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> I have perceived that to be with those I like is enough,
>
> To stop in company with the rest at evening is enough,
>
> To be surrounded by beautiful, curious, breathing, laughing flesh is enough.
>
> \- Walt Whitman

Finally, this thesis is dedicated to the memory of a man who prized higher education and whose love of science and nature provided that spark of curiousity in a child's mind which has resulted in this thesis:

to my father, Gaylord Thayer Gates
1913-1978

Bury this old Illinois farmer with respect.

He slept the Illinois nights of his life after days of work in Illinois cornfields.

Now he goes on a long sleep.

The wind he listened to in the cornsilk and the tassels, the wind that combed his red beard zero mornings when the snow lay white on the yellow ears in the bushel basket at the corncrib,

The same wind will now blow over the place here where his hands must dream of Illinois corn.

- Carl Sandburg

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ABBREVIATIONS

ANT - aminoglycoside nucleotidyltransferase

AAC - aminoglycoside acetyltransferase

APH - aminoglycoside phosphtransferase

Eco RI - Restriction endonuclease from *E. coli*

E. coli - *Escherichia coli*

P. - *Pseudomonas*

S. - *Staphylcoccus*

S. faecalis - *Streptococcus faecalis*

ATP - adenosine 5'-triphosphate

AMP - adenosine 5'-monophophate

GTP - guanosine 5'-triphosphate

CTP - cytosine 5'-triphosphate

UTP - uridine 5'-triphosphate

UDP - uridine 5'-diphosphate

dATP - 2'-deoxyadenosine 5'-triphosphate

dGTP - 2'-deoxyguanosine 5'-triphosphate

dCTP - 2'-deoxyguanosine 5'-triphosphate

TTP - thymidine 5'-triphosphate

Mg - magnesium

Mg^{2+} - magnesium ion

PPi - pyrophosphate

NADP - nicotinamide adenine dinucleotide 3'-phosphate

NADPH - reduced nicotiamide adenine dinucleotide 3'-phosphate

NAD - nicotinamide adenine dinucleotide

NADH - reduced nicotinamide dinucleotide

CoA - Coenzyme A

EDTA - ethylene diamine tetraacetic acid

SDS - sodium dodecyl sulfate

CHES - (2-N-cyclohexylamino) ethane sulfonic acid

HEPES - N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid

MES - 2-(N-morpholino) ethane sulfonic acid

MOPS - 3-(N-morpholino) propane sulfonic acid

TAPS - tris(hydroxymethyl) amino propane sulfonic acid

TRIS - tris (hydroxymethyl) amino methane

DEAE - diethylamino ethyl

Km - Micahelis constant

Ka, Kb, - Michaelis constants for substrates A and B, respectively

Ki, K_I - inhibition constnat for substrate inhibition

Vmax, V - maximal velocity

V/K - ratio of Vmax to Km

Unit, unit, I.U. - the amount of enzyme that can catalyze

the transformation of 1 micromole of substrate into products in one minute under standard conditions.

M.I.C. - minimum inhibitry concentrations

THE KINETIC MECHANISM OF AMINOGLYCOSIDE NUCLEOTIDYLTRANSFERASE 2"-I

Cynthia A. Gates

Under the supervision of Professor Dexter B. Northrop

Aminoglycoside nucelotidyltransferase 2"-I (ANT (2")I) catalyzes the transfer of a nucleotide to the 2"-hydroxyl groups of a number of clinically important aminoglycoside antibiotics. During purification, two related isozymes co-chromatographed with one another. One isozyme was found to migrate faster during polyacrylamde gel electrophoresis both in the presence and absence of SDS. The two isozymes were completely separated from one another by affinity chromatography using kanamycin-Sepharose that had been synthesized at pH 10. Determination of the aminoglycoside substrate specificity suggests that the two forms of ANT(2")I are not kinetically distinct.

The enzyme accepts eight nucleotide triphosphates in magnesium chelate form and thirteen aminoglycoside substrates. Nearly all of the aminoglycosides cause substrate inhibition. Quantitative analyses of structure-activity relationships revealed that alkylation

at the 6'-position affects binding alone. However, alkylation of the 1-amino group results in a drastic decrease in catalysis.

Using the alternative substrate diagnostic, aminoglycoside nucleotidyltransferase 2" was found to follow a Theorell-Chance kinetic mechanism. The substrates bind in an ordered fashion with the nucleotide binding first, followed by the aminoglycoside. The first product released is pyrophosphate. The rate-limiting release of the second product, the nucleotidylated aminoglycoside, controls the turnover rate of the enzyme. The Theorell-Chance mechanism was confirmed by inhibition kinetics, the effect of pH on the kinetic constants of an aminoglycoside substrate, and the effect of viscosity-variation on the kinetic parameters of the enzyme.

The Theorell-Chance mechanism of ANT(2")I raises the question as to how the enzyme continues to confer resistance during multiple turnovers since the nucleotidylated antibiotic appears to be a self-generated inhibitor. Microbial resistance conferred by

ANT(2")I was found to correlate to Vmax/Km, not to Vmax which is affected by the self-generated inhibition.

"I wish I had not known so much of this affair," said my Uncle Toby, "or that I had known more of it."

-Sterne

<u>Tristram</u> <u>Shandy</u>, vi, Ch.7

CHAPTER I

INTRODUCTION

"...a hospital is no place for a sick person to be."

- Dr. Lowell Levin
Professor of Public Health, Yale Univ. (23)

A. HOSPITAL-ACQUIRED INFECTIONS

The modern hospital is perceived as a citadel of state-of-the-art medical technology. Today, the prospective patient expects and usually receives very sophisticated treatment. Certain types of surgery, such as organ transplants and open heart surgery, have taken on heroic proportions. Fiber optics and lasers allow surgeons to perform exacting operations on minute and previously inaccessible tissues. Technical advances have also benefitted the internist in such forms as computer-assisted tomography, positron-emission tomography, and nuclear magnetic resonance imaging. The public is barraged with images of "high-tech" medicine from the newspapers, television, and magazines. As a consequence, the image of the hospital of the 1980's is of a place where a cure for all ailments may be found, and even if the pathogenic condition is very debilitating or potentially fatal, heroic measures will be taken to halt the inevitability of the disease. The patient enters the

modern hospital with some trepidation, but generally expects to receive the best modern medical treatment available and recover without any ensuing complications in a clean, aseptic environment. However, this has not always been the case.

Historically, the hospital developed as an institution where sick people could be brought together, have their diseases studied by learned physicians and then given the best contemporary treatment for their maladies. This was the ideal, but not the reality, of the pre-twentieth century hospital. The harsh reality was that the patient was much more likely to die in a hospital than recover in it. The institutions were notorious for infections and "hospital fevers". Hospitals were repositories for the poor and the destitute. Wealthier people were treated in their own homes and avoided the concentrated mass of disease found in hospitals. The staggeringly high incidence of hospital-associated infection continued into the nineteenth century. Even so recently, admission to a hospital was nearly a death sentence.

This unhappy situation began to change during the mid-nineteenth century. At this time, Louis Pasteur

convinced the scientific world that fermentations were caused by specific microbes. The implications of this proof extended beyond industrial chemistry and addressed the idea of germ theory in human disease. In 1882, Robert Koch, a German physician, demonstrated that tuberculosis in humans was caused by bacteria. By 1900, the germ theory of disease was indisputably established (65).

The ramifications of the work of Pasteur, Koch and others extended to medical practice of the time. Post-operative infection was so firmly associated with hospitals that "hospitalism" was coined to describe the role hospitals played in fostering wound infections (61). That bacteria caused these infections was not recognized, although the importance of antisepsis was demonstrated by Ignaz Semmelweiss, a Hungarian physician practicing in the Vienna Maternity Hospital. In 1847, Semmelweiss published initial results that indicated physicians' and medical students' unwashed hands were the primary cause of puerperal fever, an often fatal disease affecting post-partum women at that time. Although Semmelweiss did not realize that the disease causing material was

bacterial in origin, he demonstrated that its transmission could be prevented by washing the medical attendents' hands in chlorinated lime. Unfortunately, Semmelweiss' doctrine was ridiculed and discredited in Germany and France. However, in 1865, the English physician Joseph Lister learned of Pasteur's work and reasoned that if fermentations could be caused by germs falling from the air, then putrefaction of surgical wounds might be due to a similar source. By applying carbolic acid (phenol) to wounds, and later, by spraying the entire operating room with the same compound, Lister dramatically decreased infection in post-operative wounds. Lister's methods were at first met with resistance by the British medical establishment. However, his techniques were gradually adopted thoughout the Europeon medical community. Later, surgeons began to establish the practice of asepsis, the complete exclusion of germs from the operating room rather than their destruction after the germs had arrived there. The surgeons of the late nineteenth and early twentieth century abandoned old blood-stained frock coats, dirty bandages, and filthy instruments in favor of aseptic techniques. Thus, the early bacteriologists established

the specific cause of infection, and Lister and others demonstrated that microbial infection could be prevented in the hospital environs by following aseptic and antiseptic practices (65).

With the acceptance of germ theory and asepsis, hospitals became safer places in which to be treated for disease. Discoveries in the field of chemotherapy during the twentieth century also improved survival rates in hospitals. One of the early synthetic chemotherapeutic agents, an arsenical dye called salvarsan, was discovered by Paul Ehrlich in 1907. The dye was shown to kill treponemes, a group of microorganisms that include the causitive agent of syphylis. However, the prevailing belief at this time was that only protozoans were suseptible to chemotherapeutic agents; bacteria were thought to be unsuseptible to any drug that was not also prohibitively toxic to the human body. This notion was abolished with the advent of the sulfonamide drugs in 1935. The first of the sulfonamides, Prontosil, was synthesized by Fritz Mietzsch and Joseph Klarer at the I.G. Farben Institrie in Germany. It was shown to be effective against hemolytic streptococci in infected mice

by Gerhadt Domagk at the same laboratory. The success of the sulfonamides demonstrated that drugs could be found that would cure bacterial infections. This discovery stimulated a search for other antibacterial agents and led to the beginning of the current antibiotic era.

Although the word "antibiosis" was introduced in 1889 by Vuillemin to describe antagonism between living creatures in general, it was Selman Waksman, the discoverer of streptomycin and neomycin, who first coined the word "antibiotic". Waksman narrowed the definition of antibiotic to describe substances produced by microorganisms that are antagonistic to the growth or life of other microorganisms when the antibiotic is highly diluted. The first antibiotic to be discoverd was penicillin. In 1929, Alexander Fleming recognized penicillin's antibacterial activity. In 1940, Howard Florey and Ernst Chain recognized its therapeutic value. Florey and Chain purified penicillin and aided in the development of its large scale production by fermentation. Around that time, Waksman discovered streptomycin, an aminoglycoside antibiotic effective against tuberculosis. Waksman and his co-workers had found streptomycin and neomycin by a systematic search

for antibiotic substances in soil fungi and bacteria. This approach led to the discovery of the great majority of subsequently discovered natural antibiotics including many of the aminoglycoside antibiotics and the macrolide antibiotics, such as erythromycin (50).

Although the practices of antibiotic therapy revolutionized hospitalization in the twentieth century, hospital-acquired or nosocomial (derived from the Greek nosos - related to disease - and komein - hospital) infections remain a persistent threat to patients. Recently, the National Nosocomial Infections Study (NNIS), a division of the Centers for Disease Control, reported that during 1983, the infection rate in a sample of 54 hospitals in the United States was 3.3% of all admissions at these institutions. The same report states that out of 26,096 hospital-acquired infections noted in the survey, 1% of these caused death, and 3.6% of the infections contributed to death . The authors of this report caution that the 3.3% infection rate is very likely an underestimate and that the national nosocomial infection rate is actually around 5% to 6% of all hospital admissions (69). On a nationwide scale, this

means that some 2 million people admitted to a hospital acquire an infection not present on admission as a consequence of diagnostic and therapeutic procedures or simply because of being hospitalized. These infections increase medical cost for these patients by about $2 billion a year (15% of all hospital charges) and add an extra four days (and $800) to an infected patient's hospital stay. As many as 300,000 patients die per year as a result of hospital-acquired infections. If these deaths were reported accurately, death from nosocomial infection would rank as the tenth leading cause of mortality in the United States (23).

Nosocomial infections are the most common type of infections. The hospital environment is favorable to the pathogens involved in these infections, and patients are susceptible to endogenous hospital flora for a variety of reasons. Patients may be predisposed to infection due to intrinsic conditions such as diabetes, congestive heart failure, respiratory disease, cancer, or burns. Therapeutics, such as cytotoxic chemotherapy, steroids, immunosuppresive drugs, and radiation therapy, all increase patients' risk for acquiring an infection in the hospital. Nosocomial infections are associated with

surgical wounds, urinary and arteriovenous catheterization, and respiratory aids. The patient's own flora are frequently responsible for infection. Nosocomial infections may be transmitted by air, contaminated food, contaminated equipment, and medical personnel (87).

The four most common nosocomial pathogens are *Escherichia coli*, *Staphylococcus aureus*, the enterococci, and *Pseudomonas aeruginosa*. *E. coli* is the primary cause of urinary tract infections and is the most frequently identified pathogen in adult medical services. *S. aureus* is commonly found in pediatric wards and newborn services; coagulase-negative staphylococci are the second most frequent cause of nosocominal infection among neonates. Coagulase negative staphylococci are also an important cause of primary bacteremia or blood poisoning (69).

Treatment of a hospital-acquired infection usually requires antimicrobial chemotherapy. The antibiotics generally used for this purpose are the penicillins, cephalosporins, tetracycline, macrolide antibiotics, and the aminoglycosides. Ideally, the infecting pathogen is

identified and an appropriate antibiotic from one of these groups is carefully selected for treatment. The antibiotics should be used for the minimum amount of time neccessary to eradicate the pathogens. Unfortunately, forty to sixty percent of all antimicrobial agents are administered to patients who do not need them (109). The consequences of excessive use of antibiotics in hospitals are increased incidence of adverse reactions to the antibiotics, high financial cost to the patient (*i.e.*, more expensive health insurance premiums), and the selection of drug resistant pathogens. Continued abuse of antibiotics has resulted in a reservoir of nosocomial pathogens in which drug resistant strains predominate (87).

B. BACTERIAL RESISTANCE TO ANTIBIOTICS

The problem of antibiotic resistance emerged shortly after the discovery of antibiotics during the 1940's. In 1949, increasing prevalence of penicillin resistant *Staphylococcus aureus* was documented. By 1950, the majority of staphylococcal infections in most hospitals were penicillin resistant (50). Newer drugs were introduced, but within a short time, bacteria developed resistance to them. For example, at the close of World

War II, sulfonamides were introduced in Japan for the treatment of dysentery. Within two years, most *Shigella* strains, the organisms responsible for dysentery, were resistant to sulfonamides. Newer antibiotics, such as streptomycin, chloramphenicol and tetracyclines, were then used to treat the resistant shigellae. Four years later, it was apparent that these drugs were becoming less therapeutically potent. In 1958, shigellae were found to be simultaneously resistant to two or three different classes of antibiotics. In addition, it was observed that this multiple drug resistance was "infectious", *i.e.*, the resistance characteristics could be tranferred to sensitive strains. Japanese scientists soon demonstrated that transfer of drug resistance was due to bacterial conjugation: the transfer of genetic material involving direct cell to cell contact. The genetic material was shown to be circular molecules ofF extrachromosomal DNA or plasmids. The term "resistance factor" was proposed for this transmissible resistance property (93, 157). Infectious drug resistance of this type was also reported in Europe (34, 78).

1. Current Trends in Antibiotic Resistance

The medical community has been repeatedly warned of major increases in bacterial resistance to antibiotics. When scanning through issues of The Journal of Infectious Disease, Infection Control, Antimicrobial Agents and Chemotherapy and Reviews of Infectious Disease, one can find numerous articles that address the increased incidence of drug resistance among bacteria. Since 1971, 6383 publications have appeared on bacterial resistance to antibiotics (8). These reports cite the increased resistance of *S. aureus* to methicillin and gentamicin (an aminoglycoside antibiotic), of *Haemophilis influenzae* to ampicillin, of gram-negative bacilli (particularly *Pseudomonas aeruginosa*) to aminoglycosides, and increasing multiple drug resistance among enterococci. Resistance has been encountered with the new cephalosporins, the latest "fashionable" class of broad-spectrum antibiotics. The National Nosocomial Infection Surveillence of 1983 reported the number of strains resistant to antibiotics in the survey of 54 non-teaching, small teaching, and large teaching hospitals. When comparing these results to data obtained in 1980-1982, an increase in methicillin resistant *S. aureus* was

observed in non-teaching and small teaching hospitals. Aminoglycoside resistance among *K. pneumoniae, S. marcescens,* and *P. aeruginosa* also increased at non-teaching and small teaching hospitals. Resistance to amikacin, a semi-synthetic aminoglycoside, has also increased for the latter three organisms. *P. aeruginosa* exhibited increased resistance to cefotaxime and moxalactam at small teaching and non-teaching hospitals. In general, the highest percentage of resistant strains were found in large teaching hospitals (69).

Some infectious disease specialists consider the spread of antibiotic resistance to be of epidemic proportion, and predict that commonly used antibiotics will become obsolescent for treatment of infectious disease. Maxwell Finland of the Harvard Medical School and Boston City Hospital, suggests that "little by little, we are experiencing the erosion of the strongest bulwarks (antibiotics) against serious bacterial infection" (47).

However, the claims that the incidence of antibiotic resistance is dangerously on the rise are not without dispute. Recently, two reports appeared that claimed

bacterial suseptibility to antibiotics has remained stable for the past twelve years (8, 49). The authors of these reports analyzed a massive quantity of data on nearly 10 million bacterial strains; the data originated from the laboratories of 150-329 (mean 242) acute-care hospitals of 100 beds or more. Overall, of 53 analyses of the antimicrobial effects of different antibiotics, 8 analyses showed increase in bacterial suseptibility, 16 showed decreases, and 29 showed virtually no change in suseptibility between 1971 and 1982. In 25 of the 53 analyses, at least 90% of the strains were highly suseptible to the antibiotics that they were exposed to. From these results, Lorian and co-workers concluded that, with the exceptions of *S. epidermis* and *S. faecalis*, most species of nosocomial pathogens have shown little change in antimicrobial suseptibility during the last 12 years. The authors of these reports go on to state:

> "Although local outbreaks of bacterial resistance do arise and many have serious consequences, bacterial suseptibility on a national scale has remained virtually unchanged during the last decade. This may, perhaps, be credited to the continuous effort in U.S. hospitals to achieve balanced usage of antibiotics." (8)

The conclusions reached by these studies are highly controversial among infectious disease specialists.

Several aspects of Lorian and co-workers' investigation have been questioned. The amount of data upon which the conclusion is based is immense and was taken from a national bacteriological monitoring service for hospital laboratories. However, multiple isolates of resistant strains from the same patient are not excluded in these numbers, the clinical significance of the isolates is unknown, and the origin of the infections (hospital versus community-acquired) is not distinguished. Furthermore, overall resistance of Haemophilus influenzae to ampicillin is known to have increased by 15 to 20% and higher in many U.S. hospitals, and Gardner and Lorian's data do not reflect these changes that are known to have occurred in the decade interval of their report. Another distressing observation in Lorian and co-workers' study, is the conclusion that resistance patterns for gram-negative rods have not changed. Gardner and Lorian's data obtained for antimicrobial suseptibility of P. aeruginosa clearly indicates increasing resistance to tobramycin and gentamicin and fluctuating resistance to carbenicillin. Finally, the test used for antibiotic suseptibility (the Bauer-Kirby disk test) in the data

analyzed has changed over the past 12 years. There have been manufacturing changes in the production of the disks, modifications in the methodology of the test, and changes in the U.S. Food and Drug Administration's guidelines for interpretation of suseptibility (4).

It cannot be denied that the incidence of antibiotic resistant strains increased after the introduction of antibiotics in the mid-twentieth century. It is not clear, however, whether bacterial resistance to antibiotics is continuing to increase or whether it has stabilized. One factor that has lead to this ambiguity is the lack of large-scale epidemiological studies on resistance that encompass several nosocomial pathogens, multiple classes of antibiotics used against these pathogens, and a large cross-section of hospitals across the United States. Lorian and co-workers' have analyzed the most massive quantity of data to date. There have been epidemiological surveys that examine trends in resistance to one class of antibiotics, e.g., aminoglycoside resistance in the United States (113). Other studies on resistance have been dealt with trends in antibiotic suseptibility of a single organism, such as _Salmonella_ (127) or _Staphylococcus_ (6). Many reports on

antibiotic resistance are based on surveys conducted at a single institution. A definitive answer to this issue will be found only as a result of accurate, standardized suseptibility tests to establish baseline suseptibility data and an integrated, nationwide network for reporting incidence of antibiotic resistance among nosocomial pathogens isolated from a large number of hospitals. The assessment of nationwide trends in antibiotic resistance will require interaction of Federal agencies, academia, and individual institutions, and the development of collaborative programs to evaluate suseptibility studies designed to serve as the foundation for resistance to antibiotics.

But bacterial resistance to antibiotics has more than epidemiological significance. Resistance affects the ease in which infected patients can be treated, and, as a consequence, may affect the outcome of the patient's illness. In other words, to a patient afflicted with an antibiotic resistant pathogen, the question of whether the incidence of drug resistance is increasing or not is moot, and accurate, epidemiological surveys have little personal significance.

2. Antibiotic Resistance and Its Relation to Use of Antimicrobial Agents

Simplistic generalizations on antibiotic resistance are very misleading. Separate factors must be evaluated. These include the microbe itself, the antibiotic in question, and each social, cultural, and environmental effect that results in the emergence and spread of resistant microorganisms. However, the generalization that there is strong association between the magnitude of antibiotic use and the emergence, selection and spread of resistant bacteria is valid.

Three areas of antibiotic use that contribute to the selection of drug resistant bacteria are the addition of antibiotics to animal feed, unregulated use of antimicrobial agents in developing countries, and the uncontrolled use of antibiotics in hospitals. The first area, antibiotics as growth promoters in animal feed, is a controversial issue and the subject of a great deal of attention from the media in the United States. It has been demonstrated tat drug resistant bacteria can be transferred from animals to humans in several studies. However, the effects of addition of antibiotics to animal feed in relation to public health in general remain

unclear (58). Nonetheless, continued use of antibiotics in such a massive and widespread fashion insures the existence of a large pool of drug resistant bacteria with the potential to disseminate their resistance properties to known human pathogens. The second area, use of antibiotics in underdeveloped countries, unquestionably has relevance to human health in the United States and elsewhere. Poor practices of antibiotic use frequently occur in the so-called "Third World" nations. Potent antibiotics normally available only by prescrition in Western nations may be bought "over-the-counter", and are then self-administered (154). Another crucial problem is the export and aggressive promotion of new antimicrobial agents from U.S. pharmaceutical companies to less heavily regulated developing nations. Potent and potentially harmful antibiotics become available in these countries long before approval for their use in the United States (76). By using people of underdeveloped nations as "test populations" for new antibiotics, resistance to these antimicrobials may emerge in these countries, and by virtue of increased mobility among global populations, these resistant strains can be spread to the United

States. Thus, even if a new antibiotic is introduced in the U.S., bacterial strains that are resistant to it may only be a boat-ride or a jet-flight away. However, the third area, abuse of antibiotics in hospitals, is probably the major contributor to antibiotic resistance in the United States.

Resistance patterns in hospitals frequently reflect the use of specific antibiotics. For example, decreasing the in use of cephalosporins in the treatment of nosocomial infections caused by gram-negative bacilli resulted in a substantial decrease in cephalosporin resistant strains at the Veterans Administration Wadsworth Medical Center in Los Angeles, California (86). Conversely, the incidence of gentamicin resistant bacteria rose from 0.8% in 1971 to 7.7% in 1975 at the University of Virginia Hospital; gentamicin was first introduced to this institution in 1969 (57). It is generally accepted that bacterial resistance to antibiotics is strongly associated with use of these antimicrobial agents (86). Multiple drug resistance further complicates this problem since overuse of one class of antibiotics frequently selects for strains that are resistant to other unrelated types of antibiotics and

consequently limits choices of alternative antimicrobial therapy.

3. <u>Infectious Multiple Drug Resistance: Resistance Plasmids</u>

Although there are numerous examples of chromosomally specified resistance, the most common genetic basis of antibiotic resistance is through R factor plasmids. These are circular, extrachromosomal elements of DNA harbored in the bacterial cell that may carry genes that specify resistance to several antibiotics, and can be readily transferred among bacteria. An obvious advantage that R-factor mediated resistance offers the bacterium is that resistance to several different classes of antibiotics can be encoded on a single plasmid; selective pressure from one of these antibiotics also selects for resistance to several other antibiotics. Furthermore, the resistance genes can be amplified or de-amplified as required. These plasmids can be harbored in a small proportion of a bacterial population, but upon selective pressure due to the presence of antibiotics, can be regained by the majority (36). R-factors are frequently unstable members of the

host cell's genetic complement; in the absence of antibiotic selection, the population of R-factor containing bacteria decreases. However, the absence of antibiotics does not mean that R-factors are absent from the general bacterial population. R-factor bearing enteric bacteria were found in fecal samples taken from members of isolated, aboriginal communities of the Solomon Islands and North Borneo; neither the natives nor their environs were known to be previously exposed to antibiotics. R-factors containing resistance determinants against tetracycline and streptomycin were found in a strain of E. coli freeze-dried in 1946 - prior to the clinical introduction of these antibiotics. Similarly, R-factors mediating resistance to semisynthetic penicillins and several aminoglycoside antibiotics were found in strains of enteric bacteria isolated and preserved years before these antibiotics became available (46). R-factors, then, can be found under natural conditions without selective pressure resulting from clinically administered antibiotics.

R-factor plasmids are classified on the basis of the following properties: size, resistance characteristics, transmissibilty (conjugative vs. non-conjugative), and

compatibity range (ability to co-exist or not with another type of R-plasmid in the same cell). R plasmids are generally present as multiple copies in the cell. Most R-factors are composed of two separate DNA domains. These are the resistance transfer factor (RTF) and resistance determinants (r-determinants). Conjugative R-plasmids containing these structures may dissociate to give separate plasmids: one that consists of the RTF and contains genes for conjugation and one containing the r-determinants. The reverse sequence may also occur. A co-integrate structure of a conjugative plasmid and an r-determinant plasmid is not required for coincident transfer. However, transfer of r-determinants must be mediated by a conjugative plasmid regardless of independent or integrated existence. Extensive gene amplification and recombination of the r-determinants can occur; in some strains, this results in enlarged R plasmids containing one RTF component and multiple, tandem copies of r-determinants whereas in other bacterial genera, it results in amplification of the total number of normal sized R plasmids (125).

4. Transfer of R-Plasmids and Resistance Determinants

Transfer of drug resistance genes may occur by four mechanisms: conjugation, transduction, transformation, and transposition. Conjugation, the process of exchange of DNA from one bacterium to another *via* cell-to-cell tubes or sex pili, was recognized early on as an important mechanism of R-factor transfer. This transfer mechanism is frequently observed in Gram-negative organisms. Conjugation occurs among different species of Gram-negative bacteria as well as intraspecifically (52, 74). The host range of R-factors is very wide among Gram-negative organisms. The frequencies of transfer vary greatly among species; transfer is dependent upon whether the organism in question is an R-factor donor or a recepient. Generally, "good" donors, in descending order of their quality, are *E. coli, Shigella* sp., *Citrobacter,* and *Klebsiella-Enterobacter* species. "Good" recepients are *Klebsiella-Enterobacter* sp., *E. coli*, *Shigella* sp., *Citrobacter* sp. and *Pseudomonas aeruginosa* (46). Acceptance and maintenance of the exchanged plasmid is limited by compatibility with plasmids already present in the cell. For example, closely related, but non-identical, R-factors cannot co-exist in

the same host cell. These plasmids would then belong to the same incompatibility group. R plasmids that are not similar to each other are able to exist in the same cell and are thus compatible. As mentioned previously, R-plasmids are classified by this phenomenon into incompatibility groups (51).

Conjugation was initially believed to be very rare in Gram-positive species, such as staphylococci. Instead, R-plasmids were thought to be transferred among Gram-positive bacteria exclusively by transduction, the exchange of genetic material mediatiated by a bacterial virus vector. It was recently demonstrated that dissemination of R-factors by conjugation is clinically important in _Staphylococcus epidermis_, a Gram-positive organism frequently associated with prosthetic heart valve infection (6). Nonetheless, transduction remains an important mechanism for the transfer of resistance plasmids. Transfer of R-plasmids by transduction is limited by the size of the plasmid being tranferred (_i.e._, the ability of the plasmid DNA to fit within the "head" of the bacteriophage) and by the host range of the bacterial virus. Dissemination of R-factors by

transduction also occurs in Gram-negative organisms. The significance of transduction as a means of distribution of R-factors into the general bacterial population is not clear (46).

Transformation, the uptake of naked DNA by bacterial cells, occurs *in vitro* and requires the presence of calcium chloride and related salts. This process does not readily occur *in vivo* so its significance in R-factor transfer is not known. However, it is possible that plasmid DNA originating from lysed cells in an infection could be transformed to recepient cells under appropriate physiological conditions (36).

Perhaps the most significant transfer mechanism for resistance determinants is transposition. Transposable elements, or transposons, are discrete pieces of DNA that are capable of serial tranlocation from one DNA replicon, such as a plasmid or bacterial chromosome, to another. In the process, transposons retain their characteristic structures and leave duplicates of themselves on the replicon from which they originated. Transposition does not require *rec A* dependent homologous recombination for integration into the replicon. These extremely mobile genetic elements specify self-encoded characteristics

that include resistance to antibiotics. They insert themselves either at random sites or at preferred sites on the recepient replicon depending on the nature of the transposable element. An r-determinant transposon is typically composed of the gene encoding encoding the resistance characteristic and identical DNA sequence repeats flanking the r-determinant; these repeats may be in direct or inverted orientation with respect to each other. Transposition is in part responsible for the diversity of R plasmids. The combination of conjugative plasmids and transposable elements explains the rapid spread of antibiotic resistance among many types of bacteria. As previously noted, plasmids have a wide host range and can be conjugated to a variety of different bacterial species. Transposons carrying resistance determinants can readily "jump" onto these transmissible plasmids even if the recepient plasmid is in a different incompatibility group than that of the donor. This accounts for the many identical mechanisms of resistance found in very different kinds of bacteria (16). Transposition thus greatly increases the spread of r-determinants.

5. Clinical Significance of the Transfer of Resistance Plasmids

By far, the most clinically significant forms of resistance to antibiotics arise from R-plasmids. Gram-negative bacilli are the leading cause of nosocomial infections (69), and drug resistance among these bacteria is predominantly plasmid mediated. Similarly, *Staphylococcus* species and streptococci may harbor plasmids that encode antibiotic resistance. Many of the R-factors found in gram-negative nosocomial pathogens are conjugative. Self-transmissible R-plasmids have also been found in staphylococci. Conjugative plasmids are capable of assisting in the transfer of non-conjugative R-factors. The presence of conjugative plasmids in nosocomial strains suggests that the spread of antibiotic resistance thoughout a hospital may occur by transfer of R-factors from existing drug resistant strains to previously suseptible bacteria.

The clinical significance of conjugative R-factors is illustrated by studies undertaken by Archer and co-workers on self-transmissible plasmids in *Staphylococcus epidermis*, a coagulase-negative staphylococcus that frequently causes infections after cardiac surgery (6,

7). The studies were significant not only in their demonstration of the clinical importance of R-factor transfer, but also because they showed that conjugation of R-factors readily takes place in gram-positive bacteria. A culture survey completed in 1981 at the Medical College of Virginia Hospitals revealed that 68% of cardiac surgery patients were colonized post-surgically with coagulase-negative staphylococci that were resistant to methicillin and gentamicin. This increased incidence of resistance compared unfavorably with a 20% rate in 1978. A detailed analysis of R-plasmids isolated from a large number of coagulase-negative staphylococci from the Virginia hospital revealed that the plasmids were self-transmissible and were capable of mobilizing non-conjugative plasmids. Mapping of the staphylococcal resistance plasmids revealed extensive (greater than 80%) homology among them; differences among the plasmids were due to DNA insertions and deletions that were probably the result of transposition. The implications of the results of these reports are that conjugative transfer can rapidly spread drug resistant genes throughout a bacterial population,

and since these plasmids can mobilize the transfer of smaller, non-conjugative R-factors, the emergence of nosocomial, multiresistant staphylococci may occur. In addition, the conjugative staphylococcal R-factors are capable of transfer from coagulase-negative staphylococci to coagulase-positive strains; thus, a reservoir of R-plasmids in the coagulase-negative strains increases the likelihood of transfer of gentamicin and methicillin resistance to the more virulent *Staphylococcus aureus*. Finally, the use of antibiotics for therapy or prophylaxis selects for the trans-conjugants *in vivo* and, as a consequence, increases the hospital reservoir of these conjugative plasmids (6, 7). It must be borne in mind that infection by drug resistant nosocomial pathogens is more frequently the result of colonization of bacteria already possessing R-factors rather than the conjugative spread of plasmids during the course of antibiotic treatment. Nonetheless, conjugative transfer of R-factors and any subsequent transposition of resistance genes has epidemiological significance, especially when transfer of resistance occurs between bacterial species. Interspecific transfer can result in transfer of antibiotic reistance from non-pathogenic

strains to virulent pathogens (112).

Conjugative transfer of R-plasmids has been thought to occur only in the bowel. However, it is recognized that R-factor transfer may take place, perhaps with more facility than in the bowel, on the skin (67), in burns (124), and in peritoneal dialysis fluid (164). Conjugative transfer physically takes place within fifteen minutes (46). Even if the incidence of conjugation is rare, selection of transconjugant strains upon exposure to antibiotics and transfer of these now resistant strains to suseptible patients makes infrequent conjugative events clinically significant (50).

6. <u>Biochemical Basis of Antibiotic Resistance</u>

There are nine recognized biochemical mechanisms responsible for drug resistance in bacteria: (1) enzymatic detoxification of antibiotics; (2) genetic alteration of an enzyme so that it does not bind to the antibiotic and is thus not inhibited by it; (3) alteration of a protein component of the protein synthetic machinery such that it no longer binds the drug; (4) alteration of a ribosomal RNA through methylation of specific RNA bases, thus preventing

inhibition by the antibiotic; (5) alteration of membrane proteins such that decreased penetration of the antibiotic results; (6) increased production of an antibiotic sensitive protein; (7) increased production of a metabolite that antagonizes the antibiotic inhibitor; (8) introduction of a gene that codes an antibiotic insensitive enzyme for a bypass to an antibiotic blocked metabolic step; and (9) diversion to an alternative pathway so that the antibiotic blocked step is circumvented (72). The genes that encode for these mechanisms may be found on the bacterial chromosome or on R-plasmids. Mechanisms 6 through 9 are rare and have little clinical importance. Enzymatic detoxification and changes in cell permeability to the antibiotic are the most common biochemical means of resistance and are usually encoded on R-factors. These mechanisms will be discussed in more detail with regards to aminoglycoside resistance in section C of this chapter.

6. Origins of Antibiotic Resistance

Clearly, bacteria have developed a versatile biochemical arsenal of antibiotic defense mechanisms and equally flexible means for dissemination of these weapons

throughout the general bacterial population. However, the origins and evolution of these defense mechanisms remain obscure.

Once antibiotics were introduced as chemotherapeutic agents in the mid-twentieth century, resistance to them loomed close behind. Resistance spread rapidly throughout the microbial community. New antibiotics were introduced to counter resistance only to result in development of resistance to the newcomers. Again, this resistance was mobilized readily throughout bacterial populations. This ever-repeating pattern has led to the misconception that the biochemical means of resistance are "new" and somehow evolved in tandem with the introduction of antibiotics. On the contrary, it is most likely that resistance mechanisms evolved eons ago as general and specific countermeasures used by bacteria against antibiotic substances in their environment and were subsequently mobilized into the general bacterial population by plasmids and viruses upon selective pressures due to the clinical use of antibiotics. This notion, however, prompts the folllowing questions: (1) why would there be antibiotic substances in the

environment during the pre-antibiotic era and (2) what are the biological origins of the resistance mechanisms?

The vast majority of antibiotic producing microorganisms are soil-dwelling bacteria or fungi. Generally, it is assumed that a microorganism that produces an antibiotic does so to eliminate competitors or predators and thus, enhance its chances for survival. However, it can be argued that naturally produced antibiotics show little growth inhibitory action in natural settings for the following reasonds: high levels of antibiotic are rarely produced in nature; the antibiotics made by the organism are frequently deleterious to the producer itself; and antibiotics are usually made when the producing organism has reached stationary phase where growth no longer occurs and thus no longer faces competition. However, these arguments can be countered on several grounds. First, preventing other organisms from growing and consuming resources while the producing organism is dormant provides a selective advantage even if it is a delayed one. It is likely that a small amount of antibiotic is effective in the interstices of soil particles inhabited by an antibiotic producer whereas the same amount of antibiotic would not

be effective in aqueous or agar medium. The biosynthetic pathways of most antibiotics are very complex and occur at some metabolic cost to the organism. Therefore, antibiotic production is not an evolutionary quirk, but must provide the producing organism with a clear selective advantage. Moreover, most antibiotic producing organisms are resistant to their own antibiotics. So, not only does the microorganism make the antibiotics at some physiological cost to itself, it also provides, at additional cost, mechanisms of protection against the same antibiotics. Therefore, antibiotic production must serve a real purpose to the microorganism in the natural environment. With this in mind, then, it is very likely that producers and microorganisms at risk from the antibiotic have co-existed for a very long time. A logical evolutionary countermeasure on the suseptible soil microorganism's part would be to evolve some sort of biochemical defense mechanism against antibiotics in its environment. The resistance genes that evolved in the once suseptible soil microorganisms could then have been mobilized _via_ plasmids and other vectors into other bacterial

populations when antibiotics were introduced as chemotherapeutic agents (46, 72).

