable to detect any such occurrence in E. coli, indicative of a delicate species-specific mechanism.

In aminoglycoside research, it is increasingly evident that

$\Delta\psi$ plays a role in antibiotic uptake and activity (Bryan et al., 1977, 1983; Dalhoff, 1987; Mates et al. 1983; Taber et al. 1987; Schlessinger, 1988). The release of basic amino acids may be an indication of immediate disruption of $\Delta\psi$ by tobramycin. Positively charged amino acid efflux may reflect the cell's effort to maintain an appropriate charge-balance. Bryan et al. (1980) have examined the transport of arginine and lysine in the presence of gentamicin and dihydrostreptomycin. They found that following ribosomal binding, the transport of arginine is accelerated, but that lysine transport is decreased. Although the results presented in Chapter III indicate that basic amino acid efflux occurs either prior to, or simultaneous with, ribosomal interaction, Bryan's observation of increased arginine uptake may result from increased efflux of basic amino acids and maintenance of $\Delta\psi$. Carefully designed labelling experiments might provide an answer to some of these questions.

In following studies, Bryan and coworkers (1980, 1981, 1983) attempted to show that aminoglycoside transport and $\Delta\psi$ may be coupled to electron transport. This possibility has been the subject of much criticism because no other transport mechanism of this type is known to exist. Interestingly, anaerobic cultures exhibit higher resistance to the initial activities of aminoglycosides (Schleissinger, 1988). Bryan suggests that anaerobic cultures may possess a lower rate of electron transport which would lower the proton motive force (PMF) for

aminoglycoside uptake. However, after an initial lag period, there is a sudden increase of aminoglycoside uptake. Some investigators report that the PMF is similar in both aerobic and anaerobic cultures, but that an "oxidative insult," such as that observed with polyene antibiotics, may account for these differences (Schleissinger, 1988).

Mates et al. (1983) suggest that intracellular binding may be the key to those differences observed between aerobic and anaerobic growing cells. Classically, ribosomal binding by aminoglycosides is known to be irreversible, a unique trait for these antibiotics. In the studies by Mates, a protonophore (carbonyl cyanide-*m*-chlorophenyl hydrazone) was utilized, which collapsed the proton gradient, and caused efflux of gentamicin from anaerobically grown cells, but not from aerobic cells. This hypothesis is supported by the observation that membrane vesicles are unable to transport these drugs without cytoplasmic contents (Schlessinger, 1988).

If aminoglycoside killing is related to disruption of membrane potential, it may behave in a manner similar to that of cationic peptides. Kordel et al. (1988), have shown that a small peptide produced by Staphylococcus epidermidis (which contains eight positively charged amino acids), is able to break down the cytoplasmic membrane potential in both gram positive and gram negative bacteria, leading to increased permeation of ions, amino acids, and ATP. This activity is dependent upon a sufficient

membrane potential. These investigators were able to ascertain that it takes less than one second for this peptide to abolish the potassium gradient in Staphylococcus, which should result in cell death. These observations would easily explain why lethality by tobramycin occurs rapidly and why there is increased release of amino acids (Tables 7, 8, and 9, pages 76, 77, and 79) and potassium (Dubin and Davis, 1961).

As an explanation for amino acid excretion, increased proteolytic enzyme activity in supernatants was measured but not found following tobramycin treatment. However, proteolysis still may be involved. Indeed, if aminoglycosides block the excretion of these enzymes (Warren et al., 1985), the release of amino acids may reflect increased proteolytic activity just below the outer surface. It is known that aminoglycosides give rise to the synthesis of abnormal proteins (Davis et al., 1986). Emling and Holtje (1987) have emphasized that such abnormal peptides are rapidly degraded by proteases, and the resultant increased amino acid content would lead to an osmotic problem for the cell. Efflux would simply represent a protective measure by the cell.

Therefore, in evaluation of the data presented here, the efflux of basic amino acids may reflect the cell's response to maintain charge-balance. Such an efflux mechanism would enhance the magnitude of $\Delta\psi$, and potentiate further drug uptake. Yet, as specified earlier, the additional amount of antibiotic is not necessary for lethality. Alternatively, if aminoglycoside

stimulated proteolysis has been initiated, amino acid release may represent the cell's attempt to alleviate increased osmotic stress.