Perhaps the most convincing evidence that antibiotics have had an important role in the evolution of prokaryotes is that many of the genetic systems encoding resistance mechanisms are under inducible control. Induction is a complex interplay of genes, proteins and small molecules. Both the resistance mechanism and its regulatory system evolved under long term selective pressure. For example, a strain of <u>E. coli</u> isolated from a fecal sample 45 years ago possesses an inducible detoxification mechanism for rifampin, a semisynthetic drug derived from the product of the soil bacterium, <u>Streptomyces</u> <u>mediterranei</u>. Rifampin has been in use for about 20 years. It seems reasonable to assume that the ancestors of the 45 year old laboratory strain must have been exposed to inhibitory levels of naturally produced rifamycins or similar ansamycins to develop both a resistance mechanism and a regulatory system for its genetic expression (72).

Although it seems safe to assume that resistance mechanisms have had ample time to evolve, their biochemical origins are unknown. It is possible that

resistance mechanisms evolved *de novo* as specific responses to antibiotics in their environment. Early evolution of these mechanisms probably involved chromosomal rearrangements, mistakes in replication, and various types of mutations. Alternatively, the proteins specified by resistance determinant genes may have been derived from proteins that were more generally involved in biosynthesis or in membrane transport. For instance, the enzymes that detoxify aminoglycoside antibiotics by acetylation of the antibiotic could have arisen from an enzyme such as thiogalactoside transacetylase. This enzyme is found in *E. coli* and may aid in the active transport of galactosides into the cell where the sugar is acetylated and later metabolized; if the sugar cannot be metabolized, it is acetylated and excreted from the cell. Thus, the thiogalactoside transacetylase not only acts as a metabolic protein but also as a detoxifying enzyme since some galactosides are toxic to the cell (139). It is easy to envision an antibiotic detoxifying enzyme arising from such a protein. Similarly, the beta-lactamases, the enzymes that degrade penicillins and related compounds, may have been derived from more

general proteases or the enzymes involved in peptidoglycan synthesis (105).

An attractive hypothesis for the origin of resistance mechanisms is that the genes for resistance were somehow mobilized from antibiotic-producing organisms (11). Many antibiotic-producing strains contain detoxifying enzymes that protect them against the antibiotics that they synthesize. Some of these enzymes have very similar substrate ranges as those detoxifying enzymes found in resistant bacteria. However, antibodies prepared against enzymes isolated from resistant microorganisms do not cross-react with analogous enzymes isolated from antibiotic producing strains (39). This lack of cross-reactivity may have been due to impurities in the enzyme preparations. Recently, the DNA sequence of a gene encoding for an aminoglycoside detoxifying enzyme from Streptomyces fradiae, a neomycin producing organism, was determined; its sequence was compared to those genes that specify the analogous enzyme in gram negative bacteria, and significant nucleotide and amino acid homologies were demonstrated (145). Similarly, significant homology was observed between the genes encoding for macrolide-lincosamide-streptogramin B

resistance in *Bacillus licheniformis* and in *Streptomyces erythreus*, a macrolide antibiotic producing organism (66). However, it should be noted that one important class of aminoglycoside detoxifying enzymes, the nucleotidyltransferases, has never been found in antibiotic producing organisms. This suggests that the origin of resistance for this type of enzyme was not from antibiotic-producing soil bacteria.

The mechanisms of resistance that arose early in evolution probably included all types observed in today's resistant microorganisms. However, the simplest types of mechanisms for the cell to "create" were those that involved changes in only one protein. These mechanisms include cells with altered permeability that exclude the antibiotic, enzymatic detoxification of the antibiotic, and altered target sites. Of these, detoxification is the simplest. This is because a single gene could have been duplicated and the copy modified to encode for an enzyme that detoxifies the inhibitory substance while the other protein retained its original function. This is probably why detoxification of antibiotics is the most prevalent form of resistance. In contrast, those

resistance mechanisms that required change in more than one protein, such as altered metabolic pathways that avoid the antibiotic's inhibitory effects, are comparatively rare (72). Detoxification mechanisms, although relatively easy for the organism to produce, may have been capable of only low levels of expression in the early stages of development. This problem was later overcome by gene amplification and by inducible control.

8. Prevention of Antibiotic Resistance

It is unlikely that antibiotic resistance will ever be thoroughly eradicated. We have seen that R-plasmids exist in bacterial populations that are not clinically exposed to antimicrobial agents. However, there are

several ways in which antibiotic resistance could be controlled.

The incidence of antibiotic resistance could undoubtably be decreased by reduction of the selective pressure of antibiotics. This calls for responisible use of antibiotics on the part of the medical community. Dr. Calvin Kunin of the Ohio State University School of Medicine is one of the leading proponents for control of antibiotic use in the United States and in other countries. He notes that at least 50% of all antibiotics used in hospitals are administered inappropriately for one or more of the following reasons: (1) no infection is present; (2) the incorrect antibiotic is used; (3) the dose is excessive; (4) the duration of therapy is too long; and (5) a less expensive antibiotic could have been selected. The greatest and potentially most correctable type of antibiotic abuse is for prophylaxis before and after surgery; in fact, infectious disease specialists view surgeons as being the primary abusers of antimicrobial agents. Antibiotic abuse extends beyond the hospital and into the community when physicians in office practices prescribe antibiotics for trivial, usually viral, respiratory infections and gastroenteritis

(75, 76).

Control of antibiotic abuse within the medical community requires improved education of medical students, residents, and physicians on the basic and clinical aspects of infectious disease and the use of antibiotics. Pharmaceutical companies' massive input into advertisements for antibiotics and sales is not an appropriate source of education. Other methods of control include restrictive dispensing of antibiotics within hospitals, written justification for use of the antibiotic by the physician requesting the drug, and antibiotic use review committees within the hospital.

Antibiotic resistance can also be minimized by rigorous barrier techniques in hospitals. These techniques may be as simple as greater use of gloves, bagging sputum and wound secretions, and segregating catheterized patients. Sometimes barrier precautions must be more stringent. For example, an outbreak of gentamicin resistant *Klebsiella pneumoniae* at in a newborn intensive care unit (NICU) was controlled by establishing two NICUs at the Henry Ford Hospital. One NICU contained new "additions" among the neonates and those infants with no evidence of gentamicin resistant *Klebsiella*, and the other was used for the infected infants. Each unit was autonomous with its own medical personnel. The units were also separated by 3 floors. Within 2 months the colonization rate among the infants dropped to 63%, and in 8 months, it had dropped to 3% (131).

Resistance may also be circumvented by the use of new or modified antibiotics. Very few new and effective naturally produced antibiotics have been found in recent years although some natural products have been discovered, notably the thienamycins and cephamycins. Generally, the most common strategy is to chemically modify existing antibiotics. This approach has resulted

in a number of new penicillins, cephalosporins, and aminoglycosides. Many of the chemically modified antibiotics are less suseptible to modification by the antibiotic detoxifying enzymes produced by resistant bacteria. Unfortunately, these antibiotics are not effective against permeability type resistance (16).

Another approach for prevention of resistance is direct inhibition of the resistance mechanisms by non-antibiotics. Several beta-lactams that act as inhibitors of beta-lactamases but are not antibiotics themselves are in various stages of development. Examples of these are clavulanic acid, 6-bromopenicillanic acid, and penicillanic acid sulfone (16).

The development of more effective antibiotics through structural modification and the design of potential inhibitors of antibiotic modifying enzymes both require a detailed knowledge on the mechanism of resistance. The thesis research described herein was undertaken with this in mind.

C. <u>MECHANISMS OF RESISTANCE TO AMINOGLYCOSIDE ANTIBIOTICS</u>

C.1 <u>The Aminoglycosides</u>

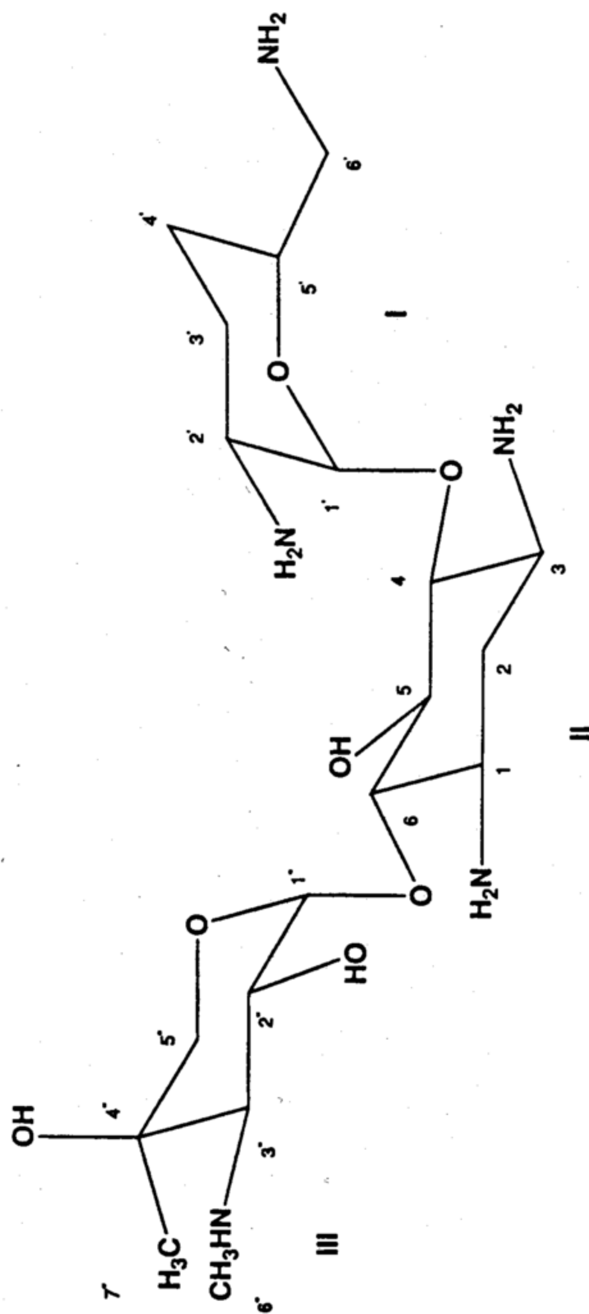
The aminoglycoside antibiotics are a diverse group of natural and semi-synthetic antimicrobial agents typified by the presence of amino sugars glycosidically

linked to an aminocyclitol. The compounds are produced predominantly by Streptomyces and Micromonaspora species. The true designation of these compounds is "aminoglycosidic aminocyclitols", but this name is cumbersome and "aminoglycosides" is the more popular usage. In general, they are stable compounds that are resistant to degradation along a wide range of pH (pH 1-11) and temperature (5 C to 120 C). The major classes of compounds have been divided by Rinehart into two groups according to the aminocyclitol contained within the antibiotic (123). Streptomycin, dihydrostreptomycin, and bluensomycin all contain the aminocyclitol streptidine, whereas the aminocyclitol contained in the neomycins, kanamycins, and gentamicins is 2-deoxystreptamine. The deoxystreptamine group can be further divided into two sub-classes on the basis of whether the deoxystreptamine ring is substituted on adjacent groups (4,5-substituted) or non-adjacent groups (4,6-substituted). The 4,5-substituted division includes the neomycins, paromomycins, and butirosin. The members of the 4,6 substituted class are the kanamycins, tobramycin and their semi-synthetic derivatives and the subgroup of gentamicin and its relatives. Figure 1 illustrates an example of a 4,6-substituted

aminoglycoside, gentamicin Cla. This antibiotic is composed of three rings: purpurosamine C (ring I), 2-deoxystreptamine (ring II), and garosamine (ring III). The carbon atoms in ring I are numbered with Arabic numerals followed by a single apostrophe or "prime"; in the 2-deoxystreptamine ring, the carbon atoms are assigned by Arabic numerals alone; ring III is numbered as in ring I, but with two apostrophes or "double prime" following the Arabic number. The functional groups attached to the carbon atoms are numbered according to which carbon atom they are associated with. For instance, the amino group bound to carbon 3 on the 2-deoxystreptamine ring is referred to as the 3-amino group. Similarly, the hydroxyl group attached to the 2"-carbon in ring III is called the 2"-hydroxyl. The kanamycins are structurally very similar to the gentamicins except that ring I is 6-amino-6-deoxy-D-glucose and ring III is kanosamine. The numbering system remains the same (94). The detailed structures of these compounds will be illustrated in Chapter IV.

The aminoglycosides are a mainstay of contemporary antibiotic therapy. They exhibit rapid bactericidal activity against a broad range of microorganisms including many gram-negative aerobic bacteria,

Figure 1: Structure of gentamicin C_{1a}



NH$_2$
H$_2$N
O
I
NH$_2$
OH
II
H$_2$N
O
HO
OH
H$_3$C
CH$_3$HN
III

staphylococci, and the tubercule bacilli. These antibiotics penetrate the bacterial cell wall and cytoplasmic membrane. They bind to ribosomes where they cause misreading of the genetic code. This results in the death of the microorganism due to the production of faulty proteins. In addition, the aminoglycosides impair the permeability control of the cytoplasmic membrane by allowing loss of small molecules through the membrane followed by the loss of macromolecules. How this occurs is not understood, although it is speculated that the transfer of the positively charged aminoglycoside into the cell somehow causes discontinuities in the cytoplasmic membrane or that synthesis of faulty membrane proteins results in defects in the membrane (16).

Aminoglycosides are used almost exclusively in the hospital environment since they must be administered intramuscularly or intravenously for systemic infections. They are particularly important in the treatment of gram-negative septacaemia as well as for treatment of urinary tract infections caused by gram-negative bacteria. They are becoming increasingly important for treatment of infections caused by staphylococci as these organisms continue to exhibit a high level of resistance to the penicillins. Aminoglycosides are also used against gram-

negative pneumonia with somewhat limited success since the cellular debris and cations present in bronchial secretions interferes with their action by restriction of bacterial uptake of the antibiotic (94, 100).

All of the aminoglycosides are potentially toxic to varying degrees. The most serious toxicity problem occurs in the inner ear where both auditory (cochlear) and balance (vestibular) functions can be impaired. Ototoxicity is both irreversible and cumulative. The aminoglycosides are also potentially toxic to kidney function. However, nephrotoxicity caused by aminoglycosides is reversible and can be detected in its early stages by monitoring urinary enzymes and other parameters. Careful monitoring during the course of aminoglycoside therapy is especially important for patients with pre-existing renal impairment. The therapeutic blood levels range from 4 to 10 micrograms per milliliter of serum for most of the aminoglycosides. Generally, toxicity is not a problem if the serum levels do not exceed 12 ug/ml. Nonetheless, accurate determination of serum levels of antibiotic is required when aminoglycosides are used due to this narrow therapeutic window (32, 126).

The potential toxicity of aminoglycoside antibiotics

has lead to the perception that the beta-lactam antibiotics, including the cephalosporins, are much safer antibiotics. Unfortunately, there are no non-toxic antibiotics. Although the penicillins are the least toxic of all antibiotics, hypersensitivity to these drugs frequently develops. High doses of penicillin are hazardous for patients with compromised renal function as the antibiotic can build up in the kidneys and result in nephrotoxicity. With considerations of toxicity aside, many penicillins are not effective against gram-negative bacteria. The newer cephalosporins are potent, broad-spectrum beta-lactam antibiotics that are effective against a range of bacteria similar to that of the aminoglycosides. However, they are also nephrotoxic and are much more expensive than the aminoglycosides: a ten-day course of therapy with cefoxitin costs $900 as opposed to $330 for tobramycin. So, in spite of a proliferation of putatively "safer" beta-lactam antibiotics with expanded antimicrobial spectra, aminoglycosides are still initial drugs of choice for most life-threatening gram-negative infections. (101, 109, 112).

C.2 Bacterial Resistance to Aminoglycosides

Bacterial resistance to aminoglycosides is based on three different mechanisms. These are alterations in the target site (ribosome) of the antibiotics, altered cellular permeability to the aminoglycoside, and enzymatic modification of the aminoglycosides.

C.2a Altered Target Site Resistance

Target site resistance to aminglycosides is exemplified by modifications in the 30S subunit of bacterial ribosomes that result in resistance to streptomycin. A chromosomal mutation results in a single amino acid change at one of two positions of the S12 protein, a protein that controls streptomycin binding, but does not bind it directly. Other mutations that give rise to resistance to spectinomycin and kasugamycin result from amino acid changes in the S5 protein and S2 protein, respectively. A mutation affecting the L6 ribosomal protein that results in gentamicin resistance has been found in association with a transport mutation (2). Other than this, resistant mutants to the 2-deoxystreptamine aminoglycosides have not been found. Presumably, this is due to these compounds' ability to bind to multiple sites on both the 30S and 50S subunits, in which case, a single protein change is unlikely to

abolish binding of the antibiotic. Target resistance is found in clinical isolates that are resistant to streptomycin and in aminoglycoside-producing bacteria (15).

C.2b <u>Transport of Aminoglycosides and Altered Permeability Resistance</u>

Clearly, aminoglycosides must somehow be transported into the bacterial cell in order to bind to ribosomes, and if such transport is prevented, the compounds cannot exert their antimicrobial action. Transport of aminoglycosides occurs in three major stages. First, the polycationic aminoglycoside binds to anionic sites on the cell surface and passively diffuses through the cell wall and extracellular layers. Second, the aminoglycoside binds to a transporter, retains its positive charge, and is "pulled" across the cytoplasmic membrane by the internal negative charge of the cell. This process is referred to as energy-dependent phase I (EDP-I) kinetics. The precise nature of the transporter molecule is unknown, but there is evidence that it is linked to a respiratory quinone participating in a functioning electron tranport chain. The transporter might be the quinone itself or a "permease" protein. Finally, the aminoglycoside is transferred from the transporter to the

ribosome to which it binds and ultimately causes disruption of protein synthesis. Once inhibition of protein synthesis is apparent, the rate of aminoglycoside accumulation increases (energy-dependent phase II; EDP-II). This is not due to an increase in the cellular internal negative charge, but rather appears to arise from an increased rate of binding to the 70S ribosomes. Energy-dependent phase II does not occur in the presence of a modified ribosome or an aminoglycoside modifying enzyme (15, 17).

Accumulation-deficient resistance may be intrinsic, as that observed for strict anaerobic bacteria. These organisms do not respire by using a functioning electron transport chain with respiratory quinones and are thus resistant to aminoglycosides. They may also lack an adequate internal cellular negativity to drive aminoglycoside accumulation. Facultative organisms also exhibit increased resistance to aminoglycosides when grown under anaerobic conditions.

Other types of permeability resistance are due to mutation. This is particularly prevalent among clinical isolates of aminoglycoside resistant *Pseudomonas aeruginosa*. This resistance is not transferable, is not eliminated by treatment of plasmid curing agents, and is

maintained in the absence of antibiotics. Furthermore, these strains are resistant to <u>all</u> aminoglycoside antibiotics. The basis for this resistance is unknown. The resistant strains have normal electron transport chains, normal electrical potential, and suseptible ribosomes. No aminoglycoside modifying enzymes have been detected in these strains. However, preliminary experiments suggest that there are major changes in the lipopolysaccharide composition of the cell wall and that this may somehow result in reduced access of the aminoglycosides to the cell (15). These resistant pseudomonads are of great clinical significance due to their very wide spectrum of aminoglycoside resistance.

Some accumulation-deficient strains owe their resistance to mutations that affect electron transport or the electrical potential of the cytoplasmic membrane. Several laboratory strains of this type have been well characterized, but the nature of this type of resistance in clinical isolates is unclear. Generally, the clinical isolates exhibit low-level resistance to a broad spectrum of aminoglycosides. They usually have a "small colony" phenotype due to their reduced rate of growth. Several of these "small colony" mutants are unstable and revert to suseptibility in the absence of aminoglycosides. Some

isolates retain significant virulence. This type of resistance is particularly insidious since the clinical isolates grow slowly and may revert prior to detection. In contrast to other forms of resistance that are established prior to antibiotic therapy, these resistant strains may arise during the course of treatment with aminoglycosides (15).

There have also been a small number of descriptions of resistance to aminoglycosides specified by R-factors in strains of P. aeruginosa that apparently contain no aminoglycoside modifying enzymes. In contrast to the accumulation-deficient strains described above, these isolates have more narrow resistance spectra. The strains have normal ribosomes. It is likely, however, that an aminoglycoside modifying enzyme is present, but is in low concentrations in the cell and is thus not readily detectable (15).

C.2c Enzymatic Modification of Aminoglycosides

The most common mechanism of aminoglycoside resistance is enzymatic modification of aminoglycoside antibiotics. The production of these enzymes is plasmid and transposon mediated and accounts for the majority of aminoglycoside-resistant bacteria found in clinical specimens.

D. AMINOGLYCOSIDE MODIFYING ENZYMES

The subject of this thesis is an aminoglycoside modifying enzyme, namely, aminoglycoside nucleotidyltransferase (2")I. Therefore, the enzymatic mechanisms of aminoglycoside resistance and the characteristics of these enzymes will be discussed in detail. The enzymes can be divided into three general categories based on the chemical reactions that they catalyze: phosphorylation of hydroxyl groups on the aminoglycoside, acetylation of amino groups on the antibiotic, and nucleotidylation of hydroxyl groups. The O-phosphorylating enzymes use adenosine triphosphate as their second substrate; the N-acetylating enzymes use acetyl-CoA and some of its analogues; and the O-nucleotidylating enzymes use a number of nucleotide triphosphates as co-substrates. Within each of the three classes, there are enzymes that modify a specific amino or hydroxyl group.

D.1 Nomenclature

The abbreviations accepted by the Plasmid Group for these enzymes are AAC for aminoglycoside acetyltranferases, APH for aminoglycoside phosphotransferases, and AAD for aminoglycoside adenylyltransferase (92). The correct and now commonly

used abbreviation for the latter enzyme class is ANT for aminoglycoside nucleotidyltransferase since the nucleotide substrate range is not limited to adenosine triphosphate. The site of modification is designated by the Arabic number of the functional group on the antibiotic that is modified by the enzyme. This number is enclosed in parentheses and follows the abbreviation for the enzyme. For example, an enzyme that acetylates the 6'-amino group of an aminoglycoside is called AAC(6') and an enzyme phosphorylating the 2"-hydroxyl group is specified as APH(2"). This nomenclature must be further refined since there may be more than one enzyme capable of modifying a specific hydroxyl or amino group. This is based primarily on differences of substrate profile. These subgroups have been designated by a Roman numeral following the type of enzyme activity and the site of modification. For example, AAC(3)I acetylates the 3-amino group of the deoxystreptamine ring of gentamicins C1, C1a, and C2, sisomicin, tobramycin, and kanamycin A whereas AAC(3)III has a similar substrate profile but also includes neomycin and paromomycin as substrates. There is evidence that these variants may have arisen by mutations in the genes encoding for these enzymes (110).

The existing nomenclature for the aminoglycoside

modifying enzymes adequately distinguishes enzymes that attack similar functional groups at different positions on the same molecule. Thus, APH(3')I can be distinguished from APH(3"). Similarly, an enzyme that catalyzes the same modification of similar functional groups at different positions of different aminoglycosides, such as ANT (4',4"), may be named by this nomenclature. However, the sometimes subtle differences among these enzymes are not adequately described by any traditional concepts in enzymology. These enzymes are not "isozymes", a term that refers to enzymes that catalyze the same reaction but are found in multiple molecular forms within the same tissue or species. Rather, the distinctions lie in the different positions of the same kind of functional group on the aminoglycoside substrate that are modified by the enzymes. Also, the duality of the nucleotidylations carried out by ANT(4',4") is not accurately described by "isozyme". An alternative classification for the aminoglycoside modifying enzymes has been proposed (120, 151). Enzymes that catalyze the same reaction on the same kind of functional group but with that functionality in a different position on the substrate are called "parazymes" (from the Greek para- "akin to"). Thus,

enzymes such as APH(3') and APH(3") are parazymes. An enzyme such as ANT(4',4") would then be called an "intraparazyme" (from the Latin intra- meaning "within" or "inside") to denote its duality in catalysis.

D.2 Distribution of Aminoglycoside Modifying Enzymes in Gram-Negative and Gram-Positive Bacteria

These enzymes are widely distributed among aminoglycoside resistant nosocomial pathogens. In gram-negative bacilli, the most common enzymes are ANT(2"), AAC(6'), APH(3'), and AAC(3). The enzymes most frequently found in the gram-positive staphylococci are ANT(4',4"), APH(3')III, and the apparently bifunctional enzyme APH(2")-AAC(6'). ANT(4') activity is found only in staphylococci. ANT 2" has been found infrequently in *Staphylococcus aureus*. Some types of modification that are unknown in gram-negative bacteria are present in gram-positive cocci, such as APH(2") which is found only in gram-positive organisms. Enzymes that are present in both gram-negative and gram-positive bacteria can be distinguished as to their source by the extent of DNA-DNA homology between plasmid genes (31). In contrast, staphylococcal and streptococcal enzymes cannot be distinguished by this criterion. This suggests that although plasmids can be exchanged between species and

genera within the gram-negative or gram-positive groups, transfer of plasmids between these two major bacterial divisions does not occur (31).

D.3 Epidemilogy of Aminoglycoside Modifying Enzymes

The occurence of aminoglycoside modifying enzymes in nosocomial pathogens is influenced by a wide variety of factors that range from the characteristics of the plasmids carrying the genes for the enzymes to antibiotic usage at the hospital from which the resistant strains are taken. Recently, aminoglycoside resistance profiles for gram-negative bacteria in the USA were compared with those found in two other geographical regions: Japan-Formosa-Korea and Chile (135). A selected group of eleven different aminoglycosides was used for suseptibility testing against the various strains of bacteria that contain the enzymes so that a unique suseptibility profile would be generated according to the enzyme or enzymes each strain possessed. The most commonly observed aminoglycoside resistance mechanisms in the USA were due to ANT(2"), AAC(3), AAC(2'), AAC(6')III and a combination of ANT(2") and AAC(6')IV. The resistance patterns in Pseudomonas aeruginosa indicated that of 155 strains, 27% of these contained ANT(2") and 31% contained substrate range variants of AAC(6'), and

26% contained variants of AAC(3). This contrasted to the patterns observed in the same organism in Japan-Formosa-Korea where 88% of the resistant strains examined contained three subgroups of AAC(6'). In Chile, 94% of the resistant *P. aeruginosa* examined contained AAC(3) enzymes. When the resistance patterns for a collection of different gram-negative bacteria, such as *Citrobacter, Echerichia, Enterobacter, Klebsiella, Salmonella,* and *Proteus mirabilis*, were examined, it was found that 82% of these bacteria in the USA contained ANT(2") whereas 46% contained ANT(2") and 51% contained various AAC(6') enzymes in the Japan-Formosa-Korea region. The AAC(3) enzyme was dominant in Chile with 97% of gram-negative strains containing this enzyme. These regional differences were correlated to the use of different types of aminoglycosides in the hospitals in each geographical region. For instance, gentamicin was widely used in the U.S. when the specimens were collected (1974-1983); the use of this particular class of aminoglycoside explains the predominance of gentamicin resistant strains due to ANT(2"), AAC(3), AAC(6')IV, and ANT(2") + AAC(6')IV observed in this country. Similarly, kanamycin and dibekacin (a kanamycin derivative) have been the most frequently used aminoglycosides in Japan-Formosa-Korea.

The most commonly observed resistance patterns, ANT(2"), AAC(6')IV, AAC(6')III, and ANT(2") + AAC(6')IV, all convey resistance to the kanamycins. Resistance due to the AAC(3) family of enzymes was rarely observed in the Asian region, presumably because these enzymes inactivate the gentamicins and related compounds more easily than the kanamycins (60, 135). A problem inherent in these studies is that some of the substrate range variants cannot be distinguished by the aminoglycoside resistance patterns that were generated, e.g., AAC(3)III, IV, and V cannot be distinguished. However, the distinction of these substrate range variants may be arbitrary as noted in Section E.3.

D.4 Mechanism of Enzyme-Mediated Resistance to Aminoglycosides

The existence of aminoglycoside modifying enzymes was confirmed by Okamoto and Suzuki. They demonstrated that a cell-free fraction of a R-factor containing streptomycin resistant strain was capable of inactivating streptomycin in the presence of Mg2+ ions and ATP (106). Initially, the aminoglycoside modifying enzymes were thought to be similar to the extracellular beta-lactamases and chloramphenicol acetyltransferases. These enzymes detoxify beta-lactams and chloramphenicol in the

culture medium, and the mechanism of resistance conferred by them requires a critical cell concentration; a single cell is vulnerable to the antibiotic since it cannot produce enough enzyme to significantly detoxify the antibiotic in the medium, and minimum inhibitory concentrations of antibiotic for these strains show very strong inoculum effects. This is characteristic of extracellular antibiotic detoxifying enzymes. In contrast, enzymatically mediated aminoglycoside resistance does not show large inoculum effects, and no modified aminoglycosides have been isolated from culture media of enzyme -containing cells grown in the presence of antibiotic. These observations indicated that the aminoglycoside modifying enzymes have an intracellular location (39). Furthermore, the vast majority of gram-negative bacteria that contain aminoglycoside modifying enzymes inactivate less than 0.5% of the extracellular drug present in the culture medium, uet they are able to grow in the presence of high concentrations of those aminoglycosides (16). These observations indicated that another dynamic process, namely transport of the antibiotic into the cell, also plays a crucial role in this mechanism of resistance to aminoglycosides.

Based on studies on the accumulation of streptomycin

and gentamicin into E. coli and P. aeruginosa, resistance to aminoglycosides depends on the outcome of two competing rates: (1) the rate of transport of the antibiotic into the cell, and (2) the rate of inactivation of the aminoglycoside by the enzyme. The latter is dependent on the kinetic properties of the enzyme for the aminoglycoside that is being transported into the cell (15). As previously noted, bacteria that possess an aminoglycoside modifying enzyme (or enzymes) do not show the energy dependent phase II transport process where the uptake of the antibiotic accelerates. This is presumably due to the modified antibiotic's inability to bind to the ribosome. If the rate of inactivation of the aminoglycoside by the enzyme exceeds or at least is equal to the rate of transport of the antibiotic into the cell, the bacterium is protected from the aminoglycoside's toxic effects. However, if the external aminoglycoside concentration is raised, the rate of transport is increased, exceeds the rate of inactivation, and the cell dies. Death of the resistant strain will also take place if the antibiotic being transported into the cell is not a substrate for the inactivating enzyme (16). However, bacterial strains may be very sensitive to an aminoglycoside even though though

it is a substrate for the modifying enzyme contained in their cells (38). This observation stimulated research that addressed the relationship of the kinetic characteristics of these enzymes to bacterial resistance against aminoglycosides.

D.5 Correlation of the Kinetic Constants of Aminoglycoside Substrates to Resistance

Williams and Northrop were the first investigators to link antibiotic resistance to enzyme kinetics (151). The kinetic constants, Vmax, Km, and Vmax/Km, were determined for 16 aminoglycoside substrates of homogeneous AAC(3)I isolated from *E.coli* C600 containing the R plasmid pJR88. In addition, inhibition constants were determined for non-substrate aminoglycosides. When comparing the literature values of aminoglycoside minimum inhibitory concentrations (MICs) for the same organism against AAC(3) activity, it was noted that the bacteria were resistant to those antibiotics that were good substrates for the enzyme, but were sensitive to those aminoglycoides that were poor substrates or inhibitors. Among the inhibitors was neomycin, an antibiotic that binds tightly to AAC(3)I (160). From this information, it was apparent that an expression of catalysis, and not binding alone, is required to convey resistance.

Comparison of the kinetic constants for aminoglycoside substrates that had diffes in MICs of greater than 10 fold revealed negligible differences in Vmax and Km, but the Vmax/Km ratios (V/K) for the same antibiotics differed by more than a factor of 10. From this, Williams and Northrop concluded that resistance was determined by V/K and not by Vmax or Km. However, even though the kinetic constants were determined with a great deal of precision, the MIC values taken from the literature contain a great deal of error. Traditionally, these values are determined by serial two fold dilutions of antibiotic, and the 50% error that is expressed in such values does not compare well with the more precisely determined kinetic parameters. Furthermore, Williams and Northrop based their conclusion on a minimum of data: MICs of pairs of good and bad substrates were compared against kinetic constants for the corresponding pair. This is not enough data for a true correlation.

Radika and Northrop extended this work with AAC(6')IV (116). This enzyme was purified to homogeneity from E. coli W677 harboring the R-factor pMH67 (117). Kinetic constants of five aminoglycoside substrates were determined for five aminoglycoside substrates (amikacin, gentamicin Cla, kanamycin A, sisomicin, and tobramycin)

at pH 6.0 and pH 7.8 and compared to MICs that were determined by close interval (20%) antibiotic dilutions for *E. coli* W677/pMH67. Of all the kinetic parameters, only V/K's determined at pH 7.8 showed a linear, positive correlation (r= +0.818). Three important conclusions were reached based on the data accrued in this study. First, the more precise determinations of the MICs revealed that antibiotic resistance is not "all or none" as frequently described, but that it is relative and determined by a kinetic feature of the aminoglycoside modifying enzyme present in the cell. Second, an antibiotic that is a substrate for a modifying enzyme *in vitro* will not necessarily be an effective antibiotic against the bacterium containing the enzyme; for bacterial resistance to be expressed, the antibiotic must be a "good" substrate for the enzyme i.e., the substrate must have a relatively high V/K. Third, correlation of resistance with an enzymatic rate process supports the hypothesis that continuing catalytic turnovers are necessary for resistance to be expressed (116).

The correlation between resistance and V/K is not surprising when the two competing rate processes, transport and enzymatic modification of the antibiotic, are considered. A correlation of resistance to Km would

not be expected since, at least for the acetylating enzymes, this is reflective of an equilibrium and not a rate. Both Vmax and V/K have the dimensions of rate constants, but Vmax requires saturating substrate concentrations for expression. At such high levels of antibiotic substrate, a proportion of the aminoglycoside would "escape" modification by the enzyme, bind to the ribosome and kill the cell. This might be expected if the enzyme failed in competition with transport or if the cell was overwhelmed with extremely high levels of antibiotic. When the cell successfully resists the antibiotic, the internal concentration of free antibiotic is maintained at a low level, and at a low concentration of antibiotic, the rate of catalytic turnover becomes expressive of V/K. Thus, a bacterium that is successfully resisting an aminoglycoside does so beacause the V/K for this substrate is high enough to allow the rate of inactivation to exceed the rate of transport. This being the case, it seems that resistance to aminoglycosides might be overcome simply by an increase in antibiotic dosage that would accelerate transport and thus overwhelm the rate of enzymatic inactivation (<u>i.e.</u>, shift from V/K to Vmax conditions). Unfortunately, the toxicity of the aminoglycosides precludes this approach

in the clinical setting.

Other attempts have been made at correlating kinetic parameters to bacterial resistance. Three studies considered only Km values (Vmax and V/K values were not even presented) and concluded that there was an inverse relationship between Km and bacterial resistance (13, 70, 153). However, two recent publications report that for the nucleotidylating enzyme, ANT(2")I, bacterial resistance to aminoglycosides correlates to V/K (12, 40). Unfortunately, the enzyme preparations used in these studies were very impure and highly unstable. In addition, the kinetic parameters were determined using a suspect radioactive assay and were evaluated by graphical analysis. The MICs were also determined using two fold serial dilutions. One of these reports claimed that bacterial resistance correlated with Km as well as with V/K (40). This conclusion was based on three Km and three MIC values, and two of the three Kms do not appear to be significantly different from each other (0.59 uM versus 0.50uM). In view of the lack of any statistical error on these values, the Km - MIC correlation is extremely tenuous.

With all of the foregoing results in mind, one of the goals of the research decribed herein was to

determine the kinetic constants for a number of aminoglycoside substrates for ANT(2")I using an accurate spectrophotometric assay and a homogeneous enzyme preparation (150) and correlate these values against bacterial resistance.

D.6 Assays for Aminoglycoside Modifying Enzymes

There are three major types of assays for aminoglycoside modifying enzymes: microbiological, radioactive, and spectrophotometric. The simplest of these is the microbiological method where a cell extract is incubated with an aminoglycoside, the appropriate co-factor, and buffer. After incubation, residual antibacterial activity is measured by standard disk sensitivity tests. Problems encountered with this type of assay are the length of time required for results (24 to 48 hours) and that the modfied antibiotic may still retain antibacterial activity (39).

The assay based on radioactivity circumvents the problems associated with the microbiological method. This assay, first described by Ozanne et al. exploits the polycationic aminoglycosides' ability to bind to anionic ion exchange paper such as phosphocellulose (107). The assay measures the transfer of radioactively labelled acetyl, nucleotidyl, and phosphate groups to the the

aminoglycoside. Unreacted nucleotides do not bind to the phosphocellulose paper, whereas both the modified and unmodified aminoglycosides remain bound to the paper. The extent of the enzymatic reaction is monitored by determining the amount of radioactivity bound to the phosphocellulose paper. This assay has the advantages that aminoglycoside substrates that do not possess antibacterial activity can be assayed, and the assays can be performed in 5 to 30 minutes. However, quantitative measurement of enzyme activity by this assay is difficult due to lack of linearity with time and enzyme concentration (53, 54).

Spectrophometric assays have been developed for all three major classes of reactions catalyzed by the aminoglycoside modifying enzymes. Acetyltransferase activity can be monitored directly by following the disappearance of the thioester bond of acetyl CoA. However, the bond is only weakly chromophoric with a molar extinction coefficient of 4,500 at 232 nm. More sensitive spectrophotometric assays for the acetyltransferases can be achieved by coupling the production of CoASH to the reduction of aromatic disulfhydryl reagents. Reduction of 5,5'-dithiobis(2-nitrobenzoic acid results in a chromophoric product with

a molar extinction coefficient of 15,700 at 412 nm (10), and similarly, reduction of 4,4'-dithiopyridine by CoASH results in a chromophore with a molar extinction coefficient of 19,800 at 324 nm (132) Phosphotransferase activity is assayed by coupling the production of ADP to pyruvate kinase and lactate dehydrogenase. The decrease in absorbance due to NADH with a molar extinction coefficient of 6,220 at 340 nm is followed (53). A coupled enzyme assay is also used for detection of nucleotidyltransferase activity. The production of pyrophosphate is linked to UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. The increase in absorbance due to NADPH with a molar extinction coefficient of 6,220 at 340 nm is measured (150).

The spectrophotometric assays offer several advantages over the previously described methods. It avoids the separation step required by the phosphocellulose binding assay. Enzyme activity can be measured for substrates that do not act as antibiotics. The assays can not only be used for measuring enzyme activity, but also for the determination of the total amount of antibiotic present, as in the measurement of serum concentrations of antibiotic (132, 162). The

spectrophotometric assays are quantitiatively reliable, and results may be obtained rapidly. However, quantititative determination of enzymatic activity in crude cell extracts is difficult, particularly for the acetyltransferase assays. This is due to backgound activity that results in reduction of DTNB or the dithiopyridine without concommitant modification of the aminoglycoside substrate. Background activity appears to be less of a problem for the nucleotidyltransferase assay since there are relatively few enzymes that generate pyrophosphate, and an excess of ATP is used inthe assays used during purification so that interference by ATP-requiring enzymes is minimized (150).

Another disadvantage of the spectrophotometric assays is a relative lack of sensitivity. This seems surprising when the high extinction coefficients for thio(2-nitro)benzene and thiopyridine are considered. However, the Michaelis constants for a number of aminoglycoside substrates for AAC(6') and AAC(3) are submicromolar (118, 160). One of the substrates for ANT(2") also has a Km in the low micromolar range (150). One approach used to enhance sensitivity for the acetyltransferase assay was to increase the light

pathlength (119). This was accomplished by the use of custom-built 5 cm and 10 cm optical glass cuvettes.

E. PURIFICATIONS, PHYSICAL PROPERTIES, AND KINETIC STUDIES OF THE AMINOGLYCOSIDE MODIFYING ENZYMES

E.1 Difficulties Encountered in the Purifications of Aminoglycoside Modifying Enzymes

Although few of these enzymes have been extensively purified and characterized, they appear to be small proteins with subunits of 17,000 to 35,000 daltons; the nucleotidyltransferases appear to be monomeric (85, 140, 150), whereas at least one aminoglycoside acetyltransferase is known to be tetrameric (161). Purification of the enzymes is difficult due to several factors. Substantial loss of activity is observed when the enzymes are exposed to gel matrices such as cellulose and Sephadex (152). The cells containing the enzymes are frequently sonicated to obtain a crude cell-free extract; inactivation of AAC(3) due to sonication has been demonstrated (152). Dialysis and ultrafiltration frequently result in low recoveries of enzyme activity; this may be due to binding of the enzymes to dialysis membranes and to hydrophobic ultrafiltration membranes (120, 152). Purified preparations of APH(3') have been shown to be unstable in the absence of antibiotic (53).

These problems, coupled with the use of the quantitatively unreliable radioactivity assay, make the determination of accurate kinetic parameters and rigorous physical characterization of these enzymes difficult.

Although many investigators routinely use affiity chromatography in attempts to purify these enzymes, the conditions for elution of enzyme activity suggest that most affinity columns are fractionating proteins on the basis of charge rather than biospecifically. Most affinity resins used in these studies were synthesized by coupling the aminoglycoside ligand to cyanogen bromide activated agarose. Enzyme activity is eluted from these resins with moderate salt concentrations; thus, the postively charged aminoglycoside ligand appears to act as an ion exchanger rather than a biospecific functionality. In our laboratory, we have found that three enzymes, APH(3'), ANT(2"), and AAC(3), require a side arm linking the antibiotic ligand to the agarose matrix for tight, biospecific binding.

Other investigators have also attempted to determine the molecular weight of the modifying enzymes with gel filtration on Sephadex. The molecular weight determined for a given enzyme in this manner vary from laboratory to laboratory even if the same resin is used.

This suggests that interactions occur between the column resin and the enzyme. However, purification of a few of these enzymes has been accomplished by the use of ion-exchange chromatography, affinity chromatography and gel filtration. The application of these techniques became possible through the use of gel matrices of agarose and polyacrylamide-agarose.

E.2 Amount and Location of Enzyme in the Gram-Negative Cell

Another difficulty frequently encountered in the study of these enzymes is the low yield of enzyme in cellular extracts. During the early studies of aminoglycoside modifying enzymes, this observation lead to the belief that these enzymes were not present in sufficient quantities to act as biochemical mediators of antibiotic resistance. However, Williams and Northrop determined the total number of AAC(3) molecules in *E. coli* C100/pJR88 to be 1763 per cell, even in the absence of antibiotic (161). This is approximately twice the calculated number of molecules of other proteins found in *E. coli* (158). When the cells containing the R-factor pJR88 are exposed to antibiotic in the medium, the number of AAC(3') molecules increases by as much as 13-fold. These concentrations are consistent with the proposal

that the aminoglycoside modifying enzymes protect the cell from the detrimental effects of the aminoglycoside antibiotics.

The fact that some researchers still find low amounts of enzyme activity in cell extracts of R-factor containing bacteria may be due to purification techniques that result in inactivation and inadequate "induction" of production of the enzymes. "Induction" is not a strictly accurate term in the case of the aminoglycoside modifying enzymes since these proteins are produced constituitively in the bacterial cell. However, the R-factor containing cell adapt to and grow in successively higher concentrations of aminoglycoside antibiotic with a concommitant increase in total enzyme activity. The increase in activity may be due to an increase in the number of resistance genes due to higher plasmid copy number (161). Most researchers tend to grow R-factor containing strains in medium with a single concentration of antibiotic rather than following step-wise increases of antibiotic. This could account for low yields of enzyme activity. By the use of current techniques of gene manipulation, perhaps the genes encoding for these enzymes can be cloned into vectors that have high level, inducible promoters, and large quantitities of enzyme can

be obtained.

In gram-negative bacteria, the cell envelope consists of an outer and an inner membrane that are separated by a rigid peptidoglycan or murein layer and the periplasmic space. It was long thought that the aminoglycoside modifying enzymes were localized in the periplasmic space. This belief is based on the fact that these enzymes can be extracted fromhe bacterial cell by osmotic shock, a technique that was thought to allow selective release of periplasmic enzymes. However, it has since been found that osmotic shock of an R-factor containing strain released 91% of the activity of an aminoglycoside modifying enzyme and 40% of beta-galactosidase, a high molecular weight cytoplasmic enzyme (41). However, when spheroplasts were formed by treating the cells with EDTA and lysozyme, a process that removes the outer membrane and peptidoglycan layer and exposes the cytoplasmic membrane, only 12% of ANT(3") activity was released. No activity was found in membrane fractions of the cell extract derived from osmotic shock. Similar results were obtained with APH(3'). In addition, the spheroplasts containing the phosphorylating enzyme could not use exogenous ATP as a substrate although it was capable of using internal ATP (111). However, the

authors of the two reports reached different conclusions as to the location of the aminoglycoside modifying enzymes. Dickie et al. concluded that although ANT(3") was not an integral protein of the cytoplasmic membrane but was "loosely" associated with the outer part of the membrane (41). Perlin and Lerner concluded that APH(3') was associated with the inner part of the cytoplasmic membrane or was free in the cytoplasm (111). From our observations of the behavior of these enzymes during purification, a membrane association seems likely (89, 121, 152). Two of the enzymes purified to homogeneity in this laboratory show characteristics consistent with hydrophobic proteins that are often associated with cell membranes. However, an association with the _outer_ part of the cytoplasmic membrane seems unlikely since the enzymes would then not have access to ATP and coenzyme A.

E.3 Substrate Specificities

The enzymes capable of modifying aminoglycosides can be divided into those that can use streptomycin or spectinomycin as substrates and those that modify the deoxystreptamine aminoglycosides. The substrate ranges of these two groups are mutually exclusive, i.e, enzymes that use the streptidine-containing antibiotics as substrates do not use deoxystreptamine-containing antibiotics and vise versa. Streptomycin is a substrate for APH(3"), APH(6), and ANT(6), whereas spectinomycin is modified by ANT(9) and by APH(3"); the latter phosphorylating enzyme is the only one that can use both streptomycin and spectinomycin as substrates. The streptidine containing antibiotics are not substrates for any acetyltransferases.

The enzymes that modify 2-deoxystreptamine aminoglycosides have much broader substrate ranges than those that modify streptidine-containing antibiotics. For example, AAC(6')IV uses the neomycins, kanamycins A and B, tobramycin, amikacin, gentamicins Cla and B, sisomicin, and netilmicin as substrates (118). Each of the nucleotidyltransferases and phosphotransferases is also capable of accepting several different

aminoglycoside substrates. In addition, some of these enzymes use a wide range of nucleotide substrates. ANT(2") accepts seven nucleotide triphosphates as substrates (150), and AAC(6')IV can use propionyl-CoA, butyrl-CoA, and N6etheno-acetyl CoA in addition to acetyl CoA (118).

As noted in section D.2, some enzymes are divided into sub-types based on substrate profiles e.g., AAC(3)I, AAC(3)II, AAC(3)III, and AAC(3)IV. The classification of these sub-types has not always been done consistently, and there has been no effort to compare directly enzymes that have been purified in different laboratories. The assays used to determine substrate ranges vary, and some methods may not detect aminoglycosides that are substrates for a given enzyme. Conversely, some antibiotics that are modified in vitro are not active as antibiotics in vivo as discussed in Section D4. However, some of the divisions are real. For instance, ANT(2")I cannot use amikacin as a substrate in vitro whereas ANT(2")II can modify this antibiotic (29). Some of the enzymes, notably the "different" AAC(6')s, do not show enough variation in substrate profile to warrant separate

classification. The presently accepted scheme of classification of these enzymes into sub-types is clearly inadequate. The designation of a given enzyme into a distinct sub-type should only be done only after kinetic studies with purified proteins have been conducted (339, 48).

E.4 The Aminoglycoside Acetyltransferases

The aminoglycoside N-acetyltransferases (AAC) are divided into three groups: AAC(3), AAC(6'), and AAC(2'). Two of the most extensive kinetic studies to date on aminoglycoside modifying enzymes have been performed on AAC(3)I and AAC(6')IV. In addition, two other sub-types of AAC(3) have been purified and partially characterised. Only those enzymes that have been purified to near homogeneity will be considered in this review.

E.4a AAC(3)I

This enzyme was first isolated from a *P. aeruginosa* strain (18) and was also found in clinical isolates of *E. coli* (149, 163). Attempts were made to purify AAC(3)I by affinity chromatography (81), but closer examination of these experiments reveal that the affinity resins were fractionating protein on the basis of ion-exchange rather than biospecific absorption.

Williams and Northrop purified AAC(3)I to homogeneity from *E. coli* C600/pJR88 as judged by six criteria (161). The protein is a tetramer, presumably composed of identical subunits, each with a molecular weight of 17,000. The total molecular weight is 63,000. The enzyme preparation is stable to a variety of conditions including lyophilization and prolonged storage at -20 C.

Initial velocity, product, dead-end, and substrate inhibition studies were performed on AAC(3)I in the forward reaction. Results were consistent with a Random BiBi mechanism (27). This is a sequential kinetic mechanism in which the two substrates bind in a random fashion, catalysis occurs, and the two products, CoA and acetyl-aminoglycoside, are released randomly from the enzyme. There is synergism between the nucleotide and antibiotic binding sites. The initial velocity patterns for good substrates, such as sisomicin, show lines with upward curvature, whereas those for poor substrates such as tobramycin, are linear. These results are consistent with a shift from non-rapid equilibrium conditions for good substrates to rapid equilibrium conditions for poor

substrates. Good substrates have a higher commitment to catalysis in this random kinetic mechanism, and when Vmax is greater than the rate constants for release of substrates, the reciprocal plots curve upward. However, with poor substrates, such as tobramycin, catalysis becomes rate-limiting, and the plots become linear. Substrate inhibition by gentamicin C1a is caused by binding of this substrate to the enzyme-CoASH complex and is expressed as a reduction in the rate of release of CoASH. Tobramycin does not cause substrate inhibition (159).

Substrate specificities and structure-activity relationships were also examined in detail by Williams and Northrop. Sixteen aminoglycoside antibiotics are substrates of AAC(3)I, including six that were previously questioned on the basis of microbiological assays or enzymatic assays with crude cell extracts. On the basis of V/K values, gentamicin C1a and sisomcin are the best substrates, and gentamcin B1 is the poorest one. Gentamicin C1 has the highest Vmax value, whereas tobramycin has the lowest.

The kinetic differences among the substrates were correlated to their structural differences. The method

of evaluation is based on the fact that, for the irreversible, random sequential kinetic mechanism of AAC(3)I, Vmax and V/K share rate constants for catalysis, but otherwise are composed of different groups of rate constants. As a consequence, structural differences in the substrates that alter catalysis will affect V and V/K similarly. In contrast, changes in the substrates that affect binding affect V and V/K differently, i.e. a structural change that results in poor binding gives a lower V/K and a higher V. The results of this study indicated that, although the enzyme modifies the 3-amino group of ring II (see Figure 1), the minimal requirements for activity include a purpurosamine ring (ring I); this reflects the binding and catalytic roles of the 2' and 6' amino groups. Methylation of the 6'-carbon or the 6'-amino group reduces binding; these changes increase the reaction velocities by shifting the rate-limiting part of the reaction from release of the acetylated antibiotic product to catalysis. Hydroxylation at the 3' and 4' positions reduces catalysis. Substitutions at the 1-N (ring II) group also reduce catalysis. The best substrates contain garosamine as ring III; this

structural feature increases catalysis and is attributible solely to the contribution of the 3"-amine. However, the identity of ring I strongly influences the kinetic contributions of ring III. The 4,5-substituted aminoglycoside inhibitors, such as neomycin, paromomycin and lividomycin, appear to bind differently to the enzyme, perhaps in a reverse orientation. Four of the structural parameters required for maximum enzymatic activity are also required for effective antimicrobial activity. If the aminoglycoside antibiotic were modified at these postions in order to make it more resistant to attack by AAC(3)I, the antibiotic would then lose antibiotic activity. This observation suggests that, at least for the acetyltransferases, chemical modification of the antibiotic as a means to combat resistance is not likely to succeed (160).

E.4b AAC(3)V

Another acetylating enzyme, AAC(3)V, was recently isolated by Coombe and George from *P. aeruginosa* (30). Gel filtration, affinity chromatography, and ion exchange chromatography yielded an enzyme preparation with a specific activity of 9.7 IU/mg. Unlike AAC(3)I, this enzyme was not inactivated by cellulose resin (DEAE-

Sephacel/Pharmacia). Polyacrylamide gel electrophoresis under non-denaturing conditions gave a single protein band. The molecular weight was determined to be 39,000 by gel filtration. A spectrophotometric assay was used to determined substrate specificities and kinetic constants. Sisomicin was the best substrate, and the enzyme is also capable of using neomycins A,B, and C as substrates (although they are poor substrates) in contrast to AAC(3)I. Another interesting feature of this enzyme that clearly separates it from its "relative", AAC(3)I,is that substrate inhibition is observed for kanamycin A and tobramycin, but not for sisomicin and gentamcin Cla.

E.4c AAC(3)IV

Purification of AAC(3)IV from *E. coli* c600/pWP701 was reported recently (13). Two fractions of activity were separated by affinity chromatography: one eluted with 0.1 M ammonium sulfate and the other eluted with buffer containing 20 mg/ml gentamicin. The first fraction appears to be bound to the affinity resin by ionic interaction whereas the second and subsequently eluted fraction appears to be bound biospecifically. SDS

polyacrylamide gel electrophoresis resulted in two protein bands with molecular weights of 27,500 and 29,500. Based on several lines of evidence, the authors tentatively concluded that two conformational forms of the enzyme exist, at least in polyacrylamide gels. The protein is apparently a monomer as indicated by gel filtration experiments. Using the radioactivity/phosphocellulose binding assay, the values of Km for seven aminoglycoside substrates are reported, but the Vmax and V/K values wereare not shown. Therefore, substrate specificities are not accurately described for this enzyme.

E.4d AAC(6')IV

Although several sub-types of AAC(6') have been described, only one has been purified to electrophoretic purity by Radika and Northrop (117). This enzyme was isolated from *E. coli* W677/pMH67; the chimeric plasmid's anscestor is R5, an R-factor that was isolated from *Shigella sonnei* (71). The enzyme was purified by dye-ligand chromatography, affinity chromatography (neomycin-Sepharose 4B), and gel filtration chromatography. Data from electrophoresis in the presence of SDS and gel filtration chromatography on Ultrogel AcA 44 indicate

that the protein is probably a tetramer with a molecular weight in the 60,000 to 70,000 range. It should be noted that, at least in our hands, determination of molecular weight of these enzymes by gel-filtration is difficult since apparent protein-column resin interactions result in asymmetrical peaks (120, 151). Both the dye-ligand and gel filtration chromatography, as well as discontinuous polyacrylamide gel electrophoresis, separated the purified enzyme equally into two active protein fractions. The less active form has an unusual absorbance spectrum near 255 nm that is not explained by the amino acid composition of this enzyme form. Chromatography of this form regenerated both enzyme forms; this suggests that the enzyme is non-covalently conjugated to an uncharged chromophore, perhaps a lipid. The usual criteria for homogeneity cannot be applied here due to the presence of two forms of active enzyme and conversion of one form to the other. Nonetheless, only two forms of AAC(6') were observed under three modes of separation. In addition, only one peak is observed with ion exchange chromatography. All of these observations argue for a high degree of purity, if not conventional

homogeneity (117). The level of purity is certainly adequate for detailed kinetic studies.

The kinetic constants of twelve aminoglycoside substrates were determined for purified AAC(6') at pH 7.8 where substrate inhibition was observed for all aminoglycosides. Substrate inhibition was not observed at pH 6.0 where the kinetic constants of six antibiotic substrates were determined. Kinetic constants for acetyl-CoA, propionyl-CoA, butyryl-CoA, and 1,N6-ethenoacetyl CoA were also determined at pH 6.0. The analytical method developed for structure-activity data for AAC(3)I (160) was applied to the kinetic data obtained for AAC(6')IV. This analysis demonstrates that most structural changes in the aminoglycoside substrates cause changes in the rate of release of the acetylated aminoglycoside product; only drastic changes near the 6'-amino group affect catalysis. As with AAC(3)I, the structural requirements for enzymatic activity are parallel to those required for antimicrobial activity. Furthermore, the structure-activity analysis of the kinetic data indicate that the turnover rate of the acylation reaction is limited by catalysis and not by the rate of release of either the acetylated antibiotic or

CoA (118).

Several attempts to determine an initial velocity pattern between amikacin and acetyl-CoA resulted in patterns of parallel lines, whereas amikacin versus propionyl CoA gave an intersecting initial velocity pattern. The difficulty in obtaining unambiguous results from traditional initial velocity patterns coupled with AAC(6')IV's ability to use several different aminoglycosides and nucleotides as substrates led to the development of a new kinetic diagnostic using alternative substrates.

E.4e The Alternative Substrate Diagnostic and AAC(6')IV

Although the notion of using alternative substrates to identify kinetic mechanisms is not new (64), the kinetic diagnostic using alternative substrates as brought forth by Radika and Northrop (115) provides a new and more sensitive method for determination of the kinetic mechanisms of bisubstrate enzyme reactions. In this diagnostic, the concentration of substrate A is varied against a fixed and saturating concentration of alternative substrates B, B', B", etc.; the prime and double prime notations designate structural variants of

B. Similarly, substrate B is varied against A, A',A", etc. Unique pairs of patterns are generated for each of the major classes of kinetic mechanisms for bisubstrate enzymatic reactions. Two sets of parallel lines are produced by the Ping-Pong mechanism, one set of parallel and one of intersecting lines for an Ordered mechanism (the set of parallel lines is reduced to a single line by Theorell-Chance), and both patterns show intersecting lines for a Rapid Equilibrium Random mechanism. The diagnostic offers several advantages over the traditional approach using initial velocity patterns: efficiency (fewer assays are necessary), accuracy (large changes in slope and intercept are possible), and precision (one substrate is always saturating).

By applying this diagnostic to AAC(6')IV, the kinetic mechanism was determined to be Rapid Equilibrium Random. The combination of these results with those obtained from the structure activity studies argue for a rapid rate of release of substrates and products relative to the rate of catalytic turnover (118, 119).

E.5 <u>The Aminoglycoside Phosphotransferases</u>

The phosphorylating enzymes are widely distributed throughout gram-negative and gram-positive species. This

group of enzymes include APH(3") and APH(6), both of which phosphorylate streptomycin. The enzymes that phosphorylate the deoxystreptamine aminoglycosides are APH(3') and APH(2"). The latter enzyme is found only in gram-positive bacteria and is apparently part of a bifunctional enzyme complex with AAC(6'); this bifunctional enzyme will be treated as a special case in the following section. APH(3') has been divided into three sub-types based on substrate range. Many attempts have been made at purifying APH(3') to homogeneity, but these have been impeded by the enzyme's instability _in vitro_. Nonetheless, some physical and kinetic charateristics have been determined for APH(3').

E.5a APH(3')I

This enzyme phosphorylates the 3' hydroxyl group of neomycin, paromomycin, and ribostamycin. It is also capable of phosphorylating the 5"-hydroxyl of lividomycin, a 3'-H aminoglycoside. Three dimensional models of the latter antibiotic show that the 5"-hydroxyl group is close to the position of the missing 3'-OH. It is suggested that the enzyme can "swivel" between phosphorylation at the 3'-OH and 5"-OH (148). Values for

molecular weight, kinetic constants for ATP, kanamycin, and lividomycin, and pH optima varied according to the bacterial strain from which the APH(3')I was isolated.

E.5b APH(3')II

Umezawa and co-workers first described a partial purification of this enzyme and later obtained a more complete purification by using affinity and hydroxyapatite chromatography (149). A single protein band was found with an apparent molecular weight of 25,000. Goldman and Northrop (53) also reported a purification procedure using ion exhange chromatography and an affinity resin with the aminoglycoside ligand covalently linked to the matrix *via* a 10 carbon spacer arm. The recovered enzyme preparation was electrophoretically 90% pure. However, the purified enzyme was highly unstable and lost 50% of its activity in less than a week. Although a spectrophotometric assay was developed for APH(3')II, kinetic parameters were not determined due to the instablity of the enzyme (53). Subsequent experiments revealed that APH(3')II activity is stabilised in the presence of aminoglycoside antibiotic (Sherman and Northrop, unpublished results).

APH(3')II was also purified to near homogeneity by Smith and Davies using DEAE-agarose chromatography, affinity chromatography, and preparative electrophoresis (138). Following affinity chromatography, the enzyme preparation was judged to be 90% pure and suitable for kinetic studies using the spectrophotometric phosphorylation assay developed by Goldman and Northrop. The best substrates for APH(3') were the neomycins, paromomycin, and ribostamycin. Kanamycins and gentamicins were found to be poor substrates of APH(3')II. The stability characteristics of this particular preparation of APH(3') were not noted (39).

E.5c <u>APH(3')III</u>

Although APH(3')III is found in many staphylococci strains, it was partially purified from *P. aeruginosa* by Umezawa and co-workers (149) using affinity chromatography. However, the enzyme preparations were highly unstable.

Recently, purification of APH(3')III from *S. aureus* was reported. DEAE-Sephadex chromatography, affinity chromatography, and gel filtration chromatography using Sephadex G-100 were the techniques used to purify this phosphotransferase. However, the enzyme activity was not

biospecifically eluted from the affinity column, and no data were given on total units recovered, specific activity or stability of the final fraction of APH(3')III (146).

E.6 <u>APH(2")-AAC(6'): A Bifunctional Enzyme From Staphylococci?</u>

Resistance to aminoglycoside antibiotics in *S. aureus* harboring the plasmid RPAL results from the presence of two inactivating reactions catalyzed by an AAC(6') and APH(2"). LeGoffic and co-workers observed that the two enzyme activities co-purified with a constant ratio between the two specific activities; the purification process included affinity chromatography with biospecific elution and molecular exclusion chromatography. The protein fraction containing the two activities was found to be a dimer with a molecular weight of 55,000 as determined by gel filtration chromatography and sucrose gradient ultracentrifugation. Two different aminoglycoside substrates were used to distinguish the acetylating reaction from the phosphorylating reaction. Gentamicin C1, an aminoglycoside whose 6'-amino group is methylated and is

thus not a substrate for AAC(6'), was used to detect APH(2") activity, whereas neamine, a kanamycin derivative without the third (kanosamine) ring and thus lacking the 2"-hydroxyl group, was used to assay for AAC(6') activity. The radioactivity/phosphocellulose binding assay was used throughout this study (88).

The kinetic mechanisms for each activity were determined by product and dead-end inhibition kinetics. Both AAC(6') and APH(2") followed a Rapid Equilibrium Random BiBi kinetic mechanism. However, substrate inhibition by neamine was observed for the acetyltransferase activity. This inhibition was linear uncompetitive against acetyl-CoA and synergistic with the product, CoASH. This is consistent with the formation of an enzyme-CoA-neamine complex. Substrate inhibition was not observed for the APH(2") activity. Substrates and co-factors of AAC(6') were tested as effectors of APH(2") and vise versa. No interaction between the two activities was observed. Thus, the two enzyme activities appear to be kinetically independent of each other. LeGoffic and co-workers also observed that gentamicin C1a, a substrate for both enzymes, can be acetylated and phosphorylated in the presence of both acetyl CoA and GTP

at the same rates as those determined for phosphorylation in the presence of GTP alone or acetylation in the presence of acetyl-CoA. These data, they argued, are consistent with the existence of two independent binding sites (88).

However, when rates of thermal inactivation of the two enzyme activities were determined in the presence and absence of substrates and co-factors, it was found that a low concentration of GTP, the co-factor of APH(2"), substantially protected both the phosphorylating and the acetylating activities from heat denaturation. If AAC(6') was an independent enzyme, this protective effect by GTP may be explained by its binding to the nucleotide site normally occupied by acetyl-CoA. In this case, the reaction catalyzed by AAC(6') should be inhibited by GTP as a competitive inhibitor. However, LeGoffic et al. found that GTP does not inhibit or activate AAC(6') even at high concentration. If the two activities are contained within a single protein or multienzyme complex, binding of GTP to the phosphorylation site should stabilize the active conformation of the acetylating site. Similarly, gentamicin C1 protected AAC(6')

activity from denaturation even though the antibiotic's Ki as a competitive inhibitor against neamine was 4.0 mM, and the concentration used for the heat denaturation protection was 0.33 mM. Although neamine and CoA protected APH(2") activity from denaturation, the concentrations used in these experiments were much higher than the inhibition constants determined for these compounds against the substrates for the phosphotransferase.

The conclusion reached from these experiments was that although the kinetic experiments argued for two separate and independent enzymes, the results of the heat denaturation experiments indicate that the two enzymes may be physically associated either as a bifunctional enzyme with independent binding sites or as a multienzyme complex. However, co-purification of the two activities and a constant specific activity ratio are not stringent proofs of a bifunctional enzyme or complex. LeGoffic et al. note that additional work is required to confirm their hypothesis.

Another group has reported the purification of this putatively bifunctional enzyme from *S. aureus* and *S. epidermis* strains that contain R-factors. The activities

co-purified on affinity chromatography and DEAE-Sephadex chromatography. As is often the case, the enzyme was eluted from the so-called affinity column non-biospecifically. However, SDS-PAGE revealed a single protein band for the final pool of purified protein. From this and gel filtration chromatography with Sephadex, a molecular weight of 56,000 was determined. The SDS-PAGE experiments in this study indicated that AAC(6')-APH(2") was a single protein and not composed of subunits in contrast to LeGoffic and co-workers' conclusion. In addition, isoelectric focusing indicated that the protein had as pI of 4.1 (146).

7. The Aminoglycoside Nucleotidyltransferases

Five nucleotidyltransferases have been identified. ANT(6) modifies streptomycin, ANT(3") modifies streptomycin and spectinomycin, and AAD(9) adenylylates spectinomycin. These nucleotidyltransferases have been found primarily in *S. aureus*. The enzymes that modify the deoxystreptamine aminoglycosides are ANT(4',4") and ANT(2"). ANT(4',4") has been detected only in *S. aureus* and *S. epidermis* whereas ANT(2") is distributed among many different species of gram-negative aminoglycoside

resistant bacteria. ANT(4',4") and ANT(2") have broad substrate ranges for both the antibiotic and the nucleotide substrates. These are the only two nucleotidyltransferases that have been purified to homogeneity.

E.7a ANT(4',4")

Two groups reported the existence of this enzyme in staphylococci (80, 130). It was purified to near homogeneity and its bifunctional catalytic behavior was confirmed by Santanam and Kayser (129). These researchers purified the enzyme using affinity chromatography and ion exchange chromatography with DEAE-Sepharose as the ion-exchange resin. Although elution from the affinity column was not biospecific, the enzyme appeared to be a single homogenous entity as judged by gel filtration and isoelectric focusing. A molecular weight of 47,000 for ANT(4',4") was determined by gel filtration. Its isoelectric point is 5.0. The purified enzyme preparation was stable for at least 5 months when stored at -70 C.

The purified enzyme uses a wide range of aminoglycosides as substrates. The enzyme appears to have greater affinity for aminoglycoside substrates with an

intact 4'-hydroxyl group. Good substrates for ANT(4',4") included kanamycin B, tobramycin, and neomycin C. In contrast, 3',4'-dideoxykanamycin was a poor substrate. ATP and GTP are good nucleotide substrates, whereas TTP, UTP, and CTP were poor substrates. However, paromomycin was found to be nucleotidylated more efficiently with UTP than with ATP or GTP. Magnesium was found to be the most efficient cation for the adenylylation reaction. The pH optimum for enzyme activity was dependent on the identity of the substrate. Substrates that contain a 4'-hydroxyl group are adenylylated optimally at an acidic pH (5.0 - 6.0), whereas those that have a hydroxyl group at the 4" position are adenylylated optimally at a neutral pH (7.5). The pH activity profiles for the 4,5-substituted aminoglycosides, neomycin and paromomycin, exhibited two pH optima for each substrate: one at 5.0 and another at 9.0. Presumably, this reflects optimal adenylylation of the 4'-OH and 4'''-OH groups at the two respective pH values. As a reminder on nomenclature, neomycin and paromomycin are <u>four</u> ring aminoglycosides, so the hydroxyl group that is modified on Ring IV is technically 4'''-OH. These studies, together with results obtained

on the structures of the adenylylated aminoglycoside products by 13C-NMR, demonstrated that the aminoglycoside nucleotidyltransferase from S. epidermis catalyzes the transfer of a nucleotide monophosphate moiety from nucleotide triphosphates to the equitorial hydroxyl groups at the 4' and 4" positions on aminoglycoside antibiotics (129, 134).

An aminoglycoside nucleotidyltransferase was isolated from Bacillus subtilis harboring the R-factor pUB110. The enzyme was purified by ion-exchange chromatography with DEAE-cellulose and gel-filtration using Sephadex G-75. SDS-PAGE gave a single protein band with a molecular weight of 34,000. The optimum pH for activity was 5.0 with kanamycin as the substrate. Although this group did not identify the enzyme in terms of the hydroxyl moiety it modifies, the enzyme is probably ANT(4',4") since the original source of the plasmid was S.aureus (128). In a more recent study, this nucleotidyltransferase, isolated from B. subtilis/pUB110 using the procedure just described, was compared to an aminoglycoside nucleotidyltransferase isolated from a thermophilic Bacillus containing the R-plasmid pTB913. The amino acid compositions of the two proteins and the

nucleotide sequences of the genes encoding for the enzymes are essentially identical except for a single base change in the nucleotide sequence from cytosine for the mesophile to adenine for the thermophile. This results in a change from threonine to lysine and significantly increases thermostability of the nucleotidyltransferase from *Bacillus stearothermophilus*/pTB913 (90).

The remaining nucleotidyltransferase, ANT(2"), is the most clinically significant aminoglycoside modifying enzyme in the United States. It has been the subject of a number of studies which will be discussed in detail in the following section.

E.7b Aminoglycoside Nucleotidyltransferase 2"

Aminoglycoside nucleotidyltransferase 2" catalyzes the transfer of a nucleotide monophosphate derived from nucleotide triphosphate to the 2"-hydroxyl group of a number of aminoglycoside antibiotics. It consists of two distinct sub-types: ANT(2")I and ANT(2")II. These enzymes differ from the other nucleotidyltransferases in two major ways: they are found primarily, if not exclusively, in gram-negative bacteria, and only 4,6-substituted

aminoglycosides composed of three rings are substrates. The existence of ANT(2")I was discovered in 1971 (10), and the presence of ANT(2")II was noted ten years later in aminoglycoside resistant, gram-negative, nosocomial bacteria from an Australian hospital (104). A partial purification and substrate range of the latter nucleotidyltransferase was also published (29). Several groups reported partial purification of ANT(2")I since its discovery, but, with one exception, all enzyme preparations had low specific activity, and were highly unstable. However, ANT(2")I was finally purified to electrophoretic homogeneity, and a quantitatively reliable spectrophotometric assay was developed (150).

E.7b (1) Clinical Significance of ANT(2")I

The predominant resistance mechanism to aminoglycoside antibiotics among gram-negative bacteria in the United States is ANT(2")I. This nucleotidyltransferase was found to confer resistance to forty-six percent of 1187 aminoglycoside resistant gram-negative strains examined in a recent survey of aminoglycoside resistance (135). The aminoglycoside resistance pattern associated with ANT(2")I indicates that the enzyme confers resistance to the gentamicins,

sisomicin, kanamycins and tobramycin. ANT(2")I apparently has a wider substrate range than AAC(3)I, but, unlike AAC(6')IV, ANT(2")I does not confer resistance to netilmicin and amikacin. However, resistant strains possessing both AAC(6') and ANT(2") have the widest resistance spectrum of all strains that carry aminoglycoside modifying enzymes. The only mechanism that confers resistance to more aminoglycosides is altered permeability. Out of a total of 1187 aminoglycoside resistant gram-negative strains in the United States, 10% had resistance patterns consistent with the presence of both AAC(6')IV and ANT(2")I. In Japan, Formosa, and Korea, 46% of the 866 aminoglycoside resistant strains contained AAC(6') and ANT(2") (135).

It has been suggested that the use of amikacin in place of gentamicin might eradicate strains containing ANT(2")I (15). However, this proposed strategy fails to take into account the nature of multiple aminoglycoside resistance and the dynamics of R-factors and resistance transposons. Although the incidence of those resistant strains that contain only ANT(2")I would be reduced by the use of amikacin, the number of strains with AAC(6')IV

would then increase due to selective pressure. Indeed, increased use of amikacin has been correlated to increased amikacin resistance due to AAC(6')IV (82). However, R-factors often carry the genes for more than one aminoglycoside modifying enzyme. Thus, it is not unlikely that the R-plasmid carrying the gene for AAC(6')IV also carries the gene for ANT(2") or has the potential to acquire it by transposition. Although the R-factors from strains containing both ANT(2")I and AAC(6')IV have not been characterised, a portion of these probably carry the genes for both enzymes. With this in mind, the following scenario can be envisioned. In an attempt to eradicate gentamicin-resistant, nosocomial strains containing ANT(2")I, amikacin is substituted as the aminoglycoside of choice for gram-negative infections. But, although amikacin is initially effective against the gentamicin-resistant strains, the incidence of amikacin-resistance begins to increase. If the use of amikacin is continued, resistance to this antibiotic may be significant enough to warrant switching over to the use of another aminoglycoside. Since strains with AAC(6')IV are suseptible to the gentamcin C complex, substitution of this antimicrobial agent would seem to be

a logical countermeasure against amikacin resistant strains. But, if the AAC(6') strains carry a gene for ANT(2")I on their R-factors, this strategy would fail, and gentamicin-resistant nosocomial flora would again predominate. Even if the R-plasmids encoding AAC(6')IV dd not initially carry the nucleotidyltransferase gene, they could readily acquire one from transposition. In summary, the use of one antibiotic can just as easily select for strains with resistance to several antibiotics, even if these antibiotics are not being used. Substituting one aminoglycoside for another will not eradicate resistance.

E.7b (2) Detection of ANT(2") Among Resistant Clinical Isolates Using a DNA Probe

In the clinical setting, identification of an aminoglycoside modifying enzyme is usually accomplished by antimicrobial suseptibility patterns and plasmid screening techniques. However, the value of resistance profiles and plasmid fingerprints as monitoring devices for the dissemination of a particular enzyme becomes limited when other aminoglycoside modifying enzymes are present in the same strain. In an effort to circumvent

this problem, a DNA probe for the structural gene of ANT(2") was constructed. The gene was cloned from the R-factor pLST1000 into pBR322. A restriction fragment from the interior of the ANT(2") gene was radiolabeled and used in Southern hybridization gels as a probe for plasmids isolated from aminoglycoside resistant bacteria. The probe proved to be highly specific and was more sensitive than the radioactive/phosphocellulose binding assay for the detection of ANT(2") in clinical isolates with complex aminoglycoside resistance phenotypes (143).