Release of β-Lactamase

In addition to amino acids, protein release by tobramycin is an immediate event (Figures 7 and 8, pages 68 and 72). SDS-PAGE of P. aeruginosa PAO1 supernatants demonstrate the release of a 29kDa protein (Figure 10, page 85). P. aeruginosa constituitively produces a low level of chromosomally-encoded β-lactamase (Nordstrom and Sykes, 1974a, 1974b; Gates et al., 1986). This particular enzyme has a molecular weight of 29kDa (Nordstrom and Sykes, 1974b). Although β-lactamase release as an initial event of aminoglycoside activity has not been previously documented, it's release from the periplasmic space has been observed in the presence of other antibiotics (Nordstrom and Sykes, 1974a, 1974b; Sanders et al., 1987). In Pseudomonas, β-lactamase was released into the surroundings following exposure to benzylpenicillin (Nordstrom and Sykes, 1974b). Two additional penicillins, amdinocillin and impenem, potentiate leakage of β-lactamase from gram negatives prior to induction (Sanders et al., 1987), demonstrating that induction is not necessary for leakage of this enzyme.

In a preliminary experiment with tobramycin, cells were preincubated with either 200 ug/ml of chloramphenicol or 1mM sodium azide before treatment (data not included). Release of the

29kDa protein was observed by SDS-PAGE. Such results, in addition to those by Sanders et al. (1987), would suggest that release activity by tobramycin does not require active uptake or protein synthesis.

To confirm that the identity of the 29kDa protein is β-lactamase, supernatant samples were applied to a gel filtration column (Figure 11, page 88). The elution profile was compared against the elution properties of standard proteins. Results allowed for a mass estimate of 28kDa for the specific protein. β-lactamase activity was associated with the pooled 28-29kDa fraction. This chromosomally-encoded enzyme is one of two commonly encountered β-lactamases in P. aeruginosa. The other is plasmid-encided (RP1), and has a reported molecular weight of 37kDa (Poole and Hancock, 1983). More definitive proof could be obtained in the future by i) elution of the 29kDa band from the gel, followed by a positive enzyme reaction, or ii) by successfully conducting an enzyme reaction directly on the gel itself.

In excellent agreement with the results presented here (Figure 13, page 94), the excretion of β-lactamase, following exposure to benzylpenicillin in P. aeruginosa, seems to be attributable to an opening into the periplasmic space rather than cell lysis (Nordstrom and Sykes, 1974a). The polycationic properties of tobramycin may contribute to the formation of rigid domains by LPS cross-bridges or to the interaction with head groups of negatively charged phospholipids. β-lactamase may

simply pass through transient cracks in the outer membrane. It would be of interest to know if β-lactamase is located at the outermost region of the periplasmic space. Perhaps this enzyme also is attached to the inner portion of the outer membrane.

From another point of view, it is known that both the outer membrane and periplasmic space have a physical dimension, determined by growth rate, and possessing a limited capacity for macromolecular components such as protein. Click et al. (1988) recently discussed translational control of outer membrane protein synthesis, which may be coupled to export. More specifically, controlled overexpression of OmpC in E. coli provoked a simultaneous elimination of the presence of OmpA and LamB in the outer membrane. Interestingly, there was no decrease in OmpA mRNA synthesis, indicating that this phenomenon is translationally controlled.

If the rapid release of β-lactamase in the presence of tobramycin or β-lactams follows the alteration of critical physical parameters in the envelope, it may be of interest to investigate a possible translational control mechanism for immediate, increased export of β-lactamase. A shared signal site on the cell surface may exist since both tobramycin and β-lactams elicit enzyme release. Also, because magnesium is able to protect cells from leakage, the competitive magnesium-tobramycin interaction site represents one likely candidate.

An attempt was made in these studies to induce

β-lactamase, and enhance enzyme yield, with a subinhibitory level of benzylpenicillin. A concentration of 20 ug/ml is necessary for induction (Nordstrom and Sykes, 1974a). However, severe morphological alterations, such as elongation and filamentous growth occur, and would probably present a false model for study.

This somewhat selective release of β-lactamase is not believed to be the reason for lethality. Gram negatives are known to survive osmotic shock of periplamic contents; loss of only one periplasmic component probably does not endanger the cell. More likely, this event signals a very sensitive cellular response following envelope damage. Efflux of β-lactamase in the case of β-lactam antibiotics, would seem to beneficially enhance the cell's defense mechanism. In future studies, β-lactamase may prove to be a useful general probe for the detection of envelope damage.