The lab group that developed this DNA probe for ANT(2") demonstrated that the cloned gene gave a protein product with a molecular weight of 35,000 as determined by SDS-PAGE. They also found that the genes for ANT(2") and ANT(3"), the streptomycin nucleotidyltransferase, have regions of homology. This concurs with the earlier findings of Yagisawa and Davies (159). However, neither gene demonstrates homology with ANT(4',4"), the gram-positive nucleotidyltransferase. Thus, the genes for ANT(2") and ANT(3") may be derived from a common anscestor (143).

E.7b (3) The R-Plasmid JR66 and Its Derivatives

The origin of the plasmids used for this thesis research was a gentamicin-resistant strain of *Klebsiella pneumoniae*. Benveniste and Davies transferred the R-factor into *E. coli* W677 and designated the plasmid JR66 (11). The presence of an aminoglycoside nucleotidyltransferase was identified in this strain. It was later determined that JR66 was actually two plasmids. JR66a encoded for APH(3')II and the streptomycin-spectinomycin adenylyltransferase ANT(3"); this plasmid was subsequently designated JR67. JR66b encoded for ANT(2")I as well as for APH(3') and AAD(3"). A derivative plasmid of JR66b is JR76.2. The strain harboring this plasmid was resistant to higher concentrations of antibiotic, and was subsequently found to contain more ANT(2") activity (151). Another plasmid, pMY10, was later constructed from *EcoR1* restriction fragments of JR66b. This plasmid encodes only ANT(2")I (167). *E. coli*/W677 harboring this R-plasmid was resistant to high concentrations of aminoglycoside and was found to produce large amounts of ANT(2") (151); the plasmid was termed a "hyper-producer" (167).

E.7b (4) Purification of ANT(2")I

The first stable preparation of ANT(2")I was reported by Goldman and Northrop (54). They partially purified the nucleotidyltransferase from *E. coli* W677/pJR76.2 using osmotic shock, nucleic acid precipitaion with streptomycin sulfate, protein precipitation with ammonium sulfate and ion exchange chromatography with DEAE-agarose. The specific activity of .07 IU/mg was higher than any previous purification by other lab groups. However, they found that the radiolabeled assay was not linear with time or enzyme.

This problem was overcome by Van Pelt and Northrop who developed a quantitatively reliable spectrophotometric assay for ANT(2")I. They also purified ANT(2") to near homogeneity from *E. coli*/W677 pMY10 (142). The early stages of purification were similar to that described by Goldman and Northrop except that the osmotic shock procedure was modified to give reproducible and higher yields of enzyme. Low molecular weight impuities were removed from the ammonium sulfate fraction by Bio-Gel P2. ANT(2") was then purified by agarose based anion exchange chromatography (DEAE-Bio-Gel

A), affinity chromatography using gentamicin Affi-Gel 10, and molecular exclusion chromatography using Ultrogel AcA 54. Purification to near homogeneity revealed the presence of two related forms of ANT(2")I. One form had a specific activity of .134 IU/mg and bound tightly and rapidly to gentamicin Affi-Gel. It migrated faster than the other form during polyacrylamide gel electrophoresis both in the presence and absence of SDS. This enzyme form was designated Ef. It has an isoelectric point of 5.7 and an apparent molecular weight of 32,500 as determined by SDS-PAGE. The second form, called Es, also bound tightly to gentamcin Affi-Gel, but the onset of binding was time dependent. ANT(2")-Es could be eluted from the affinity resin with buffer containing 0.15 M ammonium sulfate, but after overnight recycling on gentamicin Affi-Gel, it bound tightly to the resin. Es had a specific activity of .274 IU/mg and migrated slower than Ef during electrophoresis both in the presence and absence of SDS. It has an isoelectric point of 6.0, and has a molecular weight of 31,500. A molecular weight of 28,500 was estimated for Es by analytical gel filtration. An attempt was made to determine the molecular weight of Ef by gel filtration, but the enzyme interacted with the

column resin and gave an unsymmetrical peak. These results tentatively indicate that ANT(2") consists of a single polypeptide chain (150). Other investigators have reported molecular weight values for ANT(2") as determined by gel filtration chromatography. Smith and Smith reported a molecular weight of 35,000 (140), whereas Lombardini and Chen-Chu determined that ANT(2") is a 23,300 Dalton protein (85). Both groups used the plasmid HJR66 and Sephadex G-100 in their studies. The difference in these molecular weight values is probably due to interaction of ANT(2") with the dextran. Using a partially purified protein preparation, Lombardini and Chen-Chu also determined a molecular weight of 23,700 by density gradient centrifugation (85). Thus, total molecular weight of ANT(2")I remains unknown.

The exact relationship between the two enzyme forms, Ef and Es, is unclear. The amino acid compositions and polypeptide chain lengths are almost identical, so it is possible that the two proteins have a common ancestor or are the same polypeptide. Van Pelt and Northrop suggested that the two enzymes are actually one protein but are differentiated by conjugation to

either sugars or lipids. The presence of such a conjugated group could then change their chromatographic, electrophoretic, and kinetic properties (150).

The pH-activity profile gave an optimum pH of 9.5. Activity dropped off sharply on either side of this optimum peak. Smith and Smith reported an optimum pH for activity of 9.1 (140), whereas Lombardini and Chen-Chu reported an optimum of 8.1 to 8.5 (85). Van Pelt and Northrop suggest that the high pH optimum probably reflects the tight binding of AMP-aminoglycoside product, whose release would then be expressed in the rate of enzymatic turnover. As amino groups on the product lose their positive charge with increasing pH, the product binds less tightly, thus increasing the turnover rate (150).

E.7b (5) <u>Substrate Specificity of ANT(2")I</u>

The substrate specificities of ANT(2")I are not stringent, either for the nucleotide or aminoglycoside substrate. Although the nucleotidyltransferase's capability of using several nucleotide triphosphates as substrates has been known for some time. ATP was the only substrate for which kinetic constants had been determined (140). Van Pelt and Northrop were the first

to determine the kinetic constants for seven nucleotide substrates: ATP, GTP, CTP, dATP, dGTP, dCTP, and TTP. The deoxyribonucleotides are better substrates (higher V/K values) than the ribonucleotides, and pyrimidine nucleotides are better substrates than purines. Substrate inhibition was observed for all nucleotides. Since an excess of magnesium was not used in the assays for nucleotide, Van Pelt and Northrop suggested that substrate inhibition was caused by free nucleotide and that magnesium chelated nucleotides are the true substrates of ANT(2") (150).

Substrate profiles based on relative activity indicate that ANT(2")I can use a number of aminoglycosides as substrates (5, 140). Kinetic constants have been determined for several aminoglycosides (12, 85). However, these values were determined using the phosphocellulose binding assay and partially purified, unstable enzyme preparations. Generally, the gentamicin C complex, sisomcin, and tobramycin appear to be good substrates for the enzyme.

E.7b (6) <u>Characterisation of ANT(2)II</u>

Coombe and George (29) reported the partial

purification of a new sub-type of ANT(2") from aminoglycoside resistant, gram-negative clinical isolates taken from the Royal North Shore Hospital in Sydney, Australia. They used gel filtration with Sephacryl S-200, non-biospecific elution from gentamicin-Sepharose (no side arm), and ion exchange chromatography with DEAE-Sephacel, a cellulose resin in rigid bead form. The affinity resin separated the nucleotidyltransferase from APH(3')I which was also present in the cell extracts.The phophocellulose binding assay was used to monitor enzyme activity. The final specific activity was 0.04 IU/mg. The molecular weight of 29,000 was determined by gel filtration, and pH activity profile consisted of a narrow peak at 7.8 to 8.0 for five different substrates. Enzyme activity fell away sharply on either side of the optimum.

The substrate profile determined for this nucleotidyltransferase differs markedly from that of ANT(2")I. Gentamicin C2 and C1a are very poor substrates for ANT(2")II. The best substrates were gentamicin C1 and the kanamycins. Amikacin, an aminoglycoside that is not a substrate of ANT(2")I, is a good substrate for ANT(2")II (29).

When the aminoglycoside modifying enzyme content of resistant strains from the Royal North Shore Hospital was examined, ANT(2")II was always found in association with APH(3')I and not alone. This enzymatic partnership confers resistance to a broad range of aminoglycosides. (104). ANT(2")II has not yet been reported outside of Australia.

E.7b (7) Kinetic Studies of ANT(2")I

To date, there has been only one published report on the kinetic characterisation of ANT(2")I. In addition to physical characterisation of ANT(2")I, Lombardini and Chen-Chu attempted to determine the kinetic properties of ANT(2")I. A partially purified enzyme preparation of low specific activity (.013 IU/mg), and the phosphocellulose binding assay were used in this study. Slopes and intercepts were estimated by least squares linear regression analysis from which the kinetic constants of gentamicins C1, C1a, and C2 were determined. The kinetic mechanism was deduced to be sequential from an initial velocity pattern. Product inhibition by pyrophosphate and tripolyphosphate gave non-competitive inhibition versus gentamicin and competitive inhibition versus ATP. From this minimal amount of data, the kinetic mechanism

for ANT(2")I was deduced to be Ordered BiBi with ATP binding to the enzyme first, followed by gentamicin (82). Although these investigators made no mention of the order of product release, one can only presume from the logic behind their conclusions from the product inhibition kinetics that they believe the nucleotidyltated antibiotic product is released first, followed by pyrophosphate (85).

The only other kinetic study of ANT(2")I may be found in a Ph.D. thesis by Christine Green at Rutgers University (55). She partially purified ANT(2")I using the method of Goldman and Northrop and subsequently obtained a stable enzyme preparation with a final specific activity of 1.9 IU/mg. This stable enzyme preparation was used for the kinetic studies. Enzyme activity was monitored with the radiolabeled assay. Neomycin, a non-substrate of ANT(2")I, was a competitive inhibitor versus gentamicin, and a non-competitive inhibitor versus ATP. Neither phosphate nor pyrophosphate inhibited activity significantly at low levels of gentamicin. This led Green to conclude that the phosphate product inhibitors do not bind to free enzyme

but only to the enzyme-gentamicin complex. Phosphate/pyrophosphate inhibition was abolished by saturation with ATP. Green obtained an intersecting initial velocity pattern when ATP was the varied substrate. She concluded that the kinetic mechanism of ANT(2")I was Ordered BiBi with gentamicin binding to the enzyme first followed by ATP; for product release, she deduced that pyrophosphate was released from the enzyme first, followed by AMP-antibiotic. Green also observed substrate inhibition for gentamicins and other good aminoglycoside substrates. This substrate inhibition was found to be non-competitive; both intercept and slope replots were hyperbolic. Green concluded that this substrate inhibition arose from "incorrect" binding of the aminoglycoside with free enzyme; that is, antibiotic binding occurs is a position or conformation inaccessible to adenylylation.

Other investigators have noted substrate inhibition by the aminoglycoside (12, 40, 140). The presence of substrate inhibition for some antibiotics but not for others led to some confusion concerning the kinetic parameters that govern resistance. For example, it was observed that although netilmicin, a 1-N methylated

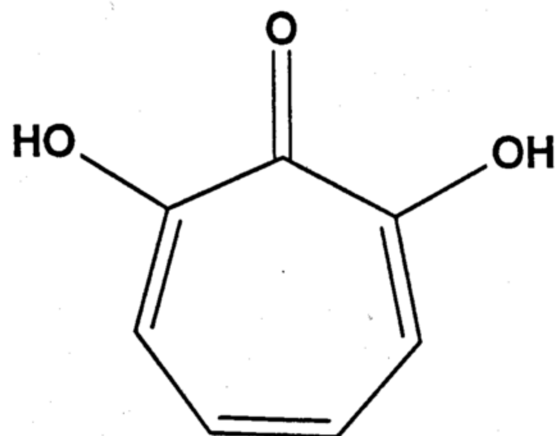
sisomicin, is an effective antibiotic against those resistant bacteria containing ANT(2")I, it was modified much more rapidly *in vitro* than equivalent concentrations of aminoglycosides known to be good substrates for ANT(2")I, such as gentamicin and tobramycin. Determination of the kinetic constants for several aminoglycosides revealed the presence of substrate inhibition for gentamicin and tobramycin, but not for netilmicin. As a consequence, good substrates at fixed concentrations equivalent to that of netilmicin are modified at lower rates *in vitro* because they are inhibiting ANT(2")I, whereas netilmicin is not (12, 40).

One way to prevent antibiotic resistance due to enzymatic modification would be to design inhibitors for the enzyme in question. Green attempted to design an irreversible, active site directed inhibitor for ANT(2")I (55). She prepared 6"-bromine derivatives of tobramycin and kanamycin A. The reasoning behind this was that the 6"-bromine group would be close enough to the active site (2"-OH) to react with a nucleophilic group on the enzyme. Incubation of ANT(2")I with 6"-bromotobramycin resulted in a decrease of activity, but

the kinetics of inactivation were complicated by the presence of substrate inhibition. Both brominated derivatives had substrate activity although it was lower than the parent compounds.

Recently, the inhibition of ANT(2")I by 7-hydroxytropolone, a natural product produced by *Streptomyces neyagawaensis*, was reported (3). Inhibition was competitive versus ATP and appeared to require the unique vicinal arrangement of oxygens found in 7-hydroxytropolone, illustrated in Figure 2. Combination of 7-hydroxytropolone (7-HT) with aminoglycoside substrates were active against resistant bacteria possessing the nucleotidyltransferase although potentiation by 7-HT varied according to the aminoglycoside being used. No potentiation was observed for resistant strains containing other aminoglycoside modifying enzymes. An interesting finding in this study was that inhibition of ANT(2")I *in vivo* facilitated penetration of tobramycin, a substrate for ANT(2"), into the cell. This observation emphasizes the significance of the role of the modifying enzyme in aminoglycoside resistance and the importance of a ribosome-drug interaction in producing enhanced antibiotic uptake (3).

Figure 2: Structure of 7-hydroxytropolone


O
HO
OH

F. GOALS OF THIS THESIS RESEARCH

Although Van Pelt and Northrop developed an excellent, reproducible purification procedure for ANT(2")I, the final total yield of enzyme was low. At best, approximately 9 units of enzyme could be recovered from 230 units of activity in the osmotic shock extract derived from approximately 80 grams of E. coli W677/pMY10 cells. One of the objectives of this research was to refine the purification procedure and increase the recovery of ANT(2")I. Part of this "fine-tuning" would include a search for a better affinity resin. Gentamicin Affi-Gel 10 is an effective affinity resin for biospecific separation of ANT(2") from other proteins. However, the resin-ligand adduct, derived from an N-hydroxysuccinamide side arm, apparently hydrolyzes during prolonged storage (152). Therefore, another activated agarose resin was investigated as an alternative to Affi-Gel 10.

Van Pelt and Northrop reported a Km of 3 uM for tobramycin. It seemed likely that the other aminoglycoside substrates would probably also have low Km values, thus increasing the difficulty of accurate determinations of the antibiotics' kinetic constants.

Therefore, one goal was to develop a more sensitive fluorimetric assay for ANT(2") activity. The existing coupled assay could be used since the final product, NADPH, is a fluorophore, but external standardization of NADPH concentrations would have to be developed since the Beer-Lambert law does not apply to fluorescence emmision spectroscopy.

Another goal was to optimize the assay conditions in terms of magnesium concentration. Since Van Pelt and Northrop surmised that the metal-chelated nucleotides are the true substrates of ANT(2") and that the free nucleotides are inhibitors, an optimal ratio of the metal-chelated nucleotide to free nucleotide needed to be determined for ANT(2") activity. This information would then provide for more accurate determination of the kinetic constants of the metal-nucleotide substrates.

Studies of the aminoglycoside acetyltransferases clearly demonstrate the interplay of <u>in vivo</u> resistance and the kinetic characteristics of the modifying enzyme. In order to determine if the same correlations occur with ANT(2"), the kinetic properties of the nucleotidyltransferase had to be elucidated. Therefore,

the primary goal of this research was the kinetic characterization of ANT(2"). This goal included determination of the aminoglycoside substrate specificity of ANT(2"). Although others have attempted to elucidate the kinetic mechanism of ANT(2"), these previous studies were not rigorous, and their results are questionable due to the suspect radiolabeled assay. Furthermore, their results are in conflict with one another. Therefore, the kinetic meachanism of ANT(2") was to be determined by using the assay developed by Van Pelt and Northrop and by application of steady-state enzyme kinetics. Knowledge of the kinetic mechanism of ANT(2") might then prove useful for the design of mechanism-based inhibitors that may then be used to combat aminoglycoside resistance conferred by this enzyme.

CHAPTER II:

EXPERIMENTAL PROCEDURES

A. MATERIALS

Substrates, Inhibitors, Enzymes, Bacterial Strains, and Miscellaneous Chemicals

Gentamicins C1, C1a, C2, A, B, B1, sisomicin, 5-episisomicin, and N-ethylsisomicin (netilmicin) were the gifts of Dr. George Miller, Schering Corporation. Dr. F. Leitner and Dr. R.P. Elander, both of Bristol Laboratories, donated kanamycins A and B and amikacin, respectively. Tobramycin and nebramycin factor 4 were the gifts of Dr. Marvin Gorman and William Fields, respectively, of Lilly Research Laboratories. Neomycin C was the gift of Dr. Joseph Grady, Upjohn Chemical Company. Tobramycin-AMP was the gift of Dr. Jean Van Pelt and Dr. Perry Frey, University of Wisconsin-Madison. Streptomycin, knamycin, gentamicin, and neomycin sulfates were purchased from Sigma Chemical Company.

Two strains of *E. coli*/W677, one harboring the R-factor JR76.2 and the other containing the chimeric plasmid pMY10, were the gifts of Dr. Julian Davies.

ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and TTP were from Sigma. (32P)-sodium pyrophosphate was purchased from New England Nuclear. NADPH, UDP-glucose, glucose 1,6-diphosphate, dithiothreitol, EDTA, UDP-glucose

pyrophosphorylase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, hexokinase, bovine serum albumin, hemoglobin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, cytochrome c, SDS, glycerol, 1,4-butanediol diglycidyl ether, N,N,N',N' tetramethylene diamine, 1-amino-2-naphthol-sulfonic acid, activated charcoal, MES, MOPS, HEPES, TAPS, CHES, TRIS base, and TRIS-HCl were all purchase from Sigma. Enzyme-grade ammonium sulfate was from Schwarz/Mann. Acrylamide and bis-acrylamide were from Bio-Rad. Ammonium persulfate was purcahsed from LKB. All other chemicals were of the highest grade available.

BioGel Desalting Gel P6DG, BioGel P2, and DEAE-BioGel A were from BioRad, and Sepharose CL-4B was from Pharmacia. Ultrogel AcA 54 was purchased from LKB.

Yeast extract, bactopeptone, and bacto-agar were from Difco. Trypticase soy was from BBL Microbiological Systems. Glucose was purchased from Fisher Scientific, and sucrose was from Eastman Chemicals. Bulk glycerol was obtained from University Stores, University of Wisconsin- Madison.

B. METHODS

B.1 Synthesis of Aminoglycoside Affinity Resins

Epoxy-activated Speharose CL-4B was prepared according to the method of Sundberg and Porath (142). The resin was activated by combining suction dried Speharose CL-4B with 1,4-butanediol diglycidyl ether (1 ml/g gel) and 0.6 M NaOH (1 ml/g gel) containing NaBH4 (2 mg/ g gel); the 1,4-butanediol diglycidyl ether as supplied by Sigma is only 70% pure so it was necessary to distill it under reduced pressure (b.p. = 145 C at 1.9 mm Hg) before use. The Sepharose resin was then stirred, together with the diglycidyl ether and other components, with a caged magnetic stirring bar for 12 hours at room temperature. The reaction was terminated by washing the resin with copius amounts of deionized, distilled water (500 ml/g gel). After suction drying, the activated resin was stored at 4 C in 1.0 M NaCl, pH 7.0. According to Sundberg and Porath, the activated resin loses about 30% of its epoxy groups over a period of 30 days when stored this way. Consequently, the resin was used within 7 days of its activation.

Neomycin-, gentamicin-, kanamycin-, tobramycin-, and amikacin-Spharose were prepared by coupling the

appropriate aminoglycoside to epoxy-activated Sepharose CL-4B. The activated resin was first washed with deiozed water and suction dried. Then it was mixed with an aminoglycoside dissolved in 0.5 M sodium carbonate at pH 10.0. The proportion of solution containing the desired ligand to activated resin was 2 ml/g gel. The following ratios of aminoglycoside to activated resin were used: 75 mg neomycin sulfate/ g gel; 20 mg gentamicin sulfate/ g gel; 23 mg kanamycin sulfate/ g gel; 19 mg tobramycin sulfate/ g gel; and 19 mg amikacin base/g gel. The mixtures were incubated in a LabLine haker with mild agitation (150 rpm) at 38 to 42 C for 21 hours for neomycin-Sepharose and 68 hours for the other resins. The solutions containing the antibitoic were then removed from the resins by filtration, and their volumes were measured. Next, the resins were washed with the following solutions (200 ml/g gel) in this order: (1) 0.5 M sodium carbonate, pH 10.0; (2) deionized water; (3) 0.1 M sodium carbonate, pH 8.0; (4) 0.1 M acetic acid, pH 4.0, containing 0.5 M NaCl; (5) deionized water until the pH of the effluent was the same as that of the water. The aminoglycoside-Sepharose resins were stored at 4 C in

deionized water containing 0.1% sodium azide. The amount of ligand bound to each resin was measured as described in section II B.8g; these values are given in Table 1.

Two other preparations of kanamycin-Sepharose were made. One was synthesized with 56 mg kanamycin sulfate/g gel in 0.5 M sodium carbonate, pH 9.0, whereas the other was made with 24 mg/g gel in sodium carbonate, pH 10.0. Both were reacted with the activated resin at 42 C for 46 hours. The resins were washed as described previously except that the resin coupled at pH 9.0 was initially washed with 0.5 M sodium carbonate, pH 9.0. The amount of aminoglycoside bound to all resins was determined by measuring the concentration of antibiotic in the initial coupling solution and in the filtrate after the coupling solution was removed from the resin; The concentration of aminoglycoside in the filtrate was determined by assays with either ANT(2") or AAC(6') as described in section II. B.9g. The amount of ligand coupled to each kanamycin-Sepharose resin is given in Table 1.

B.2 Preparation of Media

Bacterial cultures were maintained on a solid medium that contained, per liter of distilled water, 30 g

TABLE 1

AMOUNT OF AMINOGLYCOSIDE LIGAND COUPLED TO EPOXY-ACTIVATED SEPHAROSE 4B-CL

Ligand	pH of coupling solution	Amount of ligand bound to Sepharose (umole/g)[a]
neomycin[b]	10	33.3
kanamycin	10	15.9
gentamicin	10	3.7
tobramycin	10	0.5
amikacin	10	10.7
kanamycin[c]	9	45.6
kanamycin[d]	10	42.1

[a] Weight of suction dried affinity resin was measured. [b] Affinity resin used in Preparation A. [c] Affinity resin used in Preparations B and D. [d] Affinity resin used in Preparations C through C3+C4.

trypticase soy, 5 g yeast extract, 15 g Bacto-agar, 10 ml glycerol, and 100 mmoles each of dibasic and monobasic potassium phosphate. After autoclaving 0.5 L of this medium for 20 minutes at 120 C under 15 atms. steam pressure, the hot, liquified medium was allowed to cool to approximately 55 to 65 C. At this point, 10 ml of 25 mg/ml fliter-sterilized kanamycin sulfate were added to the medium to give a final concentration of 0.5 g/L kanamycin. The antibiotic was added to the maintenance medium to ensure stability of the R-factors by providing selective pressure. Immediately after the addition of the kanamycin solution, the medium was poured into sterile, plastic Petri plates (approximately 15 ml per plate) and allowed to solidify.

The solid medium was also used to prepare tubes for bacterial slants. Prior to autoclaving, the components of the solid medium were mixed with distilled water and heated on a hot plate until the agar melted. Then, 10 ml of this hot medium were pipetted into glass culture tubes. These tubes were then autoclaved under the same conditions previously described. After autoclaving, 0.3 ml of 25 mg/ml filter sterilized kanamycin sulfate were

added to each 10 ml aliquot to give a final concentration of .75 g kanamycin/L. The tubes were then positioned at a shallow angle, and the medium was allowed to solidify.

The liquid medium for bacterial growth contained 12 g bactopeptone, 5 g yeast extract, 10 ml glycerol, and 100 mmoles each of dibasic and monobasic pottasium phosphate per liter. Carboys containing 15 L of this medium were autoclaved at 120 C, 15 atms. pressure, for 40 to 50 minutes, whereas smaller quantities of liquid growth medium (1 L or less) used for preparing inocula were autoclaved for 20 minutes. In addition, liquid medium for large scale growth of bacteria (15 L/carboy) contained 6.7 g glucose per liter of medium; 100 g glucose was dissolved in approximately 300 ml distilled water and autoclaved separately from the other components of the medium.

B.3 Maintenance of Bacterial Cultures

Cultures of *E. coli* W677 harboring pMY10 or JR76.2 were maintained on slants of the solid medium. The cultures were transferred once every three months to plates of solid medium and grown over night at 37 C. Several colonies from a single plate were straked onto a single slant. Selection of multiple colonies rather that

a single colony avoids inadvertant culturing of a colony that has arisen from a single mutant cell. Culturing the colnies on plates served as an indicator of possible contamination of the strains. The slant cultures were allowed to grow overnight at 37 C and then stored at 4 C.

B.4 Determination of Minimum Inhibitory Concentrations of Antibiotics

The minimum inhibitory concentrations (M.I.C.) of six aminoglycoside antibiotics for *E. coli* W677/JR 76.2 were determined in sterile, liquid growth medium. The plasmid JR 76.2 encodes both ANT(2")I and APH(3')III. Therefore, the antibiotics used for determination of the M.I.C. values are substrates of ANT(2") but are non-substrates for APH(3')III, *i.e.* the antibiotics possess the 2"-hydroxyl functionality and lack the 3'-hydroxyl group. Thus, interference due to the presence of a second aminoglycoside modifying enzyme is minimized. The aminoglycosides used were gentamicins Cla and C2, tobramycin, sisomicin, 5-episisomicin, and netilmicin.

Matthew K. Powers performed the experiments that determined the M.I.C. values of each antibiotic. Stock solutions of antibiotics were filter-sterilized, and

diluted into 0.5 ml sterile, liquid growth medium to give a range of concentration bracketing the M.I.C. under study with a 20% difference between consecutive concentrations. The inoculating bacterial cultures was grown for approximately 6 hours in 100 ml of liquid medium containing no antibiotic at 37 C and 150 rpm in a Lab-Line shaker; the inoculating bacterial culture originated from _E. coli_ JR 76.2 grown on a slant culture containing no antibiotic. Cell populations of 1.5 x 10 to 5.2 x 10 , as determined by plate count, were added to each tube containing antibiotic. The bacteria were grown for 24 hours at 37 C, and growth was verified by observing turbidity. The M.I.C. was assessed as the lowest concentration of antibiotic at and above which there was no turbidity. The M.I.C. value for each antibiotic was determined through six trials.

B.5 Growth and Harvesting of Bacteria

Cells from a freshly grown slant culture of _E. coli_ W677/pMY10 were transferred to 100 ml sterile liquid growth medium containing 0.25 mg/ml kanamycin sulfate. This culture, the primary inoculum, was grown over night at 37 C in a Lab-Line incubator/shaker at 250 to 270 rpm. Then, 1 ml of the primary inoculum was transferred to 1 L

of liquid medium containing 0.5 mg/ml kanamycin sulfate. The base of the 2 L Erlenmeyer flask that contained the 1 L culture was indented with ridges to incrase the ratew of oxygen exchange and improve mixing of the culture. This culture, the secondary inoculum, was also grown overnight at 37 C with agitation (250 to 270 rpm) in a Lab-Line shaker.

ANT(2")I was isolated from E. coli W677/pMY10 grown at 37 C in four glass or polypropylene carboys containing 15 L of sterile, liquid medium each; kanamycin sulfate was not added to this medium. Immediately before inoculation with the 1 l secondary inoculum, the sterile glucose solution and 0.3 ml Dow-Corning antifoam were added to each carboy. After addition of the 1 L culture, the growth medium was agitated and oxygenated by continuous aeration with a flow rate of 25 L/minute. This flow rate wasmeasured by a variable area flow meter manufactured by Aalborg Instruments and purchased from Cole-Palmer.

Growth of bacteria was monitored by measuring solution turbidity at 550 nm. Cells were harvested when the optical density reached 1.0. A Dupont/Sorvall RC-5B

superspeed centrifuge equipped with a TZ-28 continuous flow rotor and feed assembly was used to collect the cells. The cells were centrifuged at 28,900 x G (16,500 rpm with an internal radius of 9.525 cm). This and all following centrifugation steps were carried out at 0 to 4 C. Medium containing the cells was introduced into the spinning rotor at a flow rate of 600 to 800 ml/minute. Approximately 70% of the cells was recovered using this flow rate and relative centrifugal force as judged by optical density readings of the medium at the time of harvest and of the supernatant. Fifteen liters of medium and cells could be harvested in 20 to 30 minutes. After collection of the cells, 5 L of washing solution containing 10.0 mM TRIS pH 7.8 and 3.0 mM NaCl were introduced into the rotor at a flow rate of 600 to 800 ml/minute. The supernatant was autoclaved before it was discarded.

B.6 Early Purification of ANT(2")

B.6a Extraction of Cellular Protein

Protein was extracted from the cells by an osmotic shock procedure originally developed by Nossal and Heppel (103) and modified by Van Pelt and Northrop (150). The collected cells were suspended in 33.0 mM TRIS buffer, pH

7.3, containing 20% sucrose and 3.0 mM EDTA; approximately 3 ml of sucrose solution were used per gram of wet cells. The cell suspension was stored at 4 C for 12 to 16 hours. Then, the suspension was centrifuged in a GSA rotor at 26,000 x G for 20 minutes. The supernatant was discarded, and the cell pellet was swabbed with cotton to remove any remaining sucrose solution. The pellets were stored at 4 C for 12 to 20 hours. After this second period of cold storage, the cells were rapidly suspended in cold, deionized water (about 4 to 7 ml/g wet weight of cells) in a chilled Waring blender at low speed for 1 minute, using four bursts of 15 seconds each; in contrast, the cells were resuspended with a single high speed burst for 25 seconds in the method of Van Pelt and Northrop (150). The cells were kept on ice for 60 to 90 minutes and then centrifuged at 26,000 x G for 20 minutes. The pellet was discarded, and the resultant supernatant, containing the cellular protein, was saved and subsequently referred to as the osmotic shock fluid.

An alternative method of extraction of cellular protein used sonication of bacterial cells. <u>E. coli</u>

W677/pMY10 cells were grown in 1 L of liquid medium containing 0.5 mg/ml kanamycin sulfate for 16 hours at 37 C and 230 rpm. Twelve grams of cells were harvested by centrifugation at 26,000 x G using a GSA rotor. These cells were resuspended in chilled 25 mM TAPS buffer, pH 8.1, containing 5.0 mM magnesium acetate and 0.125 mM EDTA. The suspended cells were kept on ice and disrupted by sonication using a Branson Instruments sonifier at 150 cps with 5, 10, and 15 second bursts. The total accumulated sonication time was 4 minutes. Aliquots of 0.1 ml were removed from the cell suspension after each burst; ANT(2") activity in each sample was determined using the spectrophotometric assay described in this chapter.

B.6b. <u>Removal of Nucleic Acids</u>

The osmotic shock fluid was adjusted to pH 7.3 with 4.0 N potassium hydroxide and maintained at 0 to 4 C throughout this procedure. Forty units of aprotinin was added to prevent proteolysis. A 50% (w/v) solution of streptomycin sulfate, pH 7.8, was added dropwise with stirring to the osmotic shock solution until a final concentration of 1% (w/v) was reached. The suspension was then centrifuged at 26,000 x G for 20 minutes, and

the precipitated nucleic acid pellet was discarded.

B.6c Precipitation of Proteins

The pH of the supernatant of the previous step was adjsuted to 8.0 with dilute ammonium hydroxide; The supernatant was kept at 0 to 4 C throughout this procedure. Enzyme-grade, crystalline ammonium sulfate was added slowly (about 60 grams every 10 minutes) to give a final concentration of 90% saturation. The suspension was stirred while the ammonium sulfate was added to the solution and was allowed to stir for 30 minutes after the last portion of ammonium sulfate was added. Then, the suspemsion was allowed to stand for 2 to 3 hours at 0 C and centrifuged at 5800 to 13,000 x G for 40 to 60 minutes; the rotor speed was derated to accomodate the added mass of the ammonium sulfate suspension. The superanatant was discarded, and the protein pellets were covered with a 95% saturated ammonium sulfate solution at pH 8.0. The pellets were then stored at -20 C.

B.7 Purification of ANT(2") Using Chromatographic Procedures

All columns were run in a glass-doored refridgerator

maintained at approximately 4 C. Conductivities of solutions were measured on a Bionometer Model 60 standardized with samples of known molarity. An LKB chrmomatographic system, consisting of a Varioperpex II peristaltic pump, a REDIRAC fraction collector, and a UVICORD S ultraviolet monitor attached to a 2 channel recorder was used for liquid chrmomatographic procedures. Protein in the eluates was monitored by absorbance at 280 nm.

The chromatographic procedures were essentially the same as those decribed by Van Pelt and Northrop (150) except for the following features: a different buffer and pH were used; different affinity resins were employed (see below under B.7e), flow of buffer or protein samples through the columns was descending; and flow rates for the columns were slower.

B.7a Removal of Low Molecular Weight Impurities

The protein pellets stored in 95% ammonium sulfate were centrifuged at 13,000 x G for 40 minutes, and the supernatant was carefully drained. The pellets were then dissolved in 25.0 mM TAPS, pH 8.1, containing 5.0 mM magnesium acetate, 0.125 mM EDTA, and 3.0 mM DTT; this buffer solution will henceforth be referred to as Buffer

1. Care was taken to dissolve the pellets in a minimal amount of buffer. One half of this, usually about 120 ml, was applied to an 800 ml BioGel P6DG or BioGel P2 column equilibrated with Buffer 1. The column was run by gravity with a flow rate of 4 ml/min (BioGel P2) to 7 ml/min (BioGel P6DG). The protein peak contained a buff-colored band that allowed visual detection. Conductivities were also measured, and fractions with conductivity equivalent to 0.01 M ammonium sulfate or less were pooled and saved. The other half of the sample was similarly chromatographed after the column had been re-equilibrated with Buffer 1.

B.7b Ion Exchange Chromatography

The pooled sample eluted from the BioGel P6DG or P2 column was loaded onto a 1.5 x 45 cm column containing DEAE-BioGel A equilibrated with Buffer 1. A 1000 ml linear gradient of ammonium sulfate from 0 to 0.3 M was used to elute ANT(2") activity from the column; the ammonium sulfate was dissolved in Buffer 1. The flow rate was 1 ml/min, and 10 ml fractions were collected.

B.7c Affinity Chromatography

During purification, pooled fractions from the DEAE-agarose column were loaded onto a 1.5 x 30 cm column of neomycin-Sepharose, kanamycin-Sepharose synthesized at pH 10.0, or kanamycin-Sepharose synthesized at pH 9.0 equilibrated with Buffer 1 containing 0.15 M ammonium sulfate. After the protein sample was loaded, the column was washed with approximately 7 column volumes (370 ml) of 0.15 M ammonium sulfate in Buffer 1 followed by 2 column volumes of 2.0 M ammonium sulfate in Buffer 1; both ammonium sulfate buffers eluted non-specifically bound protein. 2.0 M ammonium sulfate in Buffer 1. Tightly bound ANT(2") activity was eluted with 2.0 M TRIS, pH 8.1; this was referred to as specific elution. The flow rate was 1.0 ml/minute throughout this procedure, and 10 ml fractions were collected. A similar procedure was used during trial runs of runs of different affinity resins except that 1 x 5 cm columns were used, 1 ml fractions were collected, and the protein loaded onto the column was purified ANT(2") instead of the DEAE-agarose fraction.

B.7d Gel Filtration Chromatography

The activity peak eluted from the affinity column by 2.0 M TRIS was loaded onto a 2.5 x 60 cm column of Ultrogel AcA 54 equilibrated with Buffer 1. The flow rate was 0.5 ml/minute. The activity peak collected off this column was distributed into 0.5 ml to 1.0 ml aliquots and frozen at -20 C. The purified enzyme was stored for further use under these conditions.

B.7e Purification of ANT(2")I with Different Affinity Resins

Preparation A

The protein used in this preparation was extracted from 136 grams of *E. coli* W677/pMY10 cells and after early purification, desalting on BioGel-P6DG, and ion exchange chromatography, the partially purified pool of ANT(2")I activity was applied to the neomycin-Sepharose column synthesized at pH 10.0. Very little activity was eluted non-specifically; the majority of ANT(2") activity was eluted specifically with TRIS buffer subsequently applied to an Ultrogel AcA 54 column. Activity was eluted with Buffer 1.

Preparation B

The activity used in this preparation originated

from 130 grams of E. coli W677/pMY10 (see Table 2). A sample eluted from the DEAE-agarose column with a specific activity of 0.93 units/mg was loaded onto kanamycin-Sepharose synthesized at pH 9 (KS9). No ANT(2") activity eluted non-specifically, and one peak of activity was specifically eluted with TRIS. This was applied to the Ultrogel AcA 54 column and eluted with Buffer 1.

Preparation C

ANT(2") activity originating from 130 grams of cells (see preparation B) and eluted from DEAE-agarose was applied to a kanamycin-Sepharose column synthesized at pH 10 (KS10). Activity was eluted both non-specifically and speicically. The tightly bound activity was pooled loaded onto an Ultrogel AcA 54 column, and eluted with Buffer 1.

The non-specifically eluted activity, called C2, was recycled over the KS10 column for 16 hours to test for time-depndent binding. About half of the activity recycled over the column was eluted specifically. This is designated C3. After gel filtration, C3 was recombined with the non-specifically eluted activity, C4,

TABLE 2

PURIFICATION OF ANT(2")

PREPARATIONS B AND C

Fraction	Protein (mg/ml)	A280 / A280	Specific Activity (IU/mg)	Total Activity (units)	Recovery (%)	Purification Factor
osmotic shock fluid	10	0.4	0.02	499	-	1
strepto-mycin sulfate	6	0.6	0.03	253	50	1.5
P2 Pool	6	0.6	0.08	174	35	4.0
DEAE Pool	3	0.9	0.93	325	65	46

Assayed at pH 9.1 with 11.84 mM Mg:ATP and 0.49 mM tobramycin

to give the combined pool, C3 + C4.

Next, the combined C3 + C4 pool was loaded onto the KS10 column. In this case, the protein was not recycled, but simply treated as a normal affinity column run. Nearly all activity was eluted non-specifically.

Preparation D

The C3 + C4 pool was then divided into two portions, each of which was treated identically. Each portion was loaded onto the kanamycin-Sepharose column synthesized at pH 9. In each case, activity was only eluted specifically from this affinity resin. The samples eluted from KS9 were subsequently loaded onto and eluted from the gel filtration column.

B.7f Protein Determination During ANT(2") Purification

Absorbances of protein fractions at 280 nm and 260 nm were measured in a 1 cm pathlength quartz cuvette. The nomograph of A. E. Adams based on the Warburg-Christian equation (156) was used to calculate the protein concentrations based on absorbance readings.

B.8 Gel Electrophoresis

Electrophoresis was performed in a BioRad model 300A electrophoresis tank using an LKB model 300A power supply. Protein samples of 0.01 mg to 0.03 mg were

applied to polyacrylamide gels of 12% monomer concentration. The gels were made accrding to instructions provided by BioRad (129). Gel elctrophoresis of native protein was conducted for 5 hours in 0.188 M TRIS, pH 8.9, and 0.188 glycine. Electrophoresis was conducted at 4 oC, 150 V, and 20 mAmps. The protein in the gel was fixed with 40% (v/v) isopropanol and 10% (v/v) acetic acid overnight and stained for 1 hour in 0.25% (w/v) Coomassie Brilliant Blue in 7.5% acetic acid. The gels were destained overnight in 40% isopropanol and 10% acetic acid. Fixing, staining and de-staining were performed at ambient temperature.

The equipment described above was also used for electrophoresis in the presence of 0.25% SDS. Protein samples were treated with SDS and 2-mercaptoethanol after the method of Fairbanks et al. (45) prior to electrophoresis. Bovine Serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and cytochrome c were used as molecular weight standards. Protein samples of 0.02 mg to 0.03 mg were run for 5 hours at 4 oC on polyacrylamide gels of 12% monomer

concentration. Fixing, staining, and de-staining the gels followed the same procedure described for electrophoresis of native proteins.

Electrophoretic profiles of polyacrylamide gels were determined by absorbance measurements at 595 nm with a Gilford model 240 spectrophotometer using a Gilford model 2410 transport drive. A slit width of 0.05 mm and a rate of 4 cm/minute were used for scanning the gels. The data were recorded on a chart recorder. A planimeter was used to measure the relative areas under the peaks of the chart-paper tracing.

B.9 Spectrophotometric Assays of ANT(2") Activity

B.9a Coupling Assay, Instruments, and Cuvettes

The coupling assay as described by Van Pelt and Northrop was used to monitor ANT(2") activity during purification and kinetic studies (142). The formation of pyrophosphate, one of the products of the ANT(2") catalyzed reaction, is monitored by coupling it to UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. The formation of NADPH with a molar extinction coefficient of 6220 at 340 nm is monitored. One unit of ANT(2") activity was defined as the production of 1 umole of NADPH per minute.

The assay is outlined in Figure 3. For assays during purification and kinetic studies, the following concentrations of components were used per assay: 0.5 mM UDP-glucose, 1.2 mM glucose 1,6-diphosphate, 0.2 mM NADP, 0.3 mM dithiothreitol, 5 units/ml UDP-glucose pyrophosphorylase, 13 units/ml phosphoglucomutase, and 4 units/ml glucose-6-phosphate dehydrogenase. A lower concentration of EDTA, 0.01 mM, was used instead of the 0.125 mM concentration in the assay system of Van Pelt and Northrop. The components of the coupling assay were mixed together, and a variable volume of 0.1 M HEPES, pH 7.0, was added to this solution so that 0.05 ml, 0.10 ml, Or 0.20 ml Of the coupling assay components could be added to the ANT(2") assay mixtures and give the final concentrations described.

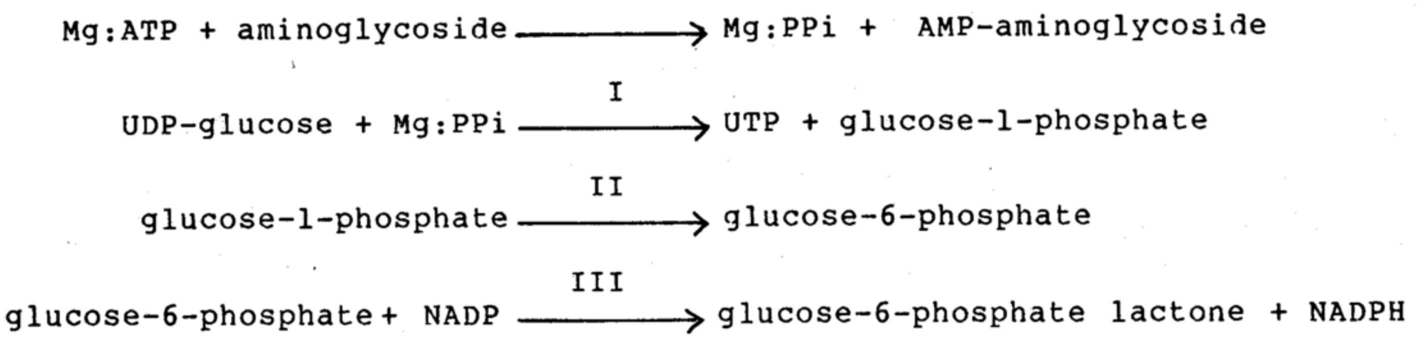
The assays were performed on either a Gilford Model 240 spectrophotometer or a Cary 118 spectrophotometer. An LKB/Bromma single channel recorder, a Linear 1201 single channel recorder, or a Leeds and Northrup Speedomax recorder was used with the Gilford. Both instruments were equipped with circulating water baths that maintained the temperature at 25 C. The custom-made

Figure 3: Assay of ANT(2")I

I: UDP-glucose pyrophosphorylase

II: phosphoglucomutase

III: glucose-6-phosphate dehydrogenase


Mg:ATP + aminoglycoside → Mg:PPi + AMP-aminoglycoside
UDP-glucose + Mg:PPi → UTP + glucose-1-phosphate
I
glucose-1-phosphate → glucose-6-phosphate
II
glucose-6-phosphate + NADP → glucose-6-phosphate lactone + NADPH
III

cuvette holders of the Gilford and Cary spectrophotometers allowed maximal contact between the walls of the holder and those of the cuvette. The temperature of the solution within the cuvette reached the constantly maintained temperature of the chamber at a rate with a half-life of 0.71 minute; the analagous half-lives with conventional cuvette holders of the Gilford and Cary spectrophotometers were 3.35 minutes and 3.85 minutes, respectively (89). The cuvettes were pre-incubated for at least 2.5 minutes in the specially constructed cuvette chamber. The cuvette holders could accomodate cells with pathlengths up to 10 cm.

Self-masking micro cuvettes with a 1cm pathlength and a 4 mm width between the interior walls were routinely used. However, for assay requiring more sensitivity, grade C optical glass cuvettes with 5 cm pathlengths were used. Since the assay components can be costly, the total assay volumes were minimized to 0.25 ml for the micro cuvettes and 1.0 ml for the 5 cm pathlength cuvettes by applying black vinyl tape to the outer walls of the cuvettes and leaving an aperature on either wall so that light could pass through. The aperatures were positioned such that light would not strike the minicus

of the solution or the side walls of the cuvette.

Enzyme assays were performed on the Gilford with a slit width of 0.32 mm where the fraction of stray light is less than 3% or on the Cary spectrophotometer with a slit width of 3.0 mm where the proportion of stray light is less than 1%. Full scale settings of 0.02 to 2.0 absorbance were used.

B.9b <u>Substrate and Enzyme Solutions</u>

Sulfate salts and free base forms of aminoglycosides were weughed out into polypropylene tubes and dissolved in distilled, deionied water. The pH of these solutions was adjusted to 7.0 to 7.5 with 0.3 N sulfuric acid. Nucleotide triphosphates in sodium salt form were also weighed into polypropylene tubes and dissolved in deionzed, distilled water; the pH of these solutions was adjusted to 7.0 with 4.0 N NaOH. All further dilutions of the substrate solutions were into distilled, deionized water. The substrate stock solutions were stored frozen at -20 C. Non-enzymatic components of the coupling mix were also dissolved in deionized, distilled water and stored frozen. Lyophilized UDP-glucose pyrophosphorylase as supplied by Sigma was dissolved in 0.1 M TAPS, pH 8.5,

and stored at 4 C. Enzyme samples were diluted into 0.1 M TAPS pH 8.5. Solutions of substrates, coupling mix, and enzyme were kept on ice during the course of the assays.

B.9c <u>Buffers</u>

All buffers were made with distilled, deionized water and were titrated to the desired pH with NaOH. The assay concentration of all buffers was 0.1 M. Buffers were maintained at 25 C for all studies. CHES, pH 9.1, was the buffer used in assays during the determination of kinetic constants of both nucleotide and aminoglycoside substrates, purification, inhibition, and viscosity experiments. The buffers used in pH studies are noted in the RESULTS chapter.

B.9d <u>Calculation of Mg:ATP Stability Constant and Determination of Total Magnesium Ion Concentration</u>

Stability constants for the Mg:nucleotide complexes were determined by the equation of Adolfsen and Moudrianakis (1):

$$K'_{ML} = K_{ML} \times ((10^{-au}) / (1 + K_{NaL}[Na^{+}] + K_{HL}[H^{+}]))$$

where K'_{ML} is the apparent metal-nucleotide stability

constant; K_{ML} is the intrinsic stability constant (= 140,000); M = metal ion; L = ligand (nucleotide); a = 3.1, a constant determined for Mg:ATP; u = the ionic strength, as contributed by Na^+;K_{NaL} is the stability constant for sodium ion:ligand complexes (= 17 for Na:ATP); and K_{HL} is the dissociation constant for protonated/unprotonated nucleotide (= 10   for HATP).

Once the stability constant for Mg:ATP was caluculated for a given pH and ionc strength, it was used to determine the concentration of toal magnesium ion necessary to give a constant concentration of free magnesium ion or a desired ratio of Mg:ATP to ATP for any given total concentration of nucleotide. The equations used for this were those described by Morrison:

$$M_t = M + MATP$$

$$ATP_t = MATP\ (1 + K_{ML}/(M))$$

$$K_{ML} = (M)(ATP)/(MATP)$$

where M_t is the total metal ion concentration, M is free metal ion concentration, ATP_t is total ATP concentration, MATP is the metal nucleotide complex, and K_{ML} is the

stability constant of the metal nucleotide complex (96). These calculations were also applied to nucleotide triphosphates other than ATP.

For the majority of the experiments on the kinetic properties of ANT(2") at pH 9.1 and for determination of activity during purification, the concentration of free magnesium ion concentration was held constant at 10.0 mM. In earlier experiments using the fluorescence assay, the ratio of Mg:ATP to free ATP was held constant at 500:1. The effect of changing the ratio of Mg:ATP to free ATP was examined by keeping the concentration of Mg:ATP constant while varying the concentrations of total ATP and total magnesium. When the effect of pH variation on kinetic constants of an aminoglycoside substrate was examined, the ratio of Mg:ATP tp ATP was held constant at 390.6:1. Magnesium acetate was the salt used in all experiments.

B.9e Determination of Enzyme Activity

During purification, an assay mixture of 0.16 ml of 0.16 M CHES, pH 9.1, 0.05 ml coupling mix, 0.01 ml of 0.51 M to 0.60 M magnesium acetate, 0.01 ml of 0.26 M to 0.35 M ATP, and 0.01 ml of enzyme was prepared in a 1 cm micro cuvette. The assay mixture was pre-incubated in

the temperature-controlled cuvette chamber for at least 2.5 minutes. The reaction was initiated by the addition of 0.01 ml of 12.25 mM tobramycin using a paddle. The total assay volume was 0.25 ml; the final concentrations of magnesium acetate, ATP, enzyme, and tobramycin were 20.4 to 24.0 mM, 10.4 mM to 14.0 mM, 0.06 to 5.0 units/ml, and 0.49 mM respectively. For the 0.5 ml total assay volume, the volumes of solutions used were simply doubled. Assays used for studies of ANT(2") stability were conducted in a similar manner.

B.9f Determination of Kinetic Constants

For determination of kinetic constants of the substrates, the assays were prepared and initiated as described above except that either the aminoglycoside or total magnesium and nucloetide concentrations were varied. For inhibition studies, 0.15 ml of 0.167 M CHES, pH 9.1, 0.05 ml coupling mix, 0.01 ml magnesium acetate, 0.01 ml ATP, 0.01 ml ANT(2"), and 0.01 ml of inhibitor solution were prepared in a 1 cm microcuvette, pre-incubated for at least 2.5 minutes, and the reaction was initiated with the aminoglycoside substrate.

The assays performed during viscosity studies were prepared and intiated as described for the kinetic constant determinations except that the CHES buffer stock contained an appropriate amount of glycerol to give the desired viscosity in the assay mixture. The coupling system was checked for linearity of velocity versus the concentration of ANT(2") at three different concentrations of glycerol. The solution viscosity was estimated by linear interpolation of data from the Chemical Rubber Company (22).

For kinetic studies as a function of pH, 0.64 ml of 0.16 M buffer, 0.20 ml coupling mixture, 0.04 ml magnesium acetate, 0.04 ml ATP, and 0.04 ml enzyme were mixed in a 5 cm cuvette and pre-incubated at least 2.5 minutes. The reaction was initiated with 0.04 ml sisomicin. The coupling system was checked for linearity of velocity versus ANT(2") concentration at pH 6.0, 7.0, 8.0, 9.0, and 9.5. Similar assays were performed in the 5 cm cuvettes for the determination of kinetic constants of aminoglycoside substrates with low values of Km except that 0.1 M CHES, pH 9.1, was used as the buffer.

B.9g Determination of Aminoglycoside and Nucleotide Concentrations

Concentrations of aminoglycosides were determined by using an assay similar to that described for those used for determination of the amount of ANT(2") activity except that the reaction was allowed to go to completion. The total assay volume was 0.5 ml. Three different dilutions of antibiotic stock were used as samples for each substrate to ensure linearity of the assay. The control contained all assay components except the aminoglycoside being assayed. The assay was initiated by the addition of an excess of enzyme (about 0.25 units); this ensured rapid completion of the reaction. The rate was not followed, but the extent of the reaction was monitored on the chart recorder. When no further change in absorbance at 340 nm occurred, the total absorbance change was read. Dilutions of aminoglycoside stock solutions were made to provide changes in absorbance of 0.2 to 0.8.

The concentrations of aminoglycosides were also measured using aminoglycoside acetyltransferase (6')IV and its spectrophotometric assay (118, 119). The assays contained 0.1 M MES, pH 6.0, 0.06 mM acetyl-CoA, 0.06 mM

4,4'-dithiopyridine, and aminolglycoside. The protocol described for ANT(2") assays was followed. Three dilutions of aminoglycoside were used, and the control assay contained all components except the antibiotic. The reaction was initiated by the addition of an excess of AAC(6')IV (0.15 units). Upon completion of the reaction the total absorbance at 324 nm was measured. This assay was especially useful for determination of the concentration of neomycin C, an aminoglycoside that is not a substrate for ANT(2").

The concentration of ATP was determined by a coupled enzyme assay. The assay used for measuring ATP concentration contained 90.0 mM TRIS-HCl, 1.0 mM NADP, 3.0 mM Mg(Cl)2, 32 units/ml hexokinase, 18 units/ml glucose-6-phosphate dehydrogenase, and three dilutions of ATP. The control contained all components except ATP. The reaction was started with 0.12 mM glucose. When the reaction was complete, the total absorbance change at 340 nm was read.

Alternatively, the ATP concentration was measured by absorbance at 260 nm at pH 7.0. Samples of ATP from a concentrated stock solution were diluted into 0.1 M

HEPES, pH 7.0, and the absorbance was read at 260 nm. The ATP concentration was calculated from measured absorbance using a molar extinction coefficient of 15,400 (108). Similarly, the concentrations of CTP, GTP, UTP, and the deoxyribonucleotide triphosphates were determined spectrophotometrically. The following molar extinction coefficients and absorbance maxima at pH 7.0 were used to calculate nucleotide concentration: for CTP - 9100 at 271 nm; for UTP - 10,000 at 262 nm, and for GTP - 13,700 at 252 nm. These extinction coefficients and absorbance maxima were also used to calculate concentrations of the corresponding deoxyribonucleotides (108).

B.10 <u>FLUORESCENCE ASSAYS OF ANT(2")</u>

B.10a <u>Instruments and Cuvettes</u>

Since the final product of the coupling system, NADPH, is a fluorophore, ANT(2") activity was monitored by measuring the increase of relative fluoresence at an excitiation wavelength of 340 nm and an emission wavelength of 462 nm. An Aminco-Bowman spectrophofluorometer equipped with a Leeds-Northrup Speedomax chart recorder was used to measure the relative fluorescence of NADPH. The temperature of the thermojacketted cuvette chamber was maintained by a

circulating water bath at 25 C. The excitation and emission slitwidths were both 5 mm. Dual pathlength optical glass cuvettes were used. The excitiation pathlength was 10 mm, and the emission pathlength was 2 mm.

B.10b Assay Components and Procedures

The same concentrations of coupling mix components as those used in the spectrophotometric assays were employed for the fluorescence assays. The total assay volume was 0.25 ml. The assays were prepared by mixing 0.16 ml 0.1 M CHES, pH 9.1, 0.05 ml coupling mix, 0.01 ml magnesium acetate, 0.01 ml ATP, and 0.01 ml enzyme in a dual pathlength cuvette. The assay components were mixed by inversion of the cuvette, placed in the cuvette holder, and allowed to pre-incubate for at least 3 minutes. The cuvette holder could accomodate only one cuvette so other cells containing assay components were pre-incubated in a thermojacketted glass chamber connected to the same circulating water bath as the cuvette holder; each cuvette was also allowed to pre-incubate 3 minutes in the instrument's cuvette holder prior to initiation of the reaction; a 0.01 ml sample of

antibiotic solution was added to the mixture, and the cuvette was inverted 4 to 6 times to mix the components before placing it back into the cuvette holder.

B.10c Standardization of the Fluoresence Assay

The full scale relative fluoresence was determined by adding a known concentration of sodium pyrophosphate to the assay mixture containing all components except aminoglycoside and allowing the reaction to run to completion. The photomultiplier (PM) microphotometer switch was set to 3, which gives a range of relative fluoresence of 0 to 3.2, and the relative fluoresence of the sample was then read. The setting on the PM microphotometer was not chnged throughout the duration of an experiment. The procedure was repeated three times to obtain an average relative fluoresence value for a given concentration of NADPH produced by the added pyrophosphate. This value was then used to set the full scale deflection on the chart paper, and the nanomoles of NADPH per division on the chart paper was calculated. This value was used to determine the rate of NADPH production from the coupled assay. Care was taken to keep the total concentration of NADPH below 20 uM where

self-absorption begins to interfere, and the assay is no longer linear.

The concentration of sodium pyrophosphate solution used for standardization was determined by a colorimetric method described by Putnins and Yamada (114).

Since dust particles and lint cause considerable interference in fluoresence assays, all buffer and substrate solutions were filtered three times each through 0.45 micron Millipore filters. The coupling mix was filtered through a Millipore Millex-GV filter unit containing a low protein-binding 0.22 micron filter. All glassware was acid washed. The dual pathlength cuvettes were cleaned daily by immersing them in 7.0 N nitric acid for 15 minutes, followed by a rinse of filtered, deionized water.

B.11 ISOTOPE EXCHANGE ASSAYS

The assay procedure used to monitor isotope exchange between pyrophosphate and ATP exploits the nucleotide's ability to bind to pyrophosphate-washed charcoal while the radioactively labeled pyrophosphate is washed away. The procedure used for separation of ATP and pyrophosphate using activated charcoal was derived from the method described by Lee and Littmann (79). One

hundred twenty grams of acid-washed, activated charcoal was washed with large volumes of distilled water until treatment of the effluent with silver nitrate gave no indication of the presence of chloride ion, i.e., no precipitate is observed. The charcoal was dried to dampness by suction filtration and then suspended in 1.0 mM sodium pyrophosphate (about 30 ml per gram dry weight charcoal). The charcoal suspension was stirred for two hours. The pyrophosphate solution was removed from the charcoal by filtration, and the charcoal was washed with 2 L of deionized water. The charcoal was dried at 110 C and then stored in glass jars at ambient temperature until ready for use.

Assays for isotope exchange contained 100 mM CHES, pH 9.1, 13 mM magnesium acetate, 3 mM ATP, 0.03 mM tobramycin, 0.016 units/ml ANT(2"), 0.4 mM sodium pyrophosphate, and 0.01 to 0.12 mCi of [32P]-pyrophosphate to give final specific activities of 0.02 mCi/mmole to 0.3 mCi/mmole [32P] PPi. The total assay volume was 1 to 2 ml. All components except the enzyme were mixed and pre-incubated for at least 5 minutes in a water bath maintained at 25 C. The reaction was

initiated by the addition of enzyme. Aliquots of 0.125 ml were removed from the assay mixture at successive timepoints and then vigorously mixed in glass test tubes containing 0.5 ml of pyrophosphate-washed charcoal suspended in cold (0 to 4 C) 10% trichloroacetic acid and 20 mM sodium pyrophosphate (24 mg charcoal per ml). A 0.125 ml sample was removed from the assay mixture prior to initiation of the reaction and added to the charcoal suspension; this served as a "background" control. The charcoal-sample mixture was kept on ice for at least 5 minutes. Then the charcoal was collected on glass fiber filter papers, rinsed twice with 2.0 ml each 0.1 M sodium pyrophosphate, and rinsed 5 times with 2 ml each deionized, distilled water. After the last water rinse, the filter papers were partially dried by suction filtration; the samples were barely damp, but neither wet nor absolutely dry. The filter paper with the collected charcoal was placed into a glass scintillation vial, and 10 ml of scintillation cocktail containing toluene, 0.3% (w/v) PPO, and 0.01% (w/v) dimethyl POPOP were added to the vial. The amount of radioactivity was measured with a Packard Tri-Carb 460 CD liquid scintillation counter.

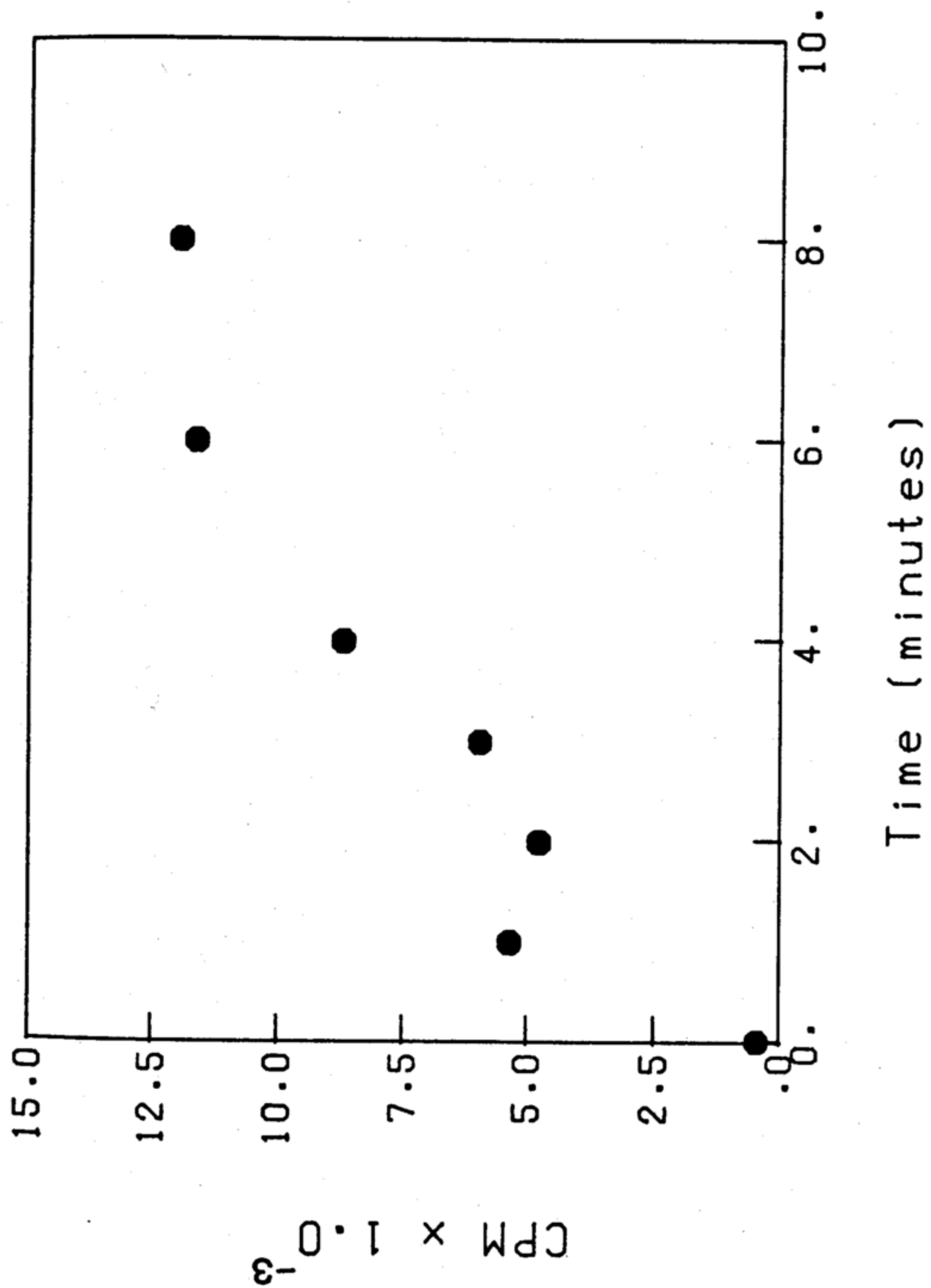
In order to assure that the PPi-treated charcoal technique was effectively binding 32P-labeled nucleotide, the production of UT32P from 32PPi as catalyzed by UDP-glucose pyrophosphorylase was measured using this technique. The assay components were identical to those used for the excahnge experiment with ANT(2")I except that tobramycin was absent and UDP-glucose and UDP-glucose pyrphosphorylase were included in the mixture. The results of this experiment are illustrated in Figure 4 and indicate that the PPi-treated charcoal does bind the radioactively labeled nucleotide.

B.12 DATA ANALYSES

Analyses of kinetic data were performed on a Northstar Horizon computer using BASIC programs developed by Northrop (Northrop, unpublished); the non-linear regression routine used in these programs was written in BASIC by R. G. Duggleby (43). Data from kinetic constant measurements were fit to Equation 1 or 2. Data from alternative substrate studies were fit to Equation 1. Data from kinetic patterns generated in the inhibition studies were initially fit to Equation 1 or 2. Graphical representations of the patterns were derived from these individual line fits. Then the data were fit to the

Figure 4: UDP-glucose pyrophosphorylase catalyzed production of $UT^{32}P$ from UDP-glucose and [^{32}P] pyrophosphate

The contents of the assay mixture were as follows: 100 mM CHES buffer, pH 9.1, 13 mM magnesium acetate, 3 mM ATP, 0.4 mM sodium pyrophosphate, 0.024 uCi/umole [^{32}P]-pyrophosphate, 1.0 mM UDP-glucose, 0.016 units/ml ANT(2"), and 0.10 units/ml UDP-glucose pyrophosphorylase. Addition of the latter enzyme to the other components of the assay mix initiated the reaction. Aliquots were removed at successive timepoints and treated the same way as those from the ^{32}PPi-ATP exchange experiment (see section III.B.10). Purified ANT(2") was from Preparation A.



CPM x 1.0^-3
15.0
12.5
10.0
7.5
5.0
2.5
.0
0.
2.
4.
6.
8.
10.
Time (minutes)

following equations: for the substrate inhibition patterns, Equations 3 and 5 were used; Equations 5 and 6 were used for dead end inhibition; for multiple inhibition, Equations 7, 8, and 9 were used; and for product inhibition, Equations 4 and 9 were used. Inhibition by TRIS buufer was evaluated with Equation 10. Graphical representation of slope and intercept replots was derived from fitting 1/Vmax to Equations 11 or 13 and K/V to Equation 12. Data from the viscosity studies were fit to Equation 2. The values of kinetic constants at different pH values were determined by fitting the data to Equation 2; then , the pH dependence of the kinetic constants was evaluated by fitting the data to Equations 14, 15, 16, or 17. The linear correlations between aminoglycoside kinetic constants and M.I.C. values were obtained by fitting the data to Equation 17 using MINITAB (copyright - Penn State Univ., 1980), a statistics software package available at the Madison Academic Computing Center.

EQUATIONS USED FOR DATA FITTING

$v = VA / (K_a + A)$ (1)

$v = VA / (K_a + A + A^2/K^I)$ (2)

$v = VA / (K_a + A(1 + I/K_{ii}))$ (3)

$v = VA / (K_a(1 + I/K_{is}) + A(1 + I/K_{ii}))$ (4)

$v = VA / (K_a + A((1 + I/K_{ii})/(1 + I/K_{id}))$ (5)

$v = VA / (K_a (1 + I/K_{is}) + A((1 + I/K_{ii})/(1 + I/K_{id}))$ (6)

$v = VA / (K_a(1 + I/K_{is}) + A(1 + A/K_I))$ (7)

$v = VA / (K_a(1 + I/K_{is}) + A(1 + A/K_I + AI/K_I K_{is}))$ (8)

$v = VA / (K_a + A(1 + K_{ii} + A/K_I))$ (9)

$v = V/(1. + I/Ki)$ (10)

$1/V_i = 1/V(I/K_{ii}) + 1/V$ (11)

$K/V_i = K/V(I/K_{is}) + K/V$ (12)

$1/V_i = 1/V ((1 + I/K_{id})/(1 + I/K_{ii}))$ (13)

$\log(V/K) = \log ((V/K) / (1 + H/K_a)^2 + (K_b/H)^2))$ (14)

$-\log(Ki) = \log (Ki(1 + (H/Ka)^2 + (K_b/H)^2))$ (15)

$\log(V) = \log ((V_l + V_h(K/H) / (1 + K/H))$ (16)

$y = mx + b$ (17)

CHAPTER III:

RESULTS

A. FLUORESCENCE ASSAY

The fluorescence assay of ANT(2") activity exploits the fluorophoric properties of NADPH, the final product of the coupled assay developed by Van Pelt and Northrop (150). However, since emission spectroscopy does not follow the Beer-Lambert law, the scale on the spectrofluorometer must be set by measuring the relative fluorescence of a known concentration of NADPH. This was accomplished by generation of NADPH from a known concentration of sodium pyrophosphate that was added to the coupled enzyme system.

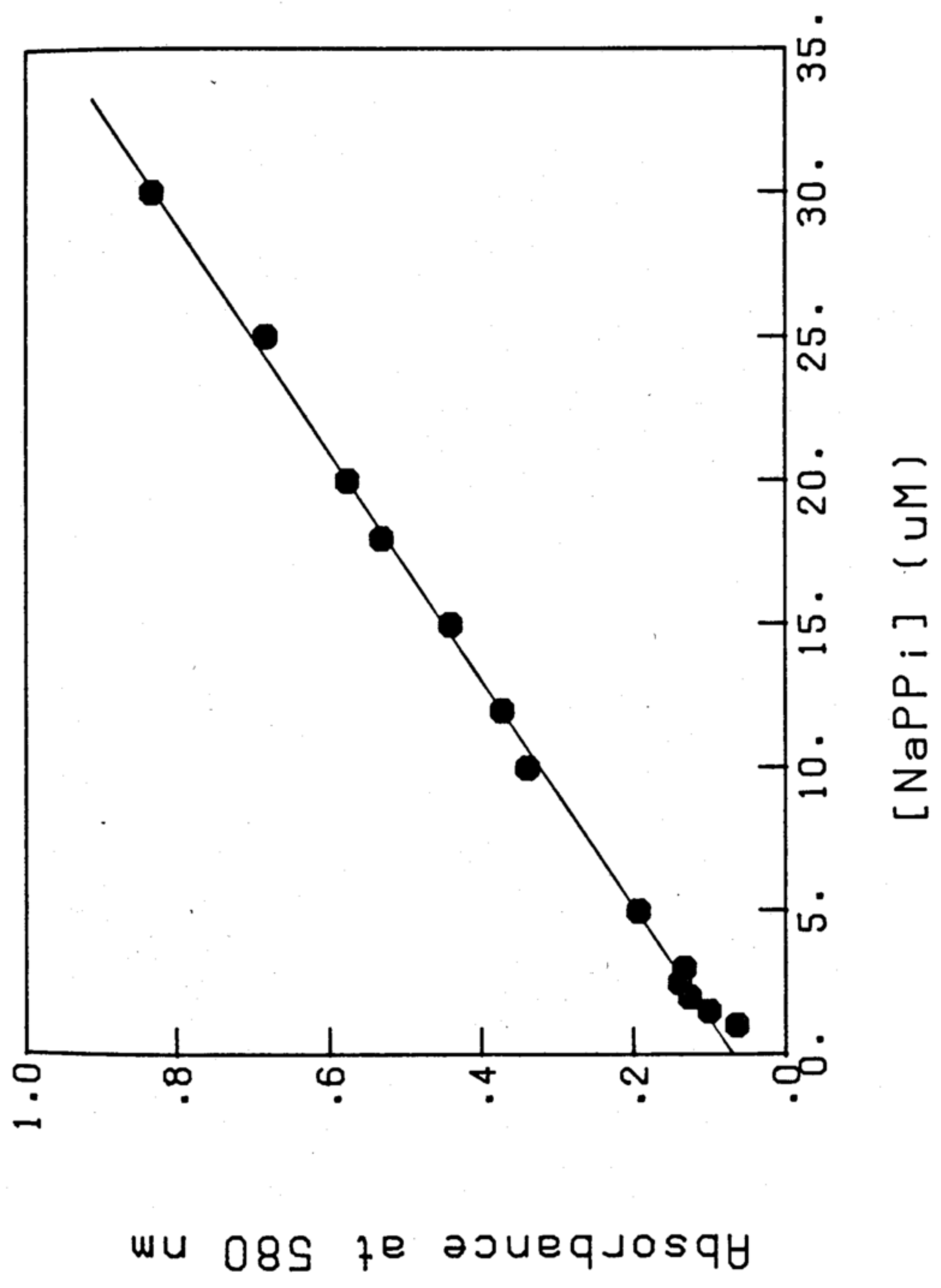
The method of Putnins and Yamada was used to determine the concentration of a stock solution of pyrophosphate (114). In this microprocedure, the concentration of PPi is determined quantitatively as the amount of chromophore formed with molybdate reagent, 1-amino-2-naphthol-4-sulfonic acid in bisulfite, and 2-mercaptoethanol; the latter reagent is required for color formation. The molar extinction coefficient at 580 nm for PPi is 27,000.

Absorbances at 580 nm were measured for a series of pyrophosphate concentrations. These samples were prepared by diluting a stock solution of pyrophosphate

into deionised, distilled water. The samples were then treated with the molybdate-ANS-thiol mixture and the absorbances were measured within an hour. Figure 5 shows that a linear relationship exists between absorbance at 580 nm and the concentration of pyrophosphate based on weight.

Aliquots of NADPH dissolved in propylene glycol were added to 0.1 M Ches buffer at pH 9.0, and the concentration of nucleotide in each sample was estimated by measuring absorbance at 340 nm. Relative fluorescence of each sample was measured using an excitation and emmission wavelengths of 340 nm and 460 nm, respectively. Figure 6 shows that a linear relationship exists between NADPH concentration and relative fluorescence. Similarly, as seen in Figure 6, fluorescence values are linear with the amount of NADPH generated <u>via</u> the ANT(2") coupled assay from known concentrations of pyrophosphate. It should be noted that the relative fluorescence of NADPH is shifted to a lower value in the presence of other assay components. This is likely due to quenching of fluorescence by proteins and magnesium acetate in the coupling mix.