Resistance in CF Isolates

Resistance to low concentrations of aminoglycosides (and other antibiotics) is attributed to outer surface alterations (Burns et al., 1985; Bush et al., 1985; Gutmann et al., 1985; McNeill et al., 1984; Moore and Hancock, 1986; Shearer and Legakis, 1985). These resistant strains rarely possess plasmids (McNeill et al., 1984; Preheim et al., 1982). Many studies are directed at the role of the LPS phenotype in low level resistance, however, it is stated that LPS cannot solely account for resistance (Shearer and Legakis,

1985). Apparently, a combination of cell surface components are involved in resistance. The role of cell surface protein appears to have as much an impact as LPS (Buschner et al., 1987; Daikos et al., 1988; Sanders et al., 1984; Werner et al., 1985). Also, the exopolysaccharide (or alginate) produced by mucoid CF isolates does not provide protection to the cells (Nichols et al., 1988; Tannenbaum et al., 1984).

The results obtained with the KB method (Table 14, page 95), were most intriguing in that aminoglycosides and polymyxins displayed separate susceptibility responses. Many researchers assume these two groups of antibiotics share the same mechanism of activity (Hancock and Wong, 1984; Rivera et al., 1988). Only recently has there been additional evidence available suggestive of different mechanisms of activity (or resistance). For example, Gilleland et al. (1984) initially proposed that polymyxin and aminoglycoside resistance in CF strains was related to phospholipid composition. Indeed, polymyxin elicits a rapid (10 minutes) alteration of lipid composition in these organisms, which in turn, eventually provides protection to the cell against polymyxin. However, no such mechanism has been identified in the case of aminoglycosides (Gilleland et al., 1988).

It is not surprising that these antibiotics would exhibit completely separate modes of activity. The aminoglycosides alone possess many pleotropic actions (Dalhoff, 1987). Not only do the antibiotics themselves elicit various properties, but each

organism should be considered as a highly diverse, individual system for resistance. For example, there is considerable strain-to-strain variation in the outer membrane protein profiles of Enterobacter cloacae (Werner et al., 1985). Continued investigation of P. aeruginosa CF isolates will most likely reveal unique biochemical properties not found in the wild-type. The role of environmental components, and growth conditions, as observed in these studies, cannot be ignored. Although magnesium in these studies was unable to reverse the lethal activity of tobramycin, it is interesting to note that addition of calcium or iron to Bacillus subtilis spores, potentiates the release of aminoglycosides, and a reversal of their inhibitory activity (Tochikubo et al., 1988).

One excellent study which exemplifies the reason(s) why general extrapolations between similar classes of antibiotics or genera of bacteria should be avoided, is that by Viljanen et al. (1986). By utilization of the bactericidal action of serum as an indicator for cell surface damage, they show that polymyxin B nonapeptide, a derivative of polymyxin B (no fatty acid tail) elicits comparable damage to that of polymyxin B in E. coli, S. typhimurium, Klebsiella spp., E. cloacae, P. aeruginosa, and Haemophilus influenzae. Unlike polymyxin B, the derivative possessed no bactericidal acitivity. Also, the outer surface of Proteus mirabilis, Niesseria gonorrhoeae, and N. meningitidis were unaffected by polymyxin B nonapeptide.

Conclusions and Hypotheses

Although the specific reason(s) for cell death by aminoglycoside antibiotics still remain unknown, this study has revealed some previuously unknown effects elicited by tobramycin. Particularly, strong evidence is presented for the immediate release of β-lactamase as evidenced by i) the appearance of a 29kDa protein by SDS-PAGE of tobramycin treated cell supernatants, ii) increased β-lactamase activity in the supernatant following tobramycin exposure, iii) β-lactamase activity in the pooled fraction from gel filtration of supernatant, associated with elution of a 29kDa protein, iv) the appearance of a 29kDa protein by SDS-PAGE of supernatants following osmotic shock, and v) the knowledge that P. aeruginosa constituitively produces a low basal level of a 29kDa β-lactamase (Nordstrom and Sykes, 1974a, 1974b). Release of this enzyme is not thought to be the direct cause of lethality, but rather a sensitive indicator of envelope damage. Since leakage occurs within a 3 minute exposure to a low concentration of tobramycin, this may represent a pre-lethal event. It is possible that β-lactamase release is the earliest detectable sign of envelope damage. Such a mechanism may serve as a useful probe for previously undetectable damage.