Figure 5: Linearity of absorbance at 580 nm with pyrophosphate concentration

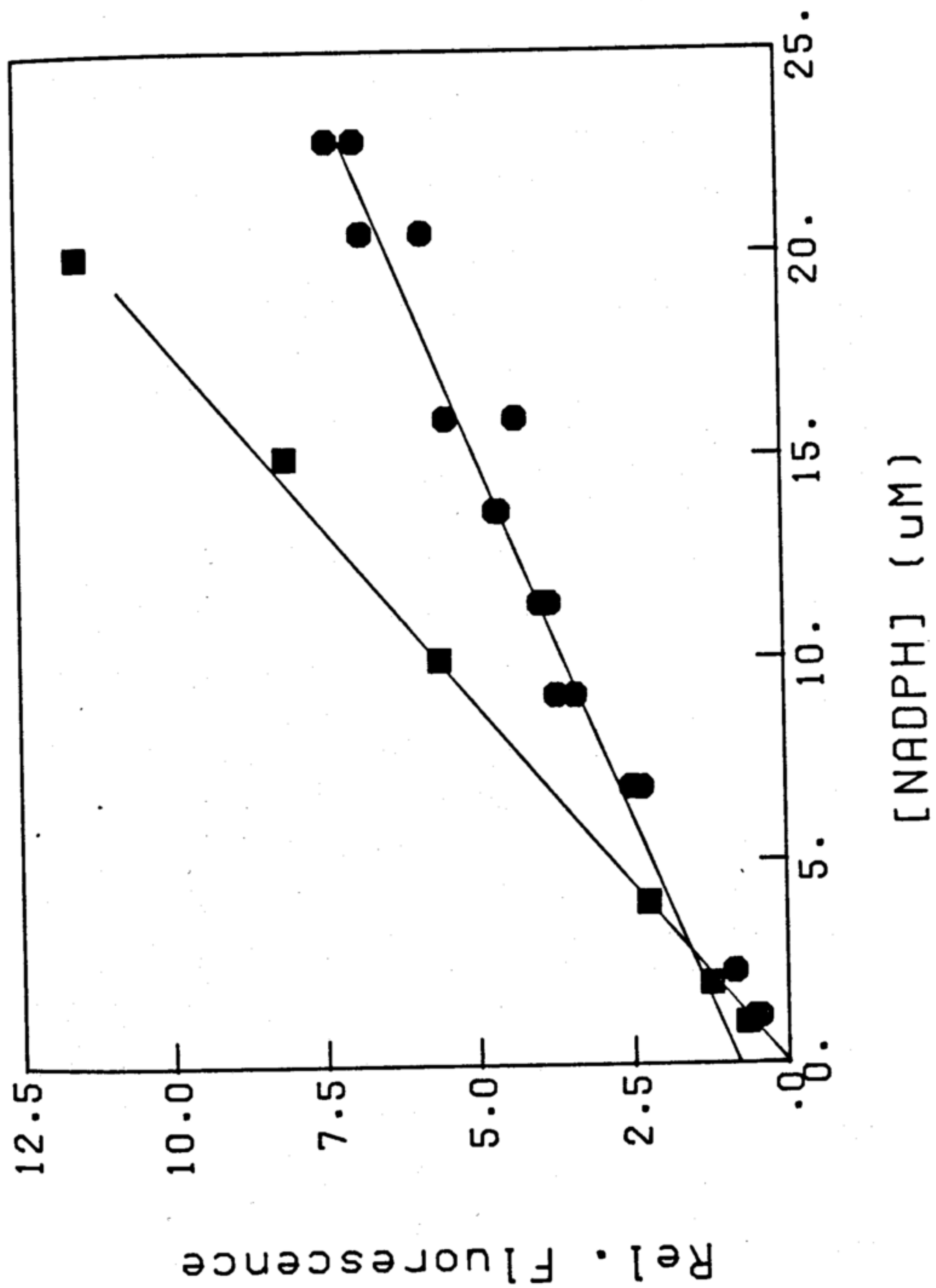


Absorbance at 580 nm
1.0
.8
.6
.4
.2
.0
[NaPPi] (uM)
0.
5.
10.
15.
20.
25.
30.
35.

Figure 6: Linearity of relative fluorescence with NADPH concentration

■ NADPH added to 0.1 M CHES buffer, pH 9.1

● NADPH generated by addition of NaPPi to ANT(2") coupling mixture (minus aminoglycoside)



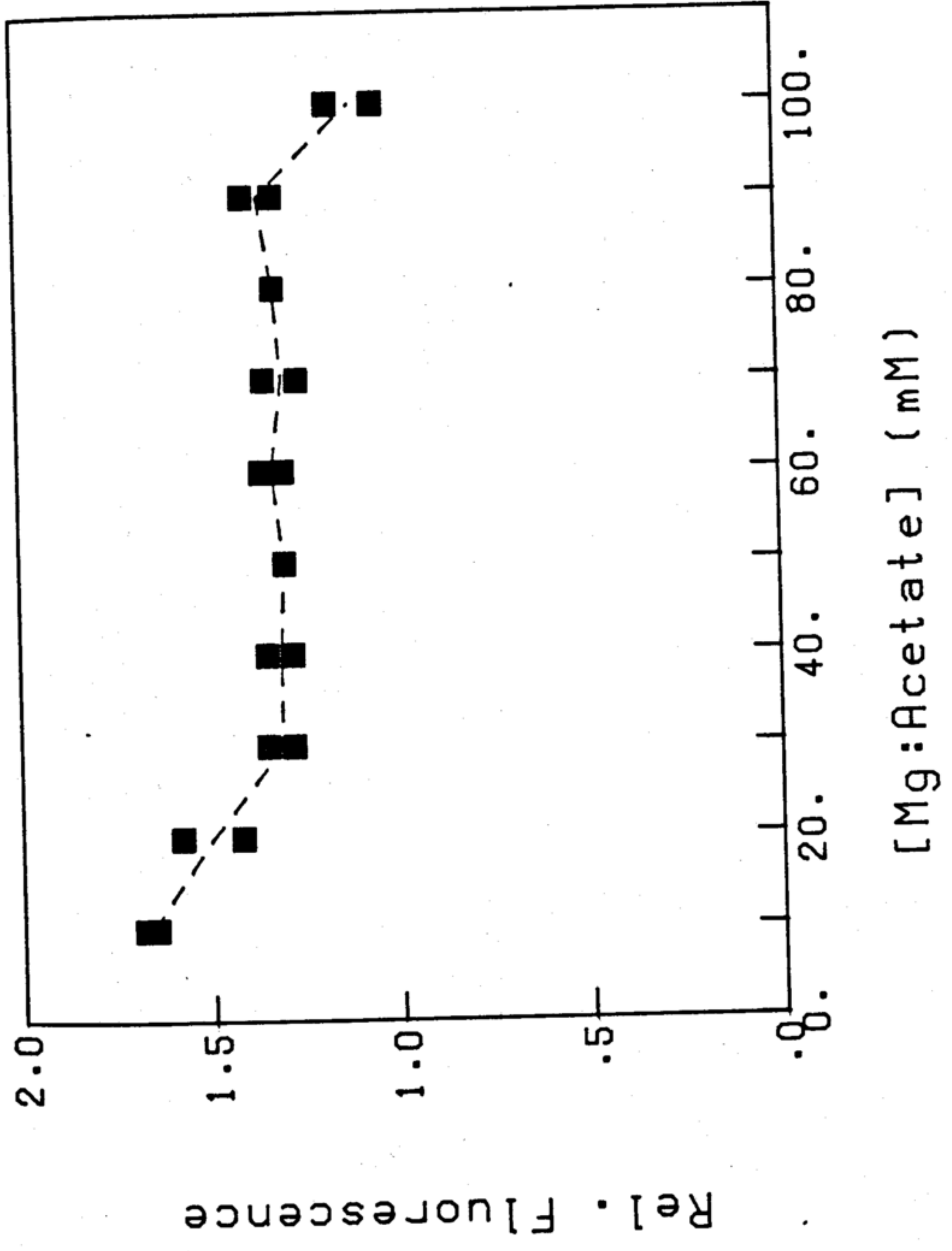
Rel. Fluorescence
12.5
10.0
7.5
5.0
2.5
0.
0.
5.
10.
15.
20.
25.
[NADPH] (uM)

Metal salts frequently act as fluorescence quenchers. Because magnesium acetate is used at fairly high concentrations in the assay, the effect of different concentrations of this metal salt on fluorescence of PPi-generated NADPH was examined. The results are shown in Figure 7. Magnesium acetate concentrations greater than 20 mM resulted in reduction of fluorescence by about 20% relative to the value determined at 10 mM. A concentration of 100 mM gave a 30 percent reduction in fluorescence. This quench curve was used to correct fluorescence values measured in assays containing concentrations of magnesium acetate greater than 20 mM.

The assay was found to be linear with changes in the amount of purified ANT(2") as shown in Figure 8. Enzyme activity was determined by the spectrophotometric assay and the fluorometric assay using identical assay components. At pH 9.1 with 0.8 mM Mg:ATP, 0.1 mg/ml tobramycin, and 0.03 mg/ml ANT(2"), the activity determined spectrophotometrically was 0.049 units/ml, and the activity determined with the fluorimetric assay was 0.047 units/ml.

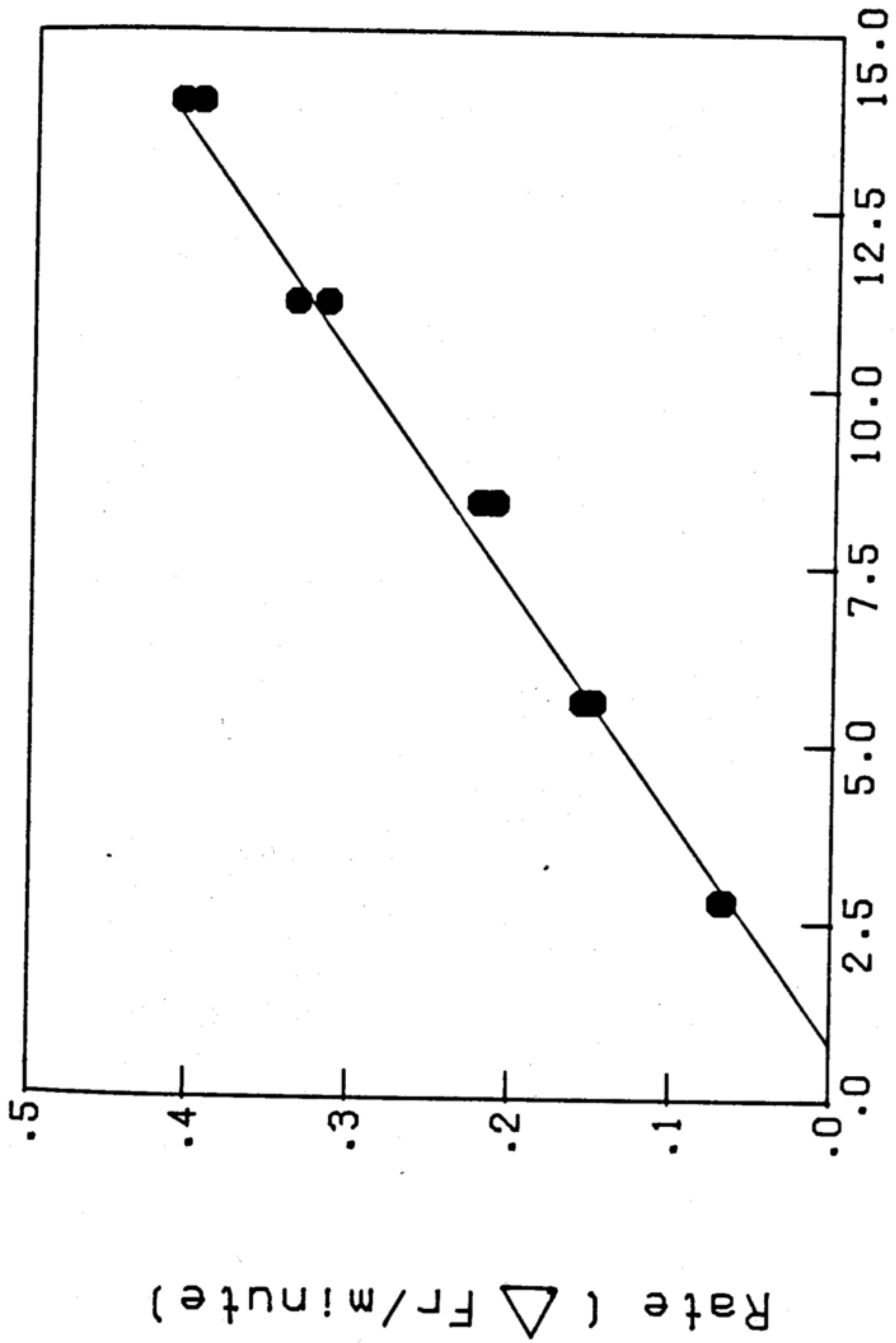
Figure 7: Effect of magnesium acetate on NADPH fluoresence

NADPH was generated by adding pyrophosphate to the ANT(2") coupling mixture (minus aminoglycoside)



Rel. Fluorescence
2.0
1.5
1.0
.5
.0
0.
20.
40.
60.
80.
100.
[Mg:Acetate] (mM)

Figure 8: Effect of enzyme concentration on the fluorescence assay



Rate (△ Fr/minute)
.0
.1
.2
.3
.4
.5
.0
2.5
5.0
7.5
10.0
12.5
15.0
ANT(2") ug

B. RATIO OF MG:ATP TO ATP REQUIRED FOR OPTIMAL ANT(2") ACTIVITY

Using the equations described in METHODS, total magnesium acetate concentrations and total ATP concentrations that would result in a fixed concentration of Mg:ATP and varied ratios of Mg:ATP to free ATP were calculated. These concentrations of metal and nucleotide were then used in the fluorometric assay, and the initial velocity of the enzymatic reaction was examined as a function of the ratio of Mg:ATP to ATP. The concentration of Mg:ATP was fixed at 1.2 mM, and the assays were performed at pH 9.1. The results of this experiment are illustrated in Figure 9. The maximum velocity occurs at a ratio of 1000:1 or higher. At a ratio of 500:1, ANT(2") activity is approximately 5-fold higher than that determined by Van Pelt and Northrop (150), who used a ratio of 10:1. The effect of increased magnesium ion appears to be specific and not due to general effects by ionic strength; ANT(2") activity increases only 1.4-fold when potassium chloride concentration is varied from 0 to 0.2 M (151). These results lend further support to the suggestion that the metal chelates of the nucleotide triphosphates are the

Figure 9: Effect of the ratio of Mg:ATP to free ATP on ANT(2") activity

Velocity (U/mg)

1.50
1.25
1.00
.75
.50
.25
.00

0. 200. 400. 600. 800. 1000. 1200.

[Mg:ATP]/[ATP]

true substrates of ANT(2")I (143). In subsequent experiments, the ratio of Mg:ATP to ATP was fixed at 400:1 to 500:1. Alternatively, free magnesium ion was fixed at 10.0 mM.

C. STABILITY OF PURIFIED ANT(2")I

The stability of ANT(2") was examined as a function of pH and of storage conditions. Samples were prepared by adding 0.05 ml (0.04 unit) of purified enzyme to 0.95 ml of 0.1 M buffer titrated to the desired pH. The activity of each sample was assayed immediately after addition of the enzyme to the buffer using the fluorometric assay, and again after storage at 4 C for four days. Differences in activity are shown in Figure 10. Optimum stability occurs at pH 8.0. In subsequent purifications, TAPS at pH 8.1 was used as the buffer component as described in METHODS.

The stability of ANT(2")I was examined as a function of storage conditions. Activity of freshly purified enzyme was determined using the spectrophotometric assay. Samples containing 0.6 unit each of enzyme were treated as shown in Table 3 to test the effects of frozen versus cold storage, freezing and thawing cycles, and time of storage. The results of this experiment indicate that

Figure 10: Stability of ANT(2") as a function of pH

ANT(2") activity represents the percentage of activity remaining after storage at 4°C for 4 days in the following buffers: MES (●), pH 5.5, 6.0, and 6.5; HEPES (■), pH 7.0, 7.5, and 8.0; TAPS (▲), pH 8.0, 8.5, and 9.0; and CHES (◆), pH 9.0, 9.5, and 10.0.

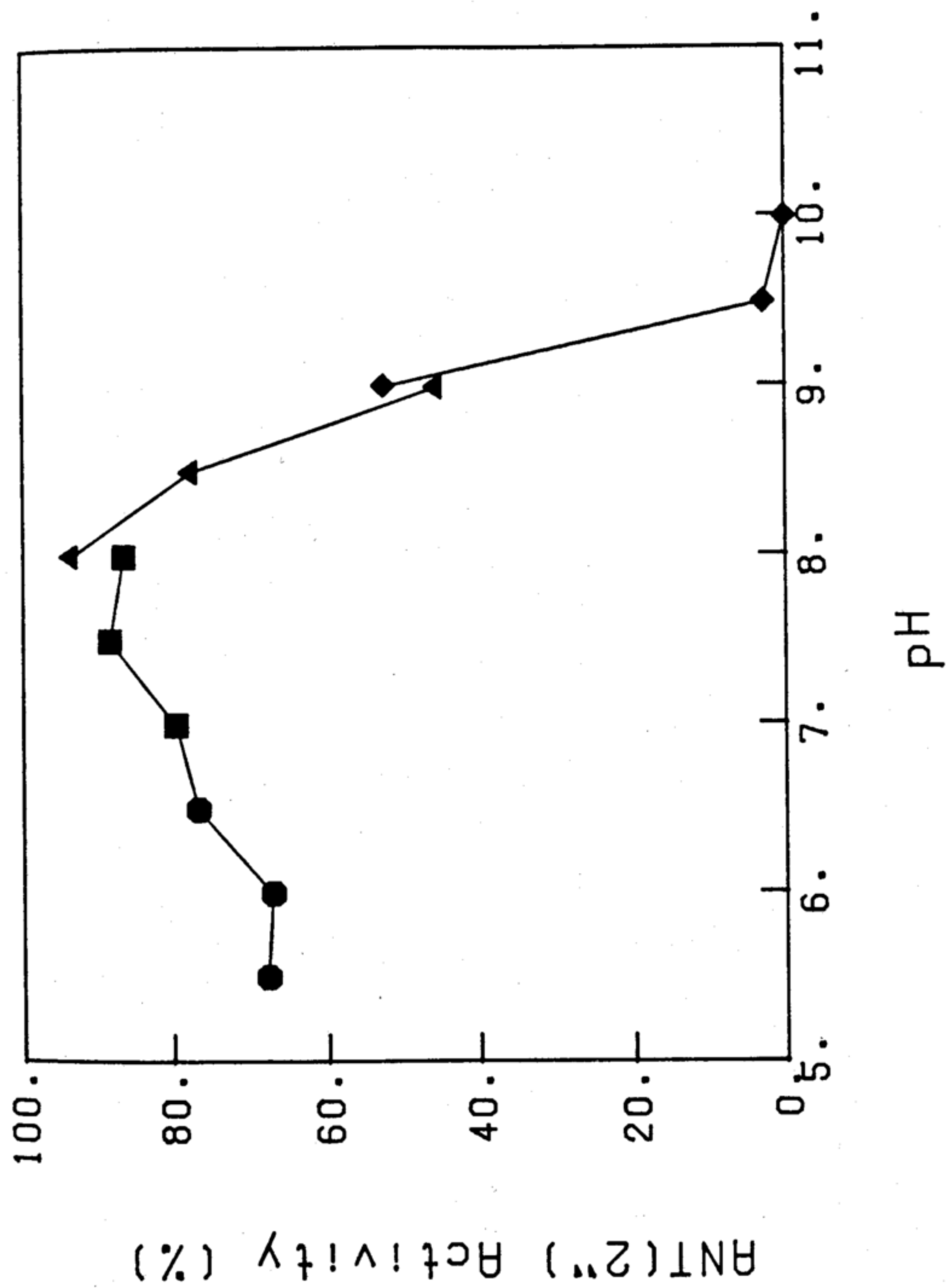

ANT(2") Activity (%)
100.
80.
60.
40.
20.
0
5.
6.
7.
8.
9.
10.
11.
pH

TABLE 3

STABILITY OF ANT(2")I

Storage and Handling	Remaining Actvity (%)
Freshly purified enzyme (control)	100
13 days at 4 °C	104
10.5 months at 4°C	42
Frozen at -20°C and thawed, twice	96
Frozen once; stored at -20°C for 10.5 months	96

Purified ANT(2") from Preparation A

storage at -20 C is the best condition for maintenance of activity over a long period of time. There appears to be no pronounced effect on enzyme activity with repeated freezing and thawing. Enzyme activity appears to be stable at 4 C for two weeks, but prolonged storage at this temperature leads to substantial loss of activity. No bacterial growth was apparent in the samples stored at 4 C.

D. EFFECT OF TRIS ON PURIFIED ANT(2") ACTIVITY

Van Pelt and Northrop note that aminoglycoside inactivating enzymes show anamolous behavior in the presence of TRIS buffer (150). ANT(2"), AAC(6'), AAC(3), and APH(3') elute in 2.0 M TRIS from affinity resins. In addition, K. Radika found that inhibition of by TRIS does not follow normal kinetics (121). Kim Marti has observed that AAC(3) is stable in the presence of TRIS, but is labile in the presence of HEPES (89). These observations indicate that TRIS buffer may interact specifically with the aminoglycoside modifying enzymes. Therefore, the effect of TRIS buffer, pH 8.1, on ANT(2") activity was examined. Different concentrations of TRIS were added to the spectrophotometric assay for ANT(2"), and the activity of ANT(2") was measured. TRIS buffer clearly

inhibits ANT(2") activity as illustrated by the Dixon plot in Figure 11; furthermore, the inhibition is linear which suggests that TRIS buffer is interacting with the enzyme at the active site. The calculated Ki was 33.0 +/- 2.1 mM. This value was used to correct for inhibition by TRIS in subsequent purification steps.

E. RELEASE OF CELLULAR PROTEIN BY SONICATION

Figure 12 illustrates the time course of release of ANT(2") activity from *E. coli* W677/pMY10 cells subjected to sonication. The amount of activity released from the cells reaches a plateau after 30 seconds of sonication and remains the same after 4 minutes of sonication. Approximately 43 units of ANT(2") activity were released from 12 grams (wet weight) of cells.

F. SEPARATION OF THE Ef AND Es FORMS OF ANT(2")I USING AFFINITY RESINS DERIVED FROM EPOXY-ACTIVATED SEPHAROSE

Van Pelt and Northrop observed that the two electrophoretic variants of ANT(2")I are partially separated using gentamicin-Affi-Gel (142). The two proteins exhibited different binding properties with this affinity resin. One form, Ef, binds rapidly and is eluted specifically with TRIS buffer, and the other, Es, elutes non-specifically. However, Es exhibits time-dependent

Figure 11: Dixon plot of activity of ANT(2") *versus* TRIS buffer

The solid line was obtained by a fit to Equation 10.

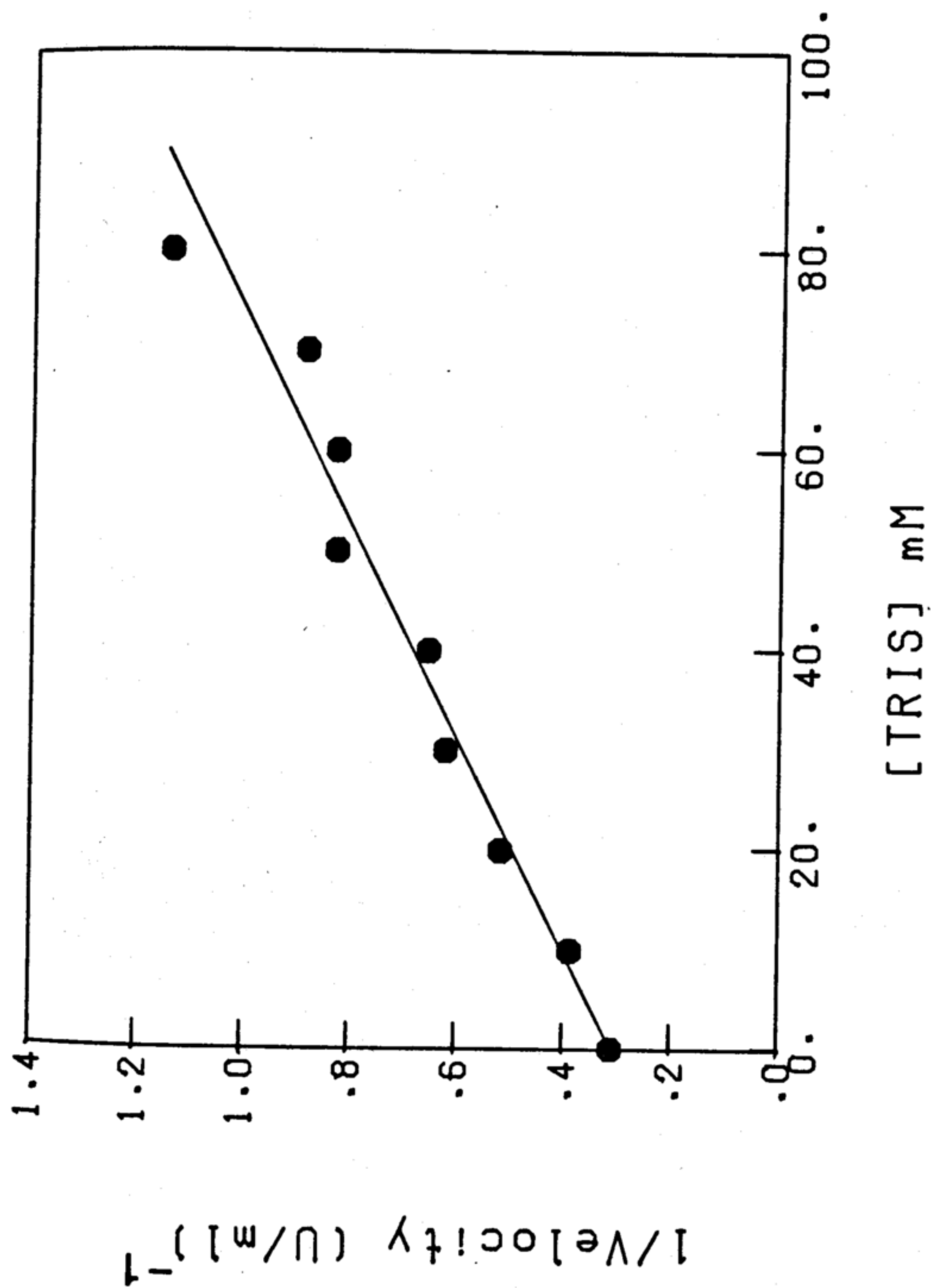
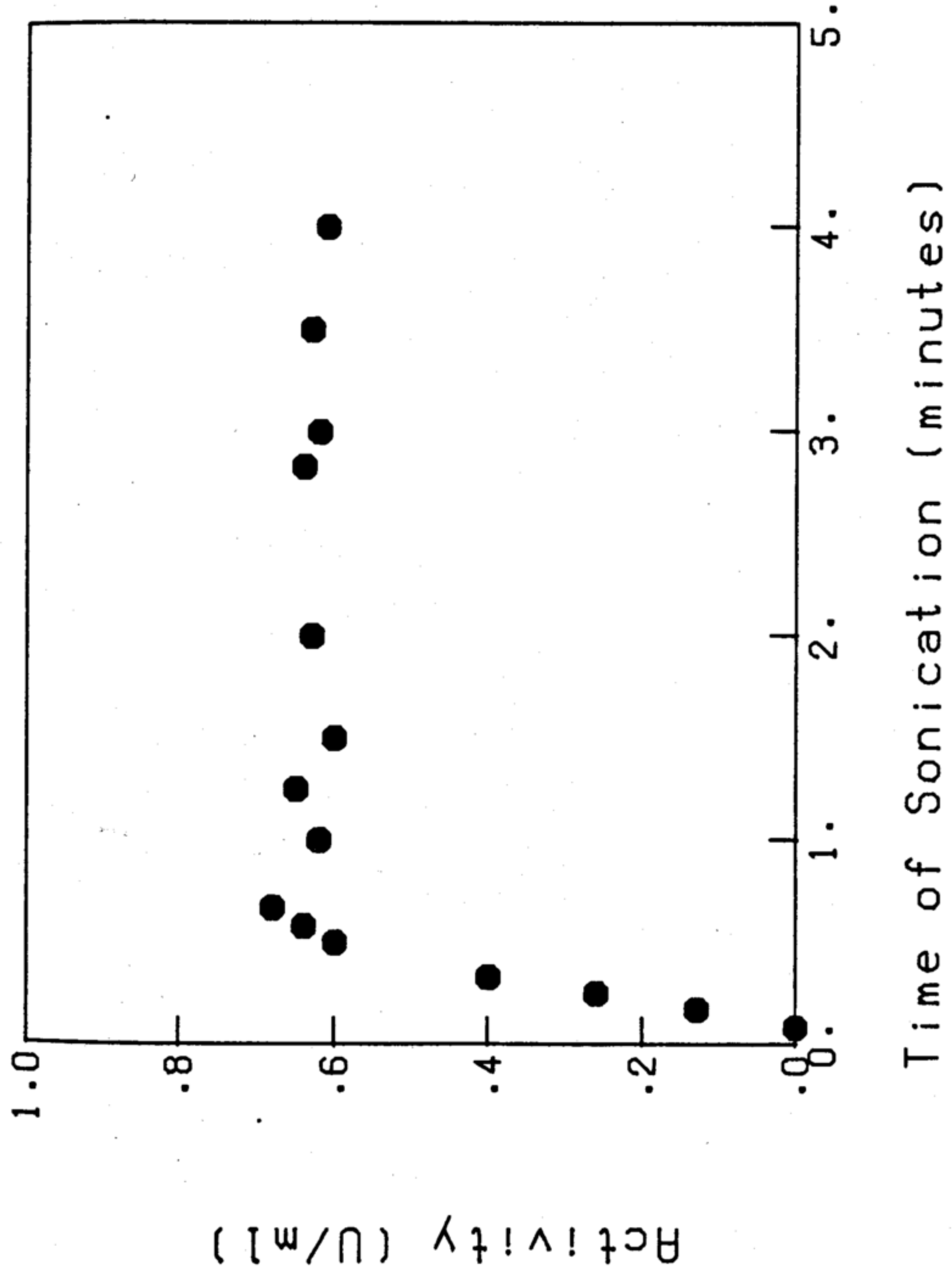

1/Velocity (U/ml)$^{-1}$
1.4
1.2
1.0
.8
.6
.4
.2
.0
0.
20.
40.
60.
80.
100.
[TRIS] mM

Figure 12: Time course of sonication of *E. coli* W677/pMY10 and release of ANT(2") activity

Twelve grams of bacterial cells, suspended in 60 ml cold Buffer 1, were sonicated using a Branson Instruments sonifier in 5, 10, 15, and 20 second bursts of 150 cps. The time course represents accumulated sonication time.



Activity (U/ml)
1.0
.8
.6
.4
.2
0.0
0.
1.
2.
3.
4.
5.
Time of Sonication (minutes)

binding and, after prolonged recycling over the resin, binds tightly and is eluted specifically. With this in mind, several affinity resins were synthesized with epoxy-activated Sepharose 4B using different aminoglycoside antibiotic ligands as described in Chapter II, section B.6e. The results of trial affinity chromatography of ANT(2")I with each of these resins are presented in Table 4. Neomycin-, tobramycin-, gentamicin-Sepharose 4B synthesized at pH 10 and kanamycin-Sepharose synthesized at pH 9 bind all of the enzyme activity added to each column; activity was recovered only by specific elution with TRIS. Recovery of ANT(2") activity is actually in excess of 100%. In contrast to the latter four resins, kanamycin-Sepharose 4B synthesized at pH 10 shows two peaks of activity: one which elutes nonspecifically with ammonium sulfate and one which elutes specifically with TRIS. However, it appears that the enzyme does not bind to the amikacin-Sepharose column since nearly all ANT(2") activity eluted non-specifically from this resin.

Table 5 shows the results of preparation A which represents a typical purification of ANT(2") beginning with crude extract and ending with purified ANT(2")I

TABLE 4

COMPARISON OF TRIAL AFFINITY RESINS

Ligand (pH of coupling solution)	Sample [a]	Total Units	Recovery[b] of activity (%)
Kanamycin (10)	NS	0.75	51
	S	0.58	40
Gentamicin (10)	NS	0.00	0
	S	1.65	114
Tobramycin (10)	NS	0.00	0
	S	1.97	136
Amikacin (10)	NS	1.07	74
	S	0.00	0
Kanamycin (9)	NS	0.00	0
	S	3.35	116

[a] NS, non-specific elution by 0.15 M and 2.0 M ammonium sulfate in Buffer 1; S, specific elution by 2 M TRIS buffer. [b] The protein samples loaded onto these columns were from Preparation A: 1.45 units of activity (1.72 mg of protein) were loaded onto each column except for the kanamycin-Sepharose resin synthesized at pH 9 where 2.9 units of activity (3 mg of protein) were applied to the column. Activity was assayed at pH 9.1 with 10.5 mM Mg:ATP and 0.49 mM tobramycin.

Neomycin-Sepharose was used for preparative affinity chromatography in preparation A. All enzyme activity bound to the affinity resin and was eluted specifically as a single peak. The profile of the neomycin-Sepharose column is illustrated in Figure 13, and the profile of the following gel filtration column is illustrated in Figure 14. The specific activity and final yield of the purified enzyme represent a 16-fold and 24-fold increase over those reported in Van Pelt and Northrop's procedure (142).

In preparation B, kanamycin-Sepharose coupled at pH 9 was used as the affinity resin. Enzyme activity was eluted specifically as a single peak from the KS9 resin as illustrated in Figure 15. The profile of the subsequent Ultrogel column is not illustrated, but resembles the one shown in Figure 14. The units of activity recovered from the KS9 column and the specific activity are shown in Table 6.

TABLE 5

PURIFICATION OF ANT(2")I USING NEOMYCIN-SEPHAROSE

PREPARATION A

Fraction	Protein (mg/ml)	Specific Activity (IU/mg)	Total Activity (units)	Recovery (%)	Purification Factor
osmotic shock fluid	20	0.03	405	100	1
strepto-mycin sulfate	14	0.06	352	87	2.2
ammonium sulfate	NM[a]	0.02	(53)	-	-
P6DG pool	9.0	0.02	(49)	-	-
DEAE pool	2.6	0.68	136	34	28
Neomycin-Sepharose					
NS[b]	NM	NM	0.7	0.1	-
S	NM	NM	133	33	28
Ultrogel AcA 54	2.3	1.03	132	32	43

Assayed at pH 9.1 with 10.5 mM Mg:ATP and 0.49 mM tobramycin
[a]NM = not measured.[b] NS, non-specific elution by ammonium sulfate in Buffer 1; S, specific elution by 2 M TRIS

Figure 13: Preparation A - Affinity chromatography of ANT(2") with neomycin-Sepharose CL-4B synthesized at pH 10

This plot represents absorbance at 280 nm (O) and ANT(2") activity (Δ). One hundred thirty-six units of ANT(2") activity eluted from the DEAE-BioGel A column were loaded onto this neomycin-Sepharose 4B column; the column was subsequently washed with 0.15 M ammonium sulfate as described in METHODS. The first 10 ml fraction collected after the 0.15 M ammonium sulfate wash (see METHODS) was initiated was designated "fraction 1". Fractions 21, 22, and 23 contained ANT(2") activity; these were pooled and subsequently applied to an Ultrogel AcA 54 column. Of the total amount of ANT(2") activity applied to the affinity column, 133 units were recovered, representing a 98 % recovery.

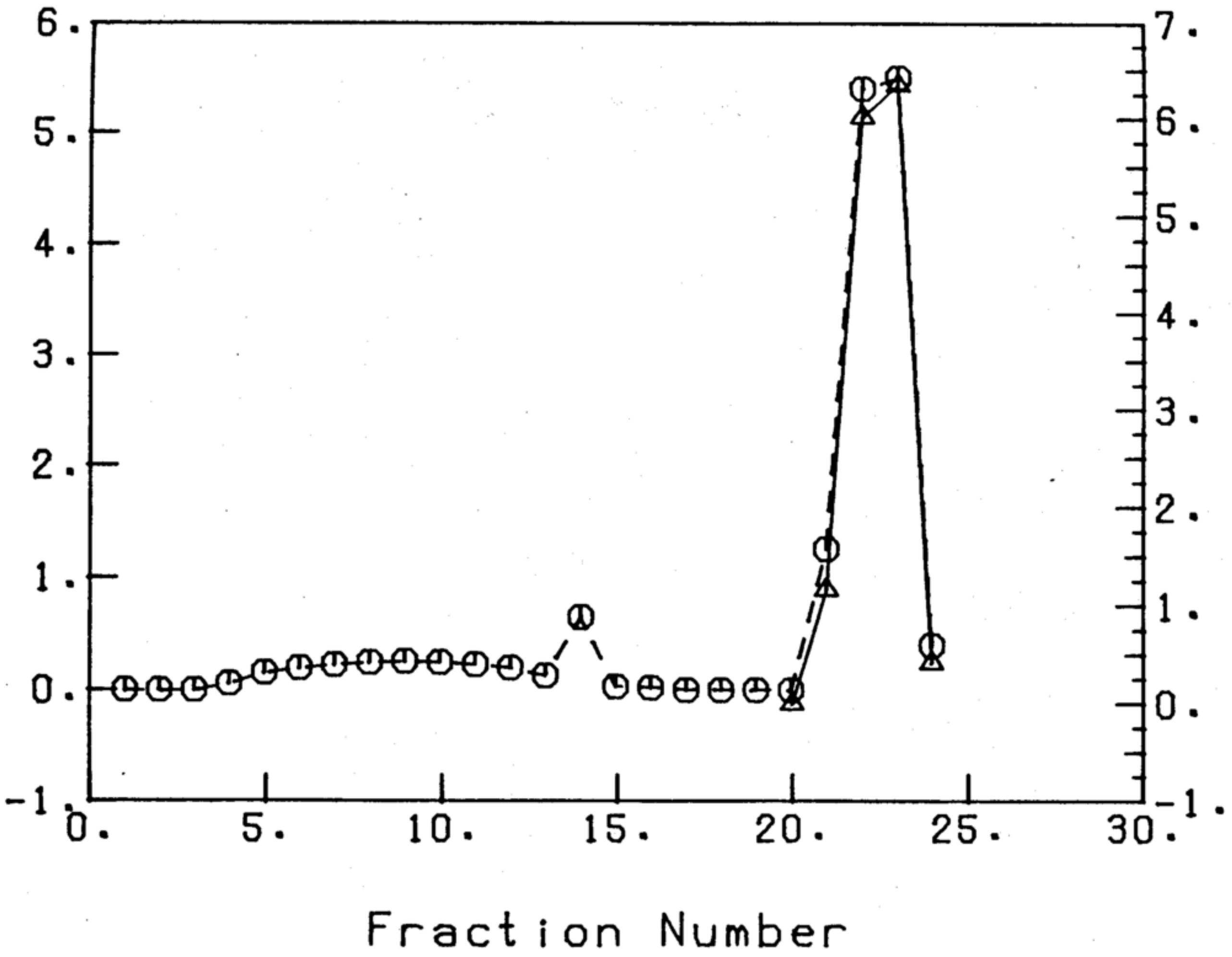
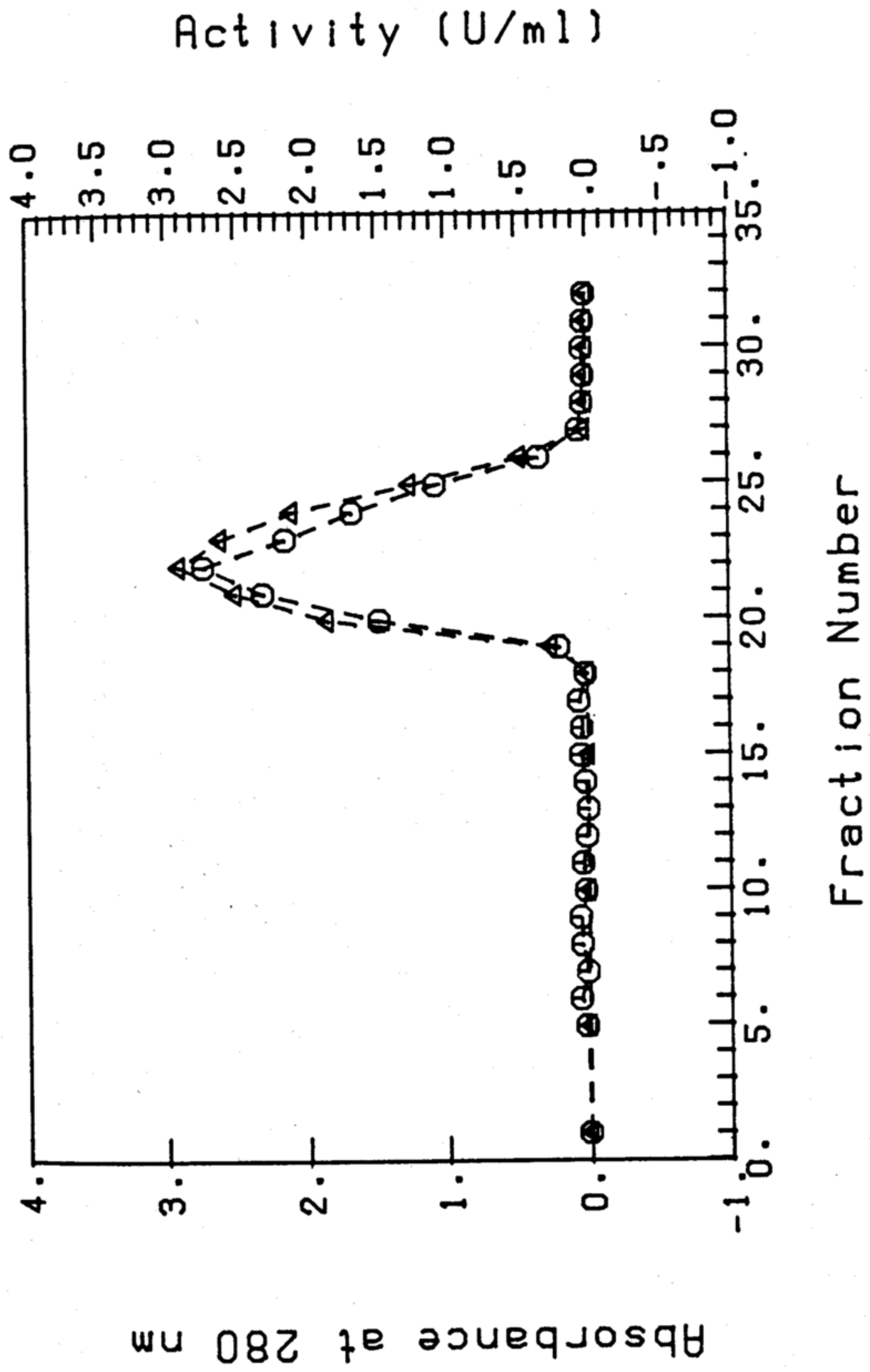

Absorbance at 280 nm
ANT(2") Activity [U/ml]
Fraction Number
-1.
0.
1.
2.
3.
4.
5.
6.
7.
0.
5.
10.
15.
20.
25.
30.

Figure 14: Preparation A - Preparative gel filtration of ANT(2") activity on Ultrogel AcA 54.

The plot represents (O) absorbance at 280 nm and (Δ) ANT(2") activity. The protein sample eluted from the neomycin-Sepharose CL-4B, containing 133 units of ANT(2") activity, was loaded onto the Ultrogel column. A total of 132 units were eluted from the column, representing a 99 % recovery from the activity applied to this column and a 33 % recovery from activity detected in the crude cellular extract. Fractions 20 through 25 were combined; the specific activity of this pool was 1.03 IU/mg.


Activity (U/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
.0
-.5
-1.0
4.
3.
2.
1.
0.
-1.
0.
5.
10.
15.
20.
25.
30.
35.
Fraction Number
Absorbance at 280 nm

In preparation C, the kanamycin-Sepharose resin synthesized at pH 10 was used for preparative affinity chromatography. As illustrated in Figure 16, one peak of activity, C1, was recovered by specific elution and the other, C2, by non-specific elution from KS10. The data from this column are presented under "C" in Table 6. Prolonged recycling of the non-specifically eluted fraction, C2, showed that time-dependent binding occurs: after 16 hours, part of the activity, C3, bound tightly to the KS10 column and was eluted specifically, and the other part, C4, remained unbound. However, after recombining C3 and C4 (see II. B.7e) and chrmomatographimg this pool over the KS10 column with no recycling, nearly all activity was recovered by non-specific elution as shown in Table 6.

Although the non-specifically eluted activity of the C3 + C4 pool did not bind rapidly to the KS10 column, it did bind rapidly and tightly to the kanamycin-Sepharose resin synthesized at pH 9 used in preparation D; activity was recovered only by specific elution in two separate trials. The results of these experiments are shown in Table 6. A profile of the KS9 column used to purify C3 + C4 in preparation D is illustrated in Figure

TABLE 6

COMPARISON OF DIFFERENT PREPARATIVE AFFINITY RESINS

Preparation[a]	Column[b]	Sample[c]	Specific Activity (IU/mg)	Total Activity[d] (units)	Recovery (%)
A	NS10	NS	-	0.7	0.1
		S	1.03	133.0	98
B	KS9	NS	1.70	235.0	85
C	KS10	NS(C2)	1.55	114.0	71
		S (C1)	1.26	69.0	43
C2	KS10	NS(C4)	2.00	43.0	38
		S (C3)	1.60	61.0	53
C3+C4	KS10	NS	1.65	89.0	83
		S	1.70	4.0	4
D	KS9	NS	0.00	0.0	0
		NS	0.00	0.0	0
		S	1.90	51.0	89
		S	2.20	36.0	100

[a] Data for Preparation A taken from Table 5. [b] NS10, neomycin-Sepharose synthesized at pH 10; KS9, kanamycin-Sepharose synthesized at pH 9; KS10 kanamycin-Sepharose synthesized at pH 10. [c] NS, non-specific elution by ammonium sulfate in Buffer 1; S, specific elution by 2 M TRIS. [d] Activity was assayed at pH 9.1 with 11.84 mM Mg:ATP and 0.49 mM tobramycin.

Figure 15: Preparation B - Affinity chromatography of ANT(2") using kanamycin-Sepharose CL-4B synthesized at pH 9

The plot represents absorbance at 280 nm (O) and ANT(2") activity (Δ). Two hundred seventy-eight units of ANT(2") activity eluted from the DEAE-BioGel A column were loaded onto this kanamycin-Sepharose column. The column was washed with 0.15 M ammonium sulfate in Buffer 1, 2.0 M ammonium sulfate in Buffer 1, and 2 M TRIS buffer, pH 8.1 as described in METHODS. The first fraction collected after initiation of loading the protein sample onto the column was designated "fraction 1". One peak of activity eluted with TRIS in fractions 41 - 43 which were then combined. This pool, containing 235 units of ANT(2") activity (84 % of the activity applied to this affinity column) was then loaded onto the Ultrogel AcA 54 column (profile not shown), Activity eluted in fractions 21 through 25 as an asymmetric peak. Two hundred ninety-nine units were recovered from the Ultrogel column, representing a 60% recovery from the osmotic shock fluid. The specific activity was 2.4 IU/mg.

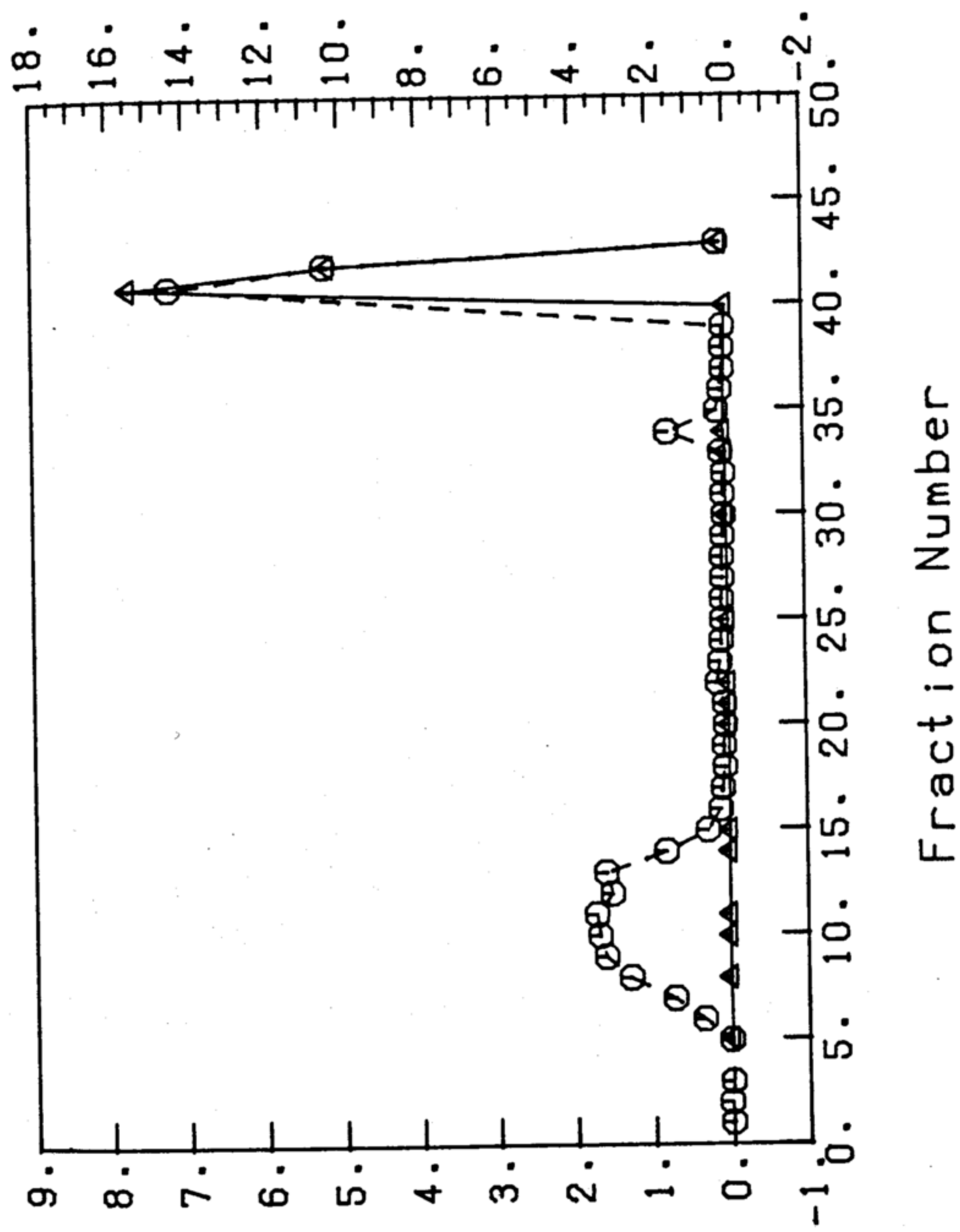
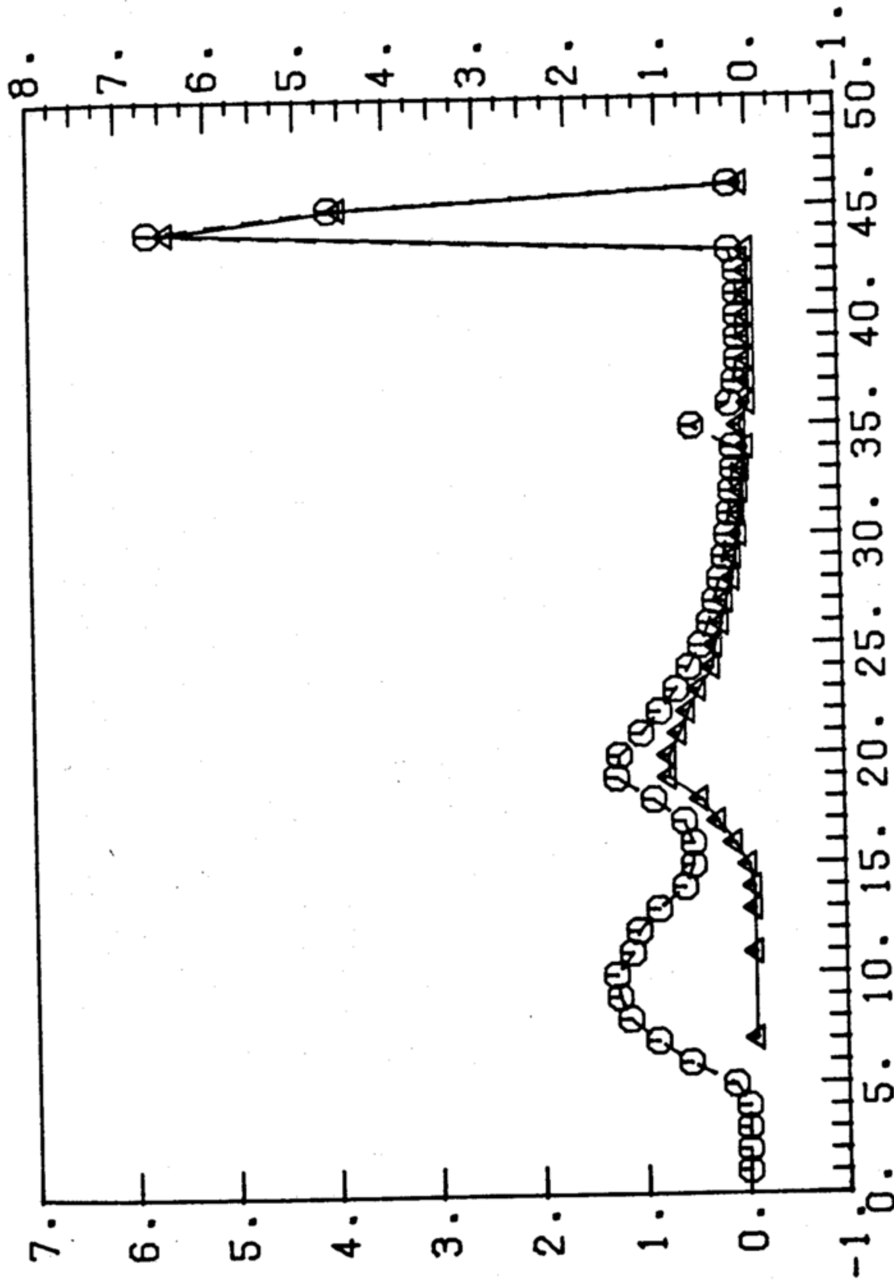

ANT(2") Activity [U/ml]
Fraction Number
Absorbance at 280 nm

Figure 16: Preparation C - Affinity chromatography of ANT(2") with kanamycin-Sepharose CL-4B synthesized at pH 10.0

This plot represents absorbance at 280 nm (O) and ANT(2") activity (Δ). One hundred sixty units of ANT(2") activity eluted from DEAE-BioGel A were loaded onto this column which was subsequently washed with 0.15 M ammonium sulfate in Buffer 1, followed by 2.0 M AS in Buffer 1, and finally, 2 M TRIS. Two peaks of ANT(2") activity eluted from this affinity resin, one with the 0.15 M ammonium sulfate wash (fractions 16 - 29) and the other with 2.0 M TRIS, pH 8.1 (fractions 44 and 45). The 69 units of ANT(2") activity eluted with TRIS were subsequently applied to an Ultrogel column. Sixty-three units of activity were were recovered from the latter column, representing a 13% recovery from the osmotic shock fluid. Fractions 24 through 26 were pooled, and the specific activity was determined to be 1.37 IU/mg.

ANT(2") Activity [U/ml]

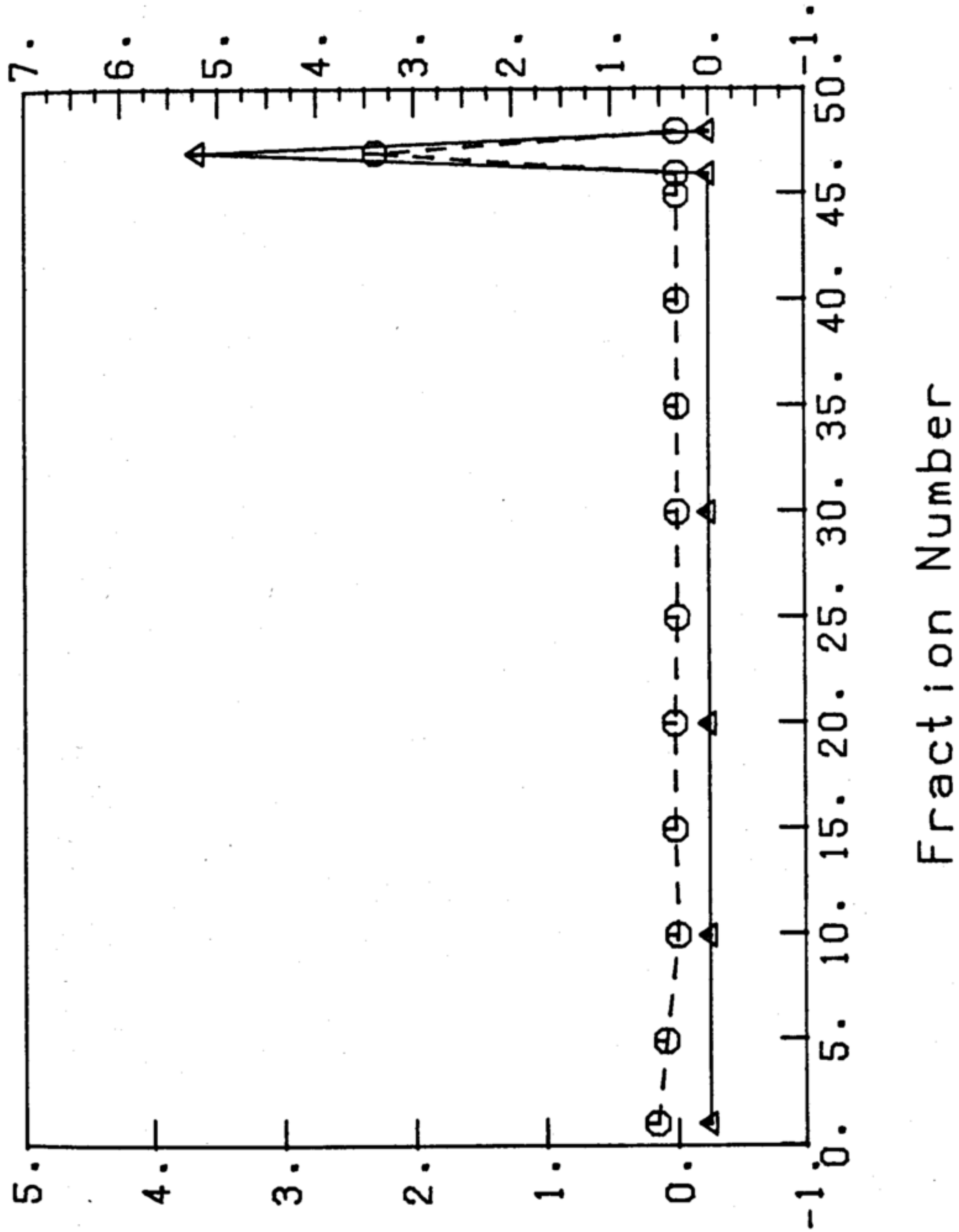


Fraction Number

Absorbance at 280 nm

Figure 17: Preparation D - Affinity chromatography of ANT(2") activity from combined fraction C3 + C4 with kanamycin-Sepharose CL-4B synthesized at pH 9

This plot represents absorbance at 280 nm (○) and ANT(2") activity (△). The sample loaded onto the affinity column contained binding (C3) and non-binding fractions from the recycling step (C4) (see METHODS for details). After loading the C3 + C4 pool onto the KS9 affinity resin, the column was washed with 0.15 M ammonium sulfate in Buffer 1, 2.0 M ammonium sulfate in Buffer 1, and 2.0 M TRIS. Of the 57 units applied to KS9, 51 units eluted as a single peak with TRIS buffer and were found in fraction 45. This fraction was then loaded onto the Ultrogel AcA 54 column from which 52 units of activity were eluted in fractions 21 and 22. This represents a 10% recovery from the osmotic shock fluid. The specific activity was 1.6 IU/mg.


ANT(2") Activity [U/ml]
7.
6.
5.
4.
3.
2.
1.
0.
-1.
Fraction Number
0.
5.
10.
15.
20.
25.
30.
35.
40.
45.
50.
5.
4.
3.
2.
1.
0.
-1.
Absorbance at 280 nm

17. The profiles of subsequent gel filtration columns are not shown but resemble that in Figure 14.

Discontinuous polyacrylamide gel electrophoresis at pH 8.9 was performed with samples of native proteins from preparation A, B, C1, and D. Figure 18 illustrates the electropherograms of these preparations, and the relative migration values of the proteins are given in Table 7. The gels of preparations A and B show two bands with one migrating faster than the other. This is consistent with the observations of two electrophoretic variants of ANT(2")I made by Van Pelt and Northrop (150). Consequently, the two form of ANT(2") observed in the current experiments are designated "Ef" for the fast migrating protein and "Es" for the slow migrating protein as proposed in Van Pelt and Northrop's work.

From the relative migration values, it is apparent that the protein from preparation C1 is Ef, and preparation D represents Es, whereas both proteins are co-purified in preparations A and B. Furthermore, the KS10 resin appears to completely separate the Ef and Es forms since a single band appears in the electropherogram of C1 or D.

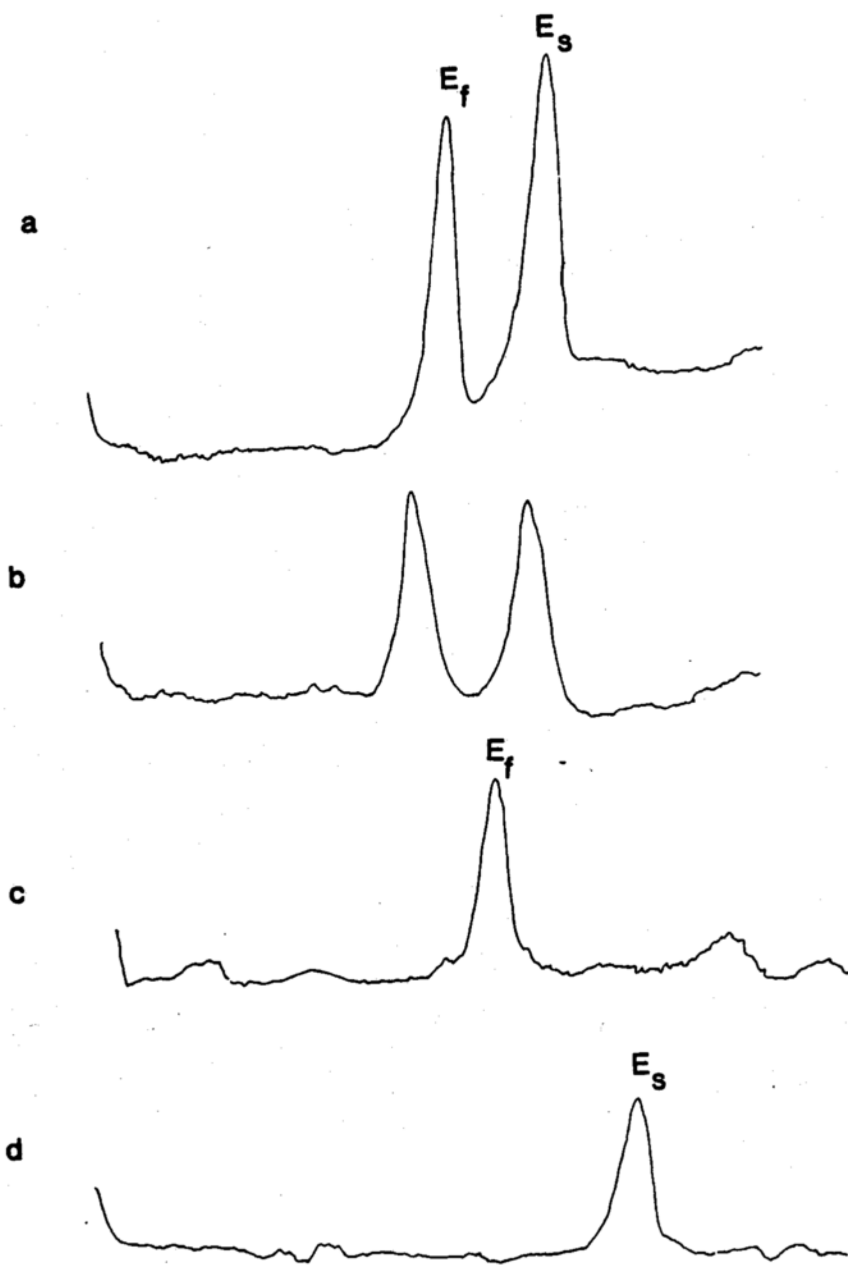
TABLE 7

RELATIVE MIGRATION VALUES OF NATIVE ANT(2")I

Preparation	Enzyme Form	Rf	% Dye
A	Ef	0.55	32
	Es	0.44	73
B	Ef	0.54	44
	Es	0.43	56
C1	Ef	0.56	-
D	Es	0.44	-

Figure 18: Electropherograms of polyacrylamide gels of native ANT(2")

The gels, containing Coomassie Brilliant Blue-stained ANT(2") samples, were scanned on a Gilford Model 240 spectrophotometer as described in METHODS. The origin lies to the right in these scans and the leading edge lies to the left. The y axis is composed of arbitrary absorbance units. Spectrophotometric scans were made of the following samples (taken from the pool of activity eluted from the Ultrogel AcA 54 column): (a) Preparation A; (b) Preparation B; (c) Preparation C1; and (d) Preparation D (see section III.6.e for descriptions of preparations). E_s designates the slower migrating electrophoretic variant of ANT(2"), and E_f designates the faster migrating variant.



E
s
E
f
a
b
E
f
c
E
s
d

The subunit molecular weights of ANT(2") purified in preparations A, B, Cl, and D were determined by gel electrophoresis in the presence of SDS. The mixture of Ef and Es from preparation A gave two bands, one consistent with a molecular weight of 27,230 and the other corresponding to a molecular weight of 25,700. Similarly, two bands were observed for ANT(2")-Ef + Es from preparation B; the subunit molecular weights determined for each band were 27,230 and 24,970. ANT(2")-Ef from preparation Cl appeared as a single band of 22,900 D. ANT(2")-Es from preparation D gave a single band consistent with a protein 26,460 D. The relative migration values of these bands are shown in Table 8.

In summary, the Ef form of ANT(2")I binds rapidly and tightly to the kanamycin-Sepaharose resin synthesized at pH 10, but Es exhibits slow, time-dependent binding to this resin. Re-chromatography of the Es form in the absence of recycling does not result in binding to KS10. However, both Es and Ef bind rapidly and tightly to neomycin-Sepharose synthesized at pH 10 and kanamycin-Sepharose synthesized at pH 9.

TABLE 8

RELATIVE MIGRATION VALUES AND SUBUNIT MOLECULAR WEIGHT OF DENATURED ANT(2")I

Preparation	Rf[a]	Subunit Molecular Weight
A	0.58	27,230 + 1120
	0.60	25,700 + 1060
B	0.58	27,230 + 1120
	0.61	24,970 + 1030
Cl	0.64	22,900 + 942
D	0.60	25,700 + 1060

[a] The relative migration values of the protein standards were fit to Eq. 17 ; the intercept was 5.16 + 0.02, and the slope was -1.26 + 0.03. This line was then used to determine the subunit molecular weight of ANT(2").

G. <u>SUBSTRATE SPECIFICTY OF ANT(2")I</u>

The substrate specificity of ANT(2")I was examined by determining the kinetic constants of various Mg:nucleotide and aminoglycoside substrates.

Using the spectrophotometric assay, experiments were performed to determine the values of the apparent kinetic constants of eight nucleotide substrates in their magnesium chelate form. The results are presented in Table 9.

Table 10 presents the apparent kinetic constants of gentamicin and kanamycin aminoglycoside antibiotics; these were obtained using the spectrophotometric assay. The kinetic constants of eight aminoglycosides were determined using the fluorometric assay; the values are shown in Appendix A.

Although the values of Vmax of the aminoglycosides shown in Table 10 vary from each other by factors of 1.5 to 4, the V/K values differ from each other by as much as 3000-fold. Another important feature of the aminoglycoside substrates is that nearly all exhibit substrate inhibition. A typical example of substrate inhibition by an aminoglycoside is illustrated in Figure 19. In contrast, the Mg:nucleotide substrates do not

TABLE 9

KINETIC CONSTANTS OF NUCLEOTIDE SUBSTRATES

Substrate	V_{max} (units/mg)	K_m (mM)	V/K
Mg:ATP	4.70 ± 0.17	1.32 ± 0.14	3.56 ± 0.26
Mg:GTP	2.97 ± 0.10	0.46 ± 0.03	6.41 ± 0.28
Mg:CTP	2.95 ± 0.11	3.19 ± 0.21	0.92 ± 0.03
Mg:UTP	3.85 ± 0.20	2.74 ± 0.31	1.41 ± 0.09
Mg:dATP	2.58 ± 0.05	0.27 ± 0.02	9.56 ± 0.49
Mg:dGTP	0.93 ± 0.02	0.067 ± 0.03	13.82 ± 0.57
Mg:dCTP	1.85 ± 0.07	1.19 ± 0.10	1.55 ± 0.08
Mg:TTP	2.05 ± 0.10	0.56 ± 0.08	3.63 ± 0.37

Assayed at pH 9.1 with 49.5 μM Gentamicin B.

TABLE 10

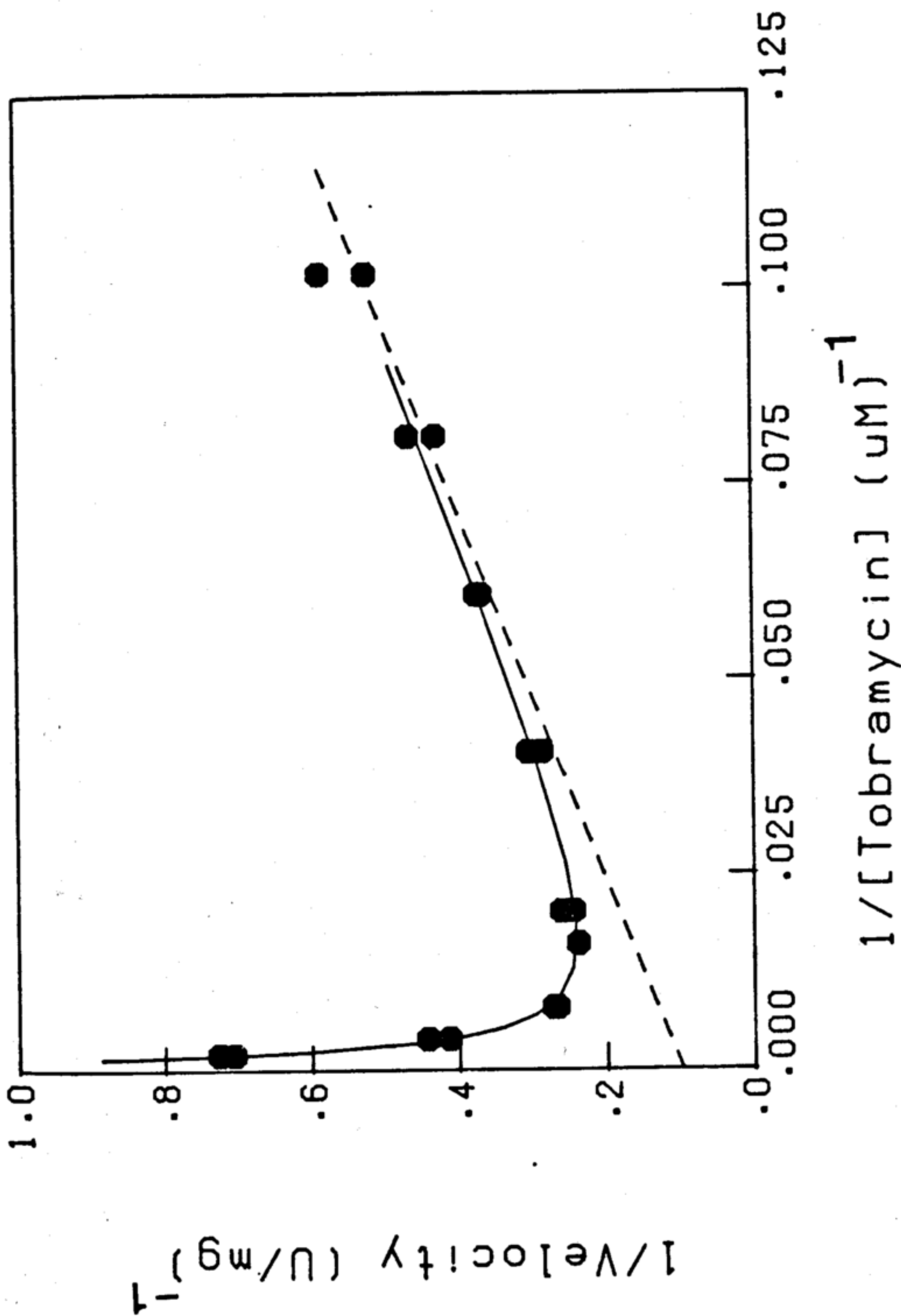
KINETIC CONSTANTS OF AMINOGLYCOSIDES

Substrate	Vmax (IU/mg)	Km (uM)	V/K (L/(mg x min))	Ki (uM)
GentamicinC_{1a}	3.5 ± 0.2	0.6 ± 0.1	5.97 ± 0.72	20.1 ± 2.5
Gentamicin C_2	3.9 ± 0.2	1.0 ± 0.1	3.74 ± 0.37	56.6 ± 5.6
GentamicinC_1	8.9 ± 0.3	24.9 ± 1.8	0.36 ± 0.01	1294 ± 194
Sisomicin	10.1 ± 0.6	18.8 ± 2.5	0.54 ± 0.04	193.5 ± 22.9
5-epiSisomicin	3.0 ± 0.2	103.7 ± 20.8	.029 ± .004	-
Netilmicin	5.7 ± 1.2	3478 ± 1030	.0016 ± .0002	5124 ± 153
Gentamicin A	5.2 ± 0.2	113.6 ± 1.5	.046 ± .002	-
Gentamicin B	4.8 ± 0.3	7.5 ± 1.2	0.64 ± 0.06	185.9 ± 26.1
Gentamicin B_1	12.9 ± 0.6	54.9 ± 4.2	0.23 ± 0.01	343.7 ± 38.3
Kanamycin A	5.3 ± 0.4	126.0 ± 18.3	.042 ± .003	484.2 ± 68.9
Kanamycin B	7.4 ± 0.7	31.7 ± 4.4	0.23 ± 0.01	85.7 ± 12.2
Tobramycin	10.2 ± 0.9	42.7 ± 0.24	0.24 ± 0.01	81.2 ± 10.9
Nebramycin 4	6.9± 1.2	50.1 ± 15.4	0.14 ± 0.02	179.9± 43.4

Assayed at pH 9.1 with 10.2 mM to 11.4 mM Mg:ATP

Figure 19: Lineweaver-Burke plot of ANT(2") reaction velocity as a function of tobramycin concentration.