Future studies directed toward defining what constitutes a lethal event by aminoglycosides may necessitate the use of mutant strains. For example, a site-specific mutation (in a susceptible strain) which would confer resistance as a result of the altered

expression of a certain physiological target, may unveil the site of lethal activity. Additionally, comparative studies with P. aeruginosa CF strains may prove to be useful tools to describe the significance of "leakage" with respect to lethality.

It is the opinion of this author that further examination of the mechanism of basic amino acid efflux may lead to a possible explanation for cell death. As was discussed earlier, efflux may be a reflection of the cell's effort to maintain charge balance following tobramycin uptake. One inconsistency with this hypothesis was the failure of tobramycin to interfere with proline uptake (Figure 4, page 62). However, this may be attributable to a function of the osmotically regulated proline transport systems (Csonka, 1988). Leakage of amino acids and β-lactamase may be due to a disruption of the outer membrane potential (Stock et al., 1977) by tobramycin. Collapse of the outer membrane proton gradient is known to change the permeability properties of the envelope (Helgerson and Cramer, 1977; Woolf and Konisky, 1984).

The reasoning behind these hypotheses is due to recent studies with a cationic peptide (Kordel et al., 1988), and studies outlined in a recent review by Schlessinger (1988). Sufficient evidence is presently becoming available that the mechanism for lethality may exist at the level of antibiotic uptake. The uptake of both the peptide and aminoglycosides requires a threshold potential across the membrane. Transport appears to be electrically "gated." The peptide studies reveal a disruption of the

membrane potential in less than one second leading to efflux of ions, amino acids, and immediate death.

To explore this possible lethal mechanism, studies could be initiated with carefully designed experiments using labelled compunds. For example, cells could be preloaded with ^{14}C-L-lysine (including chloramphenicol to inhibit protein synthesis), then tritiated tobramycin uptake could be examined and expressed as a molar ratio with labelled lysine efflux. The use of energy inhibitors, such as potassium cyanide or dinitrophenol, may serve as useful tools to further examine the role of the proton gradient in aminoglycoside uptake, release of basic amino acids, and lethality.

Another contribution of this study was in the elimination of certain previously described events as a direct cause of lethality. The data presented here show that within a lethal time frame of 1-3 minutes, i) LPS is not released, ii) excessive blebbing does not occur, and iii) there is no apparent loss of total periplasmic contents. The hypothesis that the extraction of major outer surface complexes, including protein and LPS, leads to cell death (Martin and Beveridge, 1986), is not supported by the observations in this study. This study has also shown that i) magnesium provides protection to cells at the initial tobramycin interaction site(s), and ii) tobramycin damage cannot be reversed by the addition of magnesium immediately following exposure. Therefore, it is doubtful that additional antibiotic, following the initial

amount exposed to cells, is necessary for lethality as proposed by Davis et al. (1986). This study also reveals that aminoglycoside activity at the ribosomal level cannot be clearly separated from outer surface damage. It may be of interest in future experiments to examine this release mechanism in ribosomal resistant isolates. In conclusion, this study has shown that the release of specific cellular components occurs rapidly and is associated with immediate lethality.

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VITA

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In the fall of 1979, she entered East Tennessee State University in Johnson City, Tennessee, where she majored in Microbiology and minored in Music. While attending ETSU, she was a member of the American Society for Microbiology (ASM), Tri Beta Biological Society (βββ), International Trumpet Guild (ITG), and Phi Mu Alpha Sinfonia. She was also a member of the ETSU Marching Band, Concert Band, Jazz Band, Wind Ensemble, and Jazz Singers. She was the Assistant Principal Trumpet in the Johnson City Symphony Orchestra from 1981 to 1984. She graduated *cum laude* in May 1984 with a Bachelor of Science degree.

In the fall of 1984, she began graduate study in the department of Microbiology at the University of Tennessee, Knoxville, and was awarded the Doctor of Philosophy degree in December, 1988. While at UT, she was a Graduate Student Council representative for the College of Liberal Arts, 1984-1988.