The solid line represents the data fit to Eq. 2, and the dashed line designates the positions of 1/V and K/V. The concentrations of Mg:ATP and free Mg^{2+} were fixed at 10.4 mM and 10.0 mM, respectively. The concentration range of tobramycin was from 9.8 uM to 491.2 uM. Purified ANT(2")I from preparation A was used.



1/Velocity (U/mg)$^{-1}$
1.0
.8
.6
.4
.2
0.
.000
.025
.050
.075
.100
.125
1/[Tobramycin] (uM)$^{-1}$

cause substrate inhibition.

The kinetic constants of six aminoglycoside substrates were determined for each electrophoretic variant of ANT(2") with the spectrophotometric assay. The enzyme preparations used in these experiments were Ef from preparation C1 and Es from D. When the kinetic constants were determined, it was noted that the Vmax and V/K values of Es were consistently about 50% less than the values for Ef; both Vmax and V/K are functions of total enzyme concentration. This prompted the question as to whether the total enzyme concentration was measured accurately by assuming that an absorbance of 1.0 at 280 nm was equal to 1 mg/ml for each enzyme form. The different physical characteristics of Es and Ef introduce uncertainty into the value of the extinction coefficient of each protein. By plotting the V/K values of five antibiotics for Ef or Es against each other and obtaining a fit of these data to the equation for a straight line, a slope of 0.49 was obtained; the V/K of gentamicin C1a was excluded from this analysis due to a large standard error. The value of 0.49 was in turn used to normalize the apparent extinction coefficient for Es and resulted

TABLE 11

KINETIC CONSTANTS OF AMINOGLYCOSIDES FOR EACH ENZYME FORM

Substrate	Enzyme Form	Vmax[a] (units/mg)	Km (uM)	V/K[a] (L/(mg x min))	Ki (uM)
Sisomicin	E_f	9.2 ± 1.6	19.1 ± 6.5	0.48 ± 0.09	110.3 ± 24.4
	E_s	9.0 ± 1.8	21.2 ± 7.9	0.42 ± 0.09	139.0 ± 38.2
5-epi Sisomicin	E_f	5.0 ± 0.1	121.4 ± 10.1	.041 ± .003	-
	E_s	5.6 ± 0.3	162.5 ± 23.5	.036 ± .003	-
Netilmicin	E_f	4.5 ± 0.4	1449 ± 253	.0031 ± .0003	5762 ± 870
	E_s	6.3 ± 1.0	2931 ± 684	.0023 ± .0001	4028 ± 895
Gentamicin C_{1a}	E_f	1.5 ± 0.2	0.4 ± 0.2	4.23 ± 1.49	8.2 ± 2.1
	E_s	1.5 ± 0.4	1.2 ± 0.7	1.20 ± 0.40	8.7 ± 4.7
Gentamicin C_2	E_f	1.6 ± 0.1	2.3 ± 0.4	0.70 ± 0.08	263.1 ± 57.0
	E_s	1.8 ± .05	3.5 ± 0.3	0.50 ± 0.03	369.5 ± 48.5
Tobramycin	E_f	8.0 ± 0.7	27.7 ± 4.2	0.29 ± 0.02	86.3 ± 11.7
	E_s	4.7 ± 0.6	14.5 ± 4.4	0.33 ± 0.06	170.0 ± 44.0

Assayed at pH 9.1 with 12.86 mM Mg:ATP

[a] The Vmax and V/K values for the aminoglycoside substrates of E_f were determined by assuming an absorbance of 1.0 at 280 nm was equivalent to a protein concentration of 1 mg/ml. Vmax and V/K for the E_s form were calculated by assuming an absorbance of 1.0 at 280 nm was equivalent to 0.67 mg/ml protein.

in an absorbance of 1.0 at 280 nm being equal to 0.67 mg/ml. The Vmax and V/K values for the six aminoglycoside substrates of Es were calculated using this extinction coefficient. The results are presented in Table 11 and show that the Vmax and V/K values are very similar for the two enzyme forms. Note that the substrate inhibition constants and Michaelis constants, both of which are unaffected by enzyme concentration and thus not normalized, are also very similar.

H. THE KINETIC MECHANISM OF ANT(2")I

The first step in the determination of the kinetic mechanism of ANT(2")I was application of the alternative substrate diagnostic as developed by Radika and Northrop (115) Then, several approaches involving steady-state enzyme kinetics were taken to corroborate the results of the alternative substrate diagnostic. These included inhibition kinetics, non-equilibrium isotope exchange, determination of the kinetic parameters as a function of pH, and examination of the effect of changes in solution viscosity on the kinetic constants of three aminoglycoside antibiotic substrates.

F.1 Alternative Substrate Diagnostic

The spectrophotometric assay was employed to determine the kinetic constants of gentamicin C1 with saturating concentrations of Mg:ATP, Mg:dATP, or Mg:dGTP. The Vmax and V/K values of the aminoglycoside vary by a factor of 2.5 and 7.6, resepectively, as listed in Table 12. The resulting pattern of intersecting lines is illustrated in Figure 20. The V/K and Vmax values of the alternative nucleotides used in the diagnostic vary over a of 4-fold and 5-fold range, respectively, as shown in Table 9. A similar experiment was performed using the fluorometric assay. The results are presented in Appendix A.

Next, the kinetic constants of Mg:ATP were determined with gentamicin A, C1, or C1a as the alternative substrate. Activity was measured spectrophtometrically in 1 cm cells with gentamicin A or C1 and in 5 cm cuvettes with gentamicin C1a. As shown in Table 13, Vmax and V/K of the nucleotide are identical with gentamicin A or C1 as an alternative substrate; the values of Vmax and V/K of Mg:ATP with gentamicin C1a vary from the other constants by about 2-fold. However, the V/K values of the alternative aminoglycoside substrates

TABLE 12

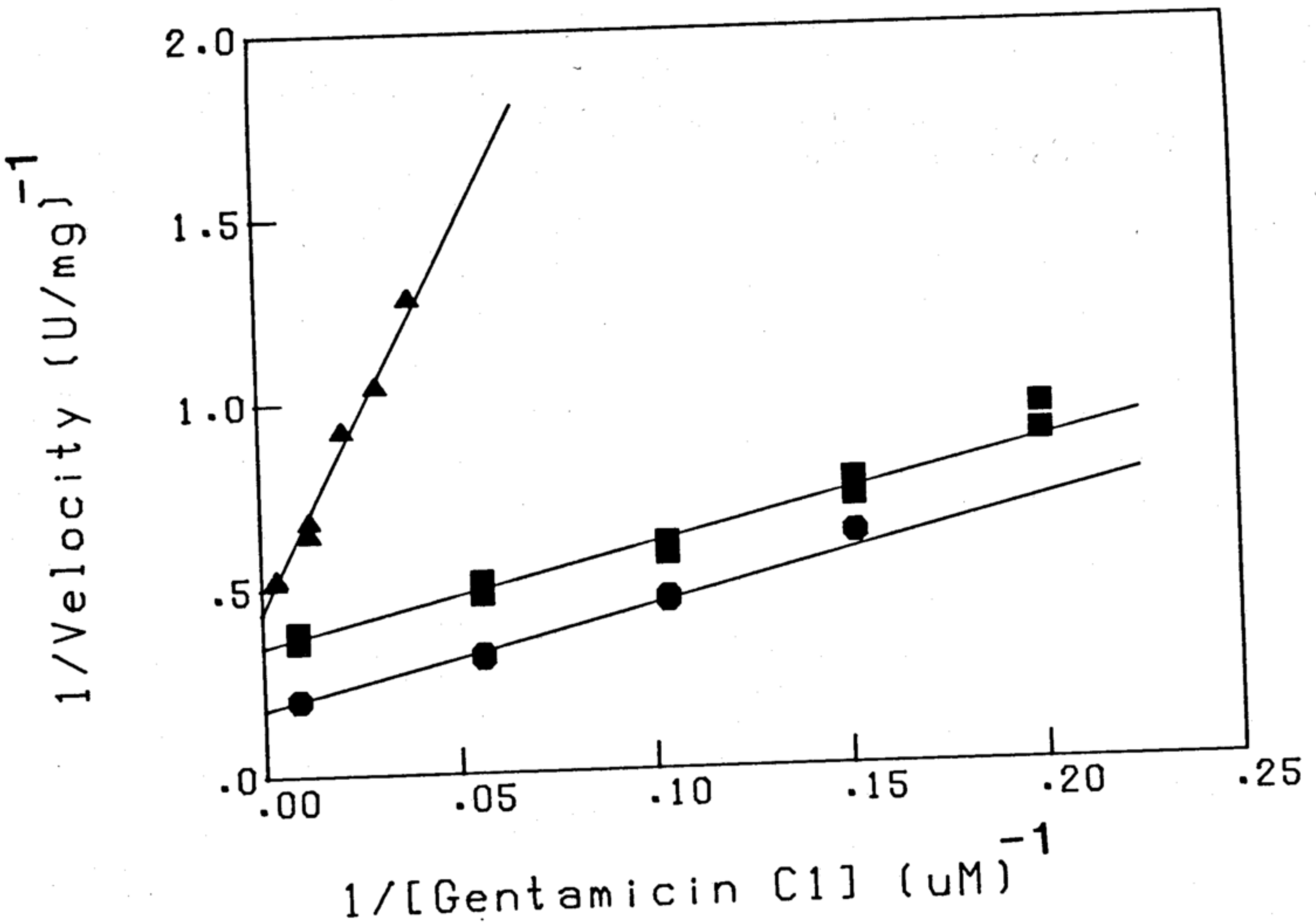
GENTAMICIN C1 KINETIC CONSTANTS USING ALTERNATIVE NUCLEOTIDES

Vmax (IU/mg)	V/K (L/(mgxmin))	Nucleotide[a]	Conc. (mM)
5.57 ± 0.11	0.38 ± 0.01	Mg:ATP	11.32
2.89 ± 0.07	0.38 ± 0.02	Mg:dATP	2.42
2.30 ± 0.07	0.050 ± 0.002	Mg:dGTP	0.67

[a] Relative values of Vmax of alternative nucleotides are 5:2:1, respectively; relative values of V/K are 1:2.7:4, respectively.

Figure 20: Alternative substrate diagnostic with gentamicin C_1 as the variable substrate

The alternative nucleotide substrates were Mg:ATP (●) at 11.3 mM, Mg:dATP (■) at 2.4 mM, or Mg:dGTP at (▲) 0.7 mM. The solid lines are drawn from the data fit to Eq. 1. The concentration range of gentamicin C_1 was from 6.6 uM to 112.4 uM with Mg:ATP, 5.0 uM to 112.4 uM for Mg:dATP, and 25.3 uM to 253.0 uM for Mg:dGTP. The free Mg^{2+} concentration was fixed at 10.0 mM. Purified ANT(2") from Preparation A was used.



2.0
1.5
1.0
.5
.0
.00
.05
.10
.15
.20
.25
1/Velocity (U/mg)^-1
1/[Gentamicin C1] (uM)^-1

PHARMACY LIB.
SCHOOL OF PHARMACY
UNIV. OF WISCONSIN

SCHOOL OF [illegible]
UNIV. OF WISCONSIN

PHARMACY LIB.
SCHOOL OF PHARMACY
UNIV. OF WISCONSIN

SCHOOL OF [illegible]
UNIV. OF WISCONSIN

UNIV. OF WISCONSIN

SCHOOL OF [illegible]
UNIV. OF WISCONSIN

UNIV. OF WISCONSIN

SCHOOL OF PHARMACY
UNIV. OF WISCONSIN

SCHOOL OF PHARMACY
UNIV. OF WISCONSIN

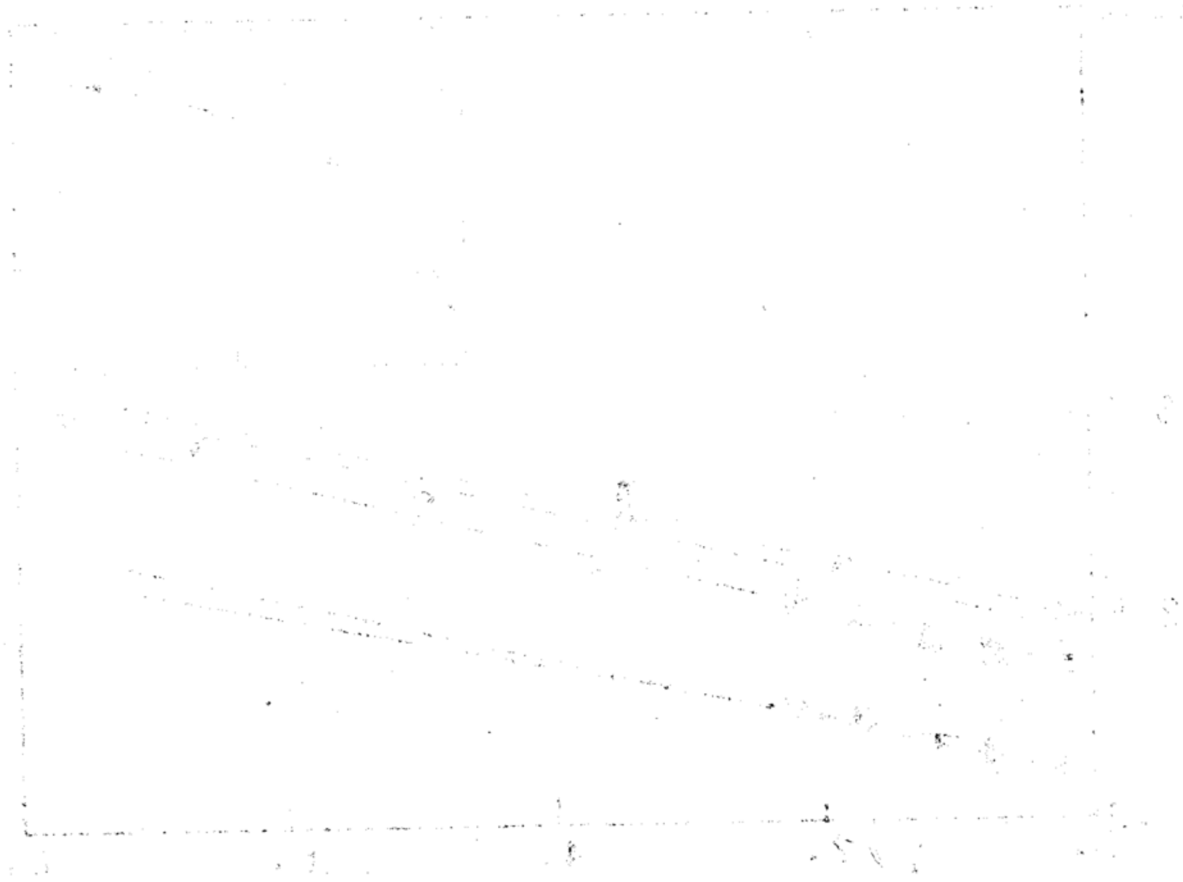


Figure 24: Structure of neomycin C

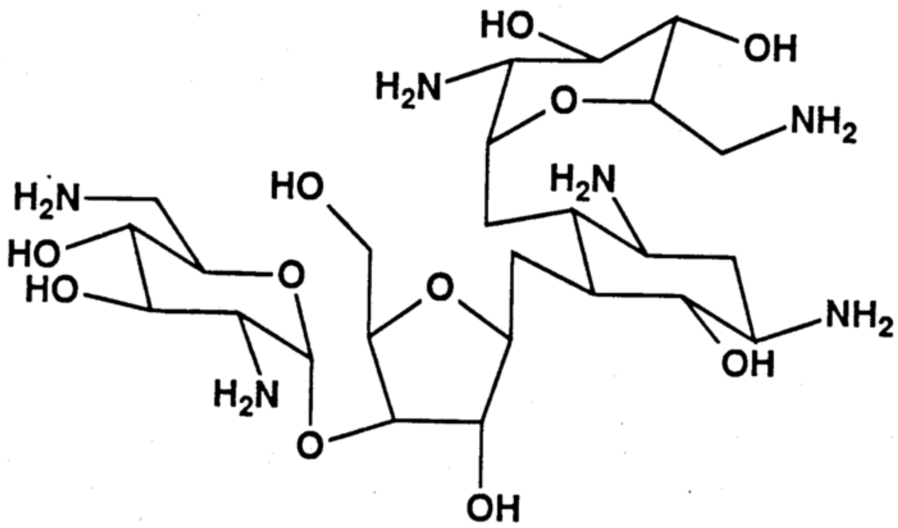
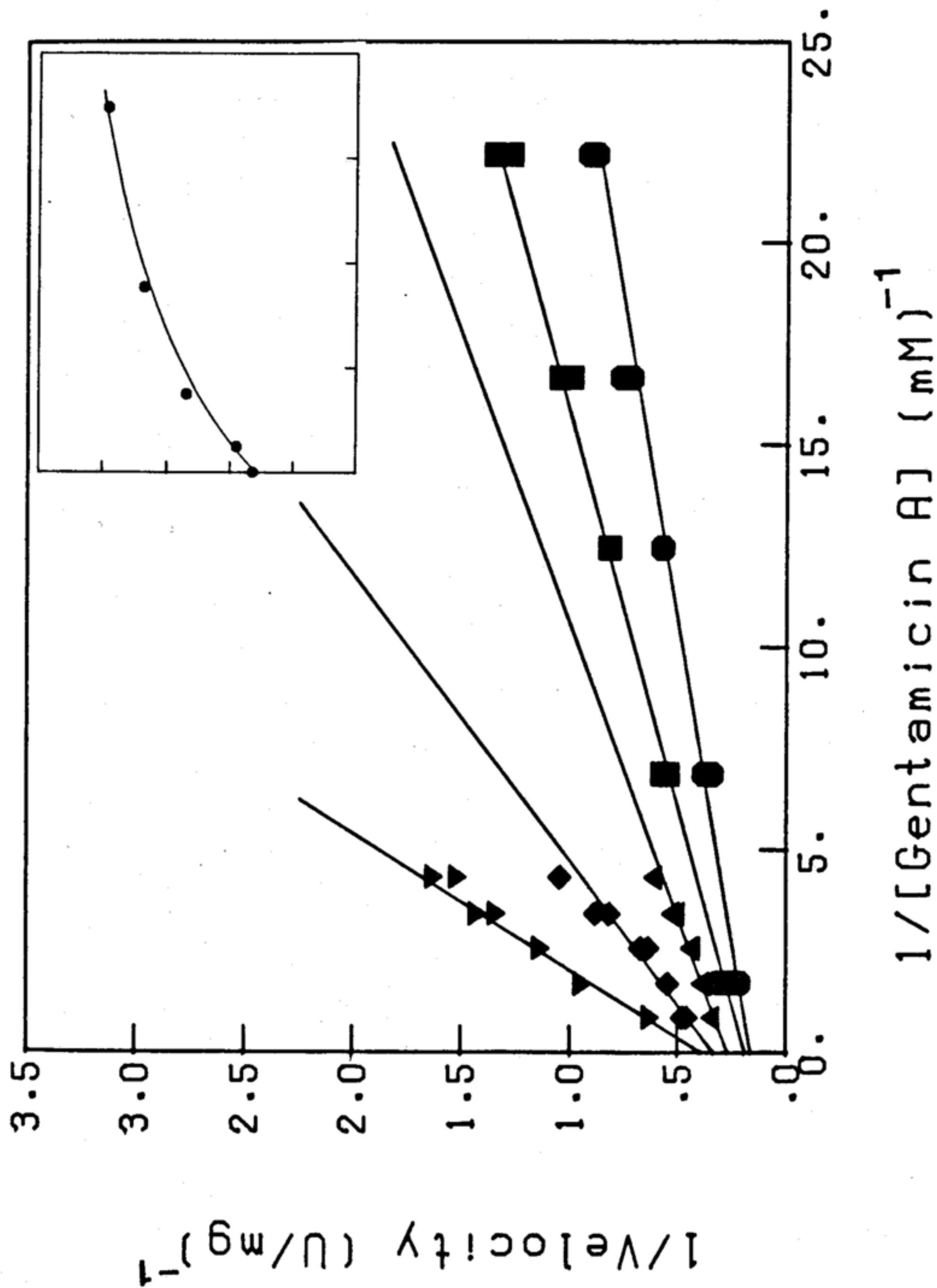

HO
OH
H2N
O
NH2
H2N
HO
H2N
HO
O
HO
O
NH2
OH
H2N
O
OH

Figure 25: Noncompetitive inhibition by neomycin C with gentamicin A as the variable substrate

Each solid line represents the data fit to Eq. 1. The concentrations of neomycin were 0 mM (●), 0.025 mM (■), 0.075 mM (▲), 0.177 mM (◆), and 0.348 mM (▼). The concentration ranges of gentamicin A were from 0.045 mM to 0.580 mM for 0 mM and 0.025 mM gentamicin A and 0.230 mM to 1.155 mM for 0.075 mM, 0.177 mM, and 0.348 mM gentamicin A. The Mg:ATP and free Mg^{2+} ion concentrations were fixed at 10.24 mM and 10 mM, respectively. Purified ANT(2") from Preparation A was used.

Inset: Intercept replot of gentamicin A versus neomycin C

The solid line represents the fit of apparent 1/Vmax values of gentamicin A to Eq. 13.



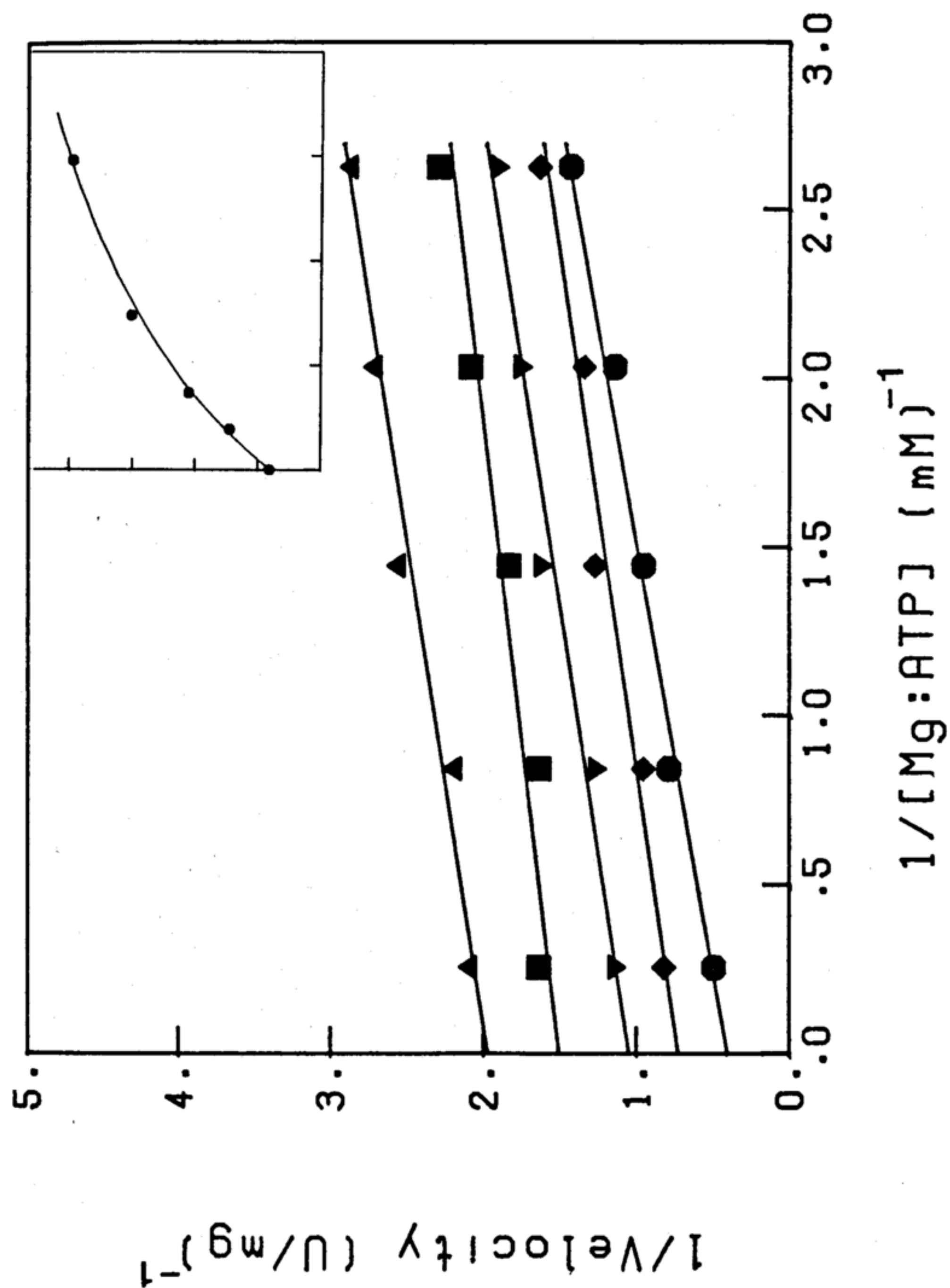
1/Velocity (U/mg)$^{-1}$
1/[Gentamicin A] (mM)$^{-1}$
0.
.5
1.0
1.5
2.0
2.5
3.0
3.5
5.
10.
15.
20.
25.

Figure 26: Uncompetitive inhibition by neomycin C with Mg:ATP as the variable substrate

Each solid line is drawn from the data fit to Eq. 1. The concentrations of neomycin C were 0 mM (●), 0.08 mM (◆), 0.15 mM (▼), 0.30 mM (■), and 0.59 mM (▲). The concentration range of Mg:ATP was 0.38 mM to 3.94 mM; free Mg ion concentration was fixed at 10 mM for each total nucleotide concentration. The concentration of gentamicin A was fixed at 0.22 mM. Enzyme from Preparation A was used.

Inset: Intercept replot of Mg:ATP *versus* neomycin C

The solid line represents the fit of apparent 1/Vmax values of Mg:ATP to Eq. 13.



1/Velocity (U/mg)$^{-1}$
0.
1.
2.
3.
4.
5.
.0
.5
1.0
1.5
2.0
2.5
3.0
1/[Mg:ATP] (mM)$^{-1}$

F.2c Multiple Inhibition

When tobramycin was the variable substrate, neomycin C acted as an apparent competitive inhibitor as illustrated in Figure 27. The slope effect of inhibition by neomycin C is linear (inset, Fig. 27). In contrast to neomycin C as a non-competitive inhibitor versus gentamicin A, the intercept effect is very slight. An important feature of inhibition by neomycin C versus tobramycin is that the Ki value of substrate inhibition by tobramycin becomes larger with increasing concentrations of neomycin C. This is illustrated in Figure 28. These results indicate that neomycin C and tobramycin in its inhibitory mode may bind at the same site on the enzyme. The data were fit to Equation 7 which describes competitive inhibition with substrate inhibition where the binding of the inhibitors is mutually exclusive. The inhibition constants obtained from this fit are presented in Table 14. A fit of these data to Equation 8 where inhibitor binding is independent was attempted but would not converge.

Figure 27: Inhibition by neomycin C with tobramycin as the variable substrate

Each curved, solid line represents the data fit to Eq. 2, and each dashed line designates the position of 1/Vmax and K/V. The concentrations of neomycin C were 0 mM (▼), 0.12 mM (■), 0.24 mM (▲), and 0.59 mM ( ). The concentration ranges of tobramycin were from 0.010 mM to 0.610 mM for 0 mM neomycin C and 0.019 mM to 0.610 mM for 0.12 mM, 0.24 mM, and 0.59 mM neomycin C. Mg:ATP and free Mg^{2+} were fixed at 11.86 mM and 10 mM, respectively. Enzyme from Preparation A was used.

Inset: Slope replot of tobramycin *versus* neomycin C

The solid line is drawn from a fit of apparent K/V values of tobramycin to Eq 12.

Inset b: Intercept replot of tobramycin *versus* neomycin C

The solid line was drawn from the fit of apparent 1/Vmax values of of tobramycin to Eq. 10.

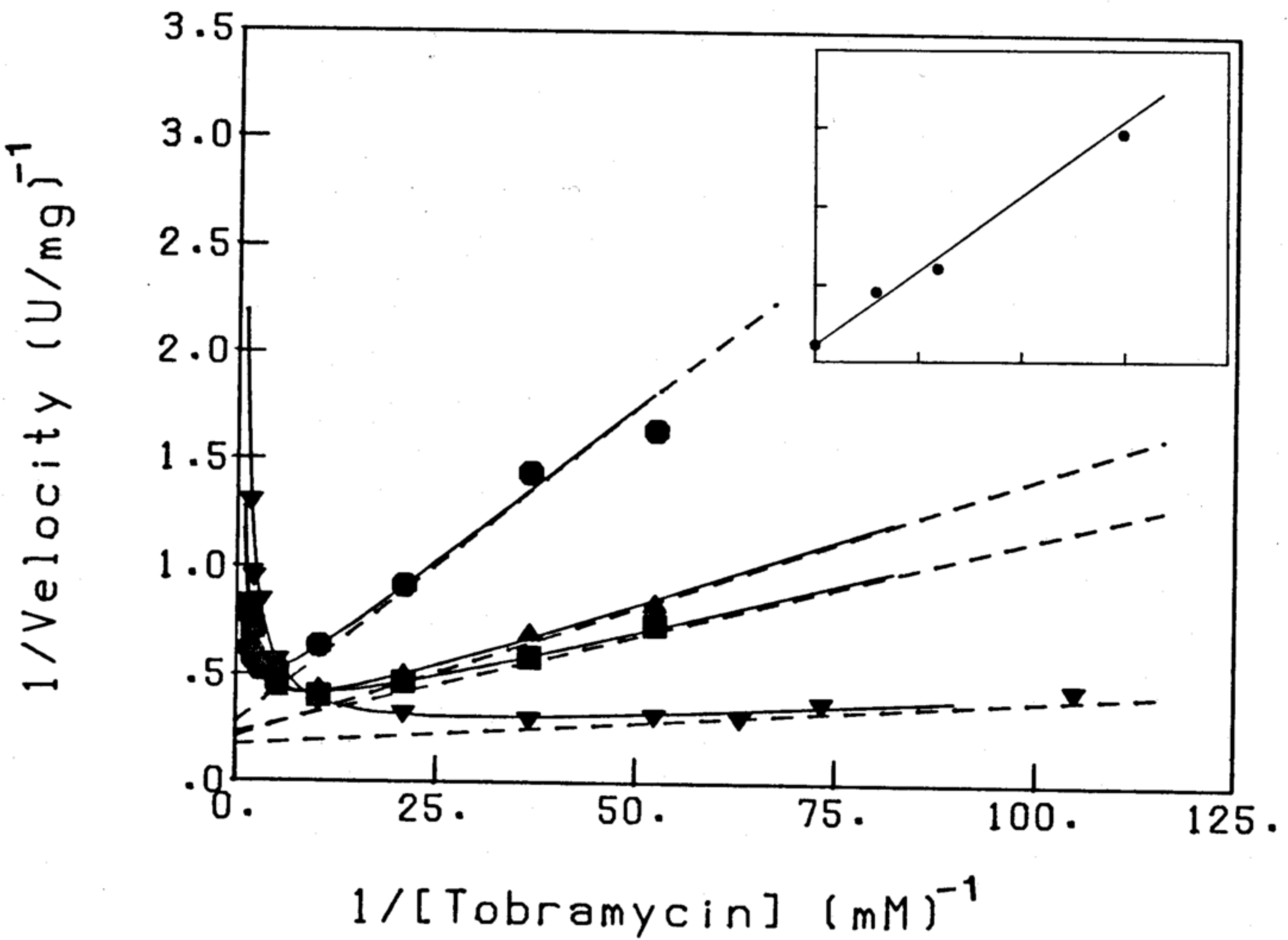
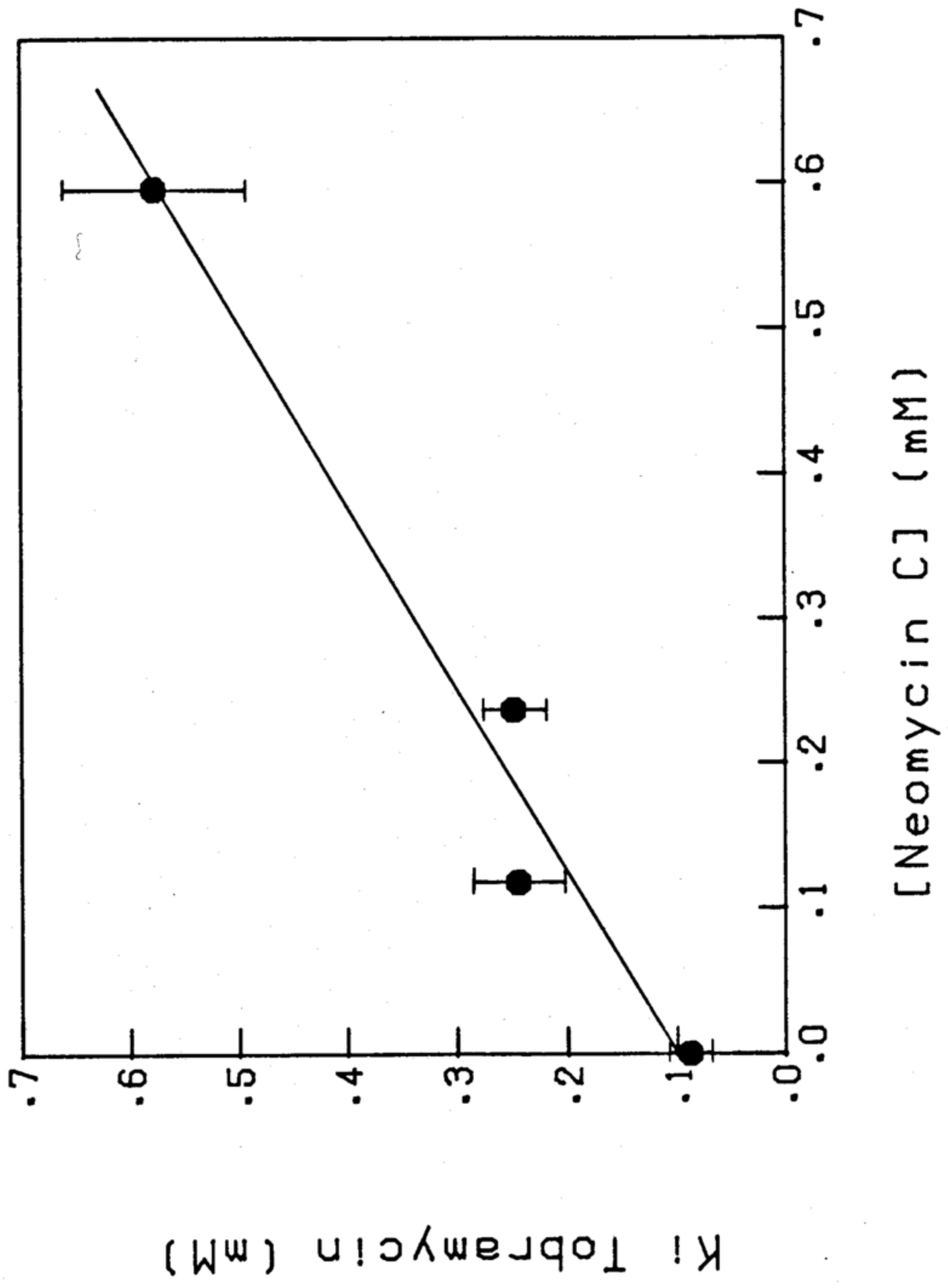

3.5
3.0
2.5
2.0
1.5
1.0
.5
.0
1/Velocity (U/mg)^-1
0.
25.
50.
75.
100.
125.
1/[Tobramycin] (mM)^-1

Figure 28: Replot of substrate inhibition constant as a function of the concentration of neomycin C

The solid line represents the data fit of the K_I of tobramycin and the concentrations of neomycin C to Eq. 17.



Ki Tobramycin (mM)
.0
.1
.2
.3
.4
.5
.6
.7
[Neomycin C] (mM)

F.2d Product Inhibition

When Mg:ATP was the variable substrate, tobramycin-AMP acted as a non-competitive inhibitor as shown in Figure 29. Inhibition by tobramycin-AMP had a linear intercept effect (inset b, Fig. 29) and a slight linear slope effect (inset a, Fig. 29). The inhibition constants of the slope and intercept are presented in Table 14.

Figure 30 shows that tobramycin-AMP acted as an uncompetitive inhibitor when tobramycin was the variable substrate. The inhibitory effect on the intercepts was linear (inset, Fig. 30). Note that the value of Ki for tobramycin increases with increasing tobramycin-AMP concentration; this is illustrated in Figure 31. The intercept inhibition constant is given in Table 14.

F.3 Non-Equilibrium Isotope Exchange

Figure 32 illustrates the results of a non-equilibrium isotope exchange experiment in which (32P) pyrophosphate was incubated with Mg:ATP as described in METHODS. No exchange from pyrophosphate back into Mg:ATP was apparent even after 18 hours. This experiment was repeated several times, all with the same result (data not shown).

Figure 29: Noncompetitive inhibition by tobramycin-AMP with Mg:ATP as the variable substrate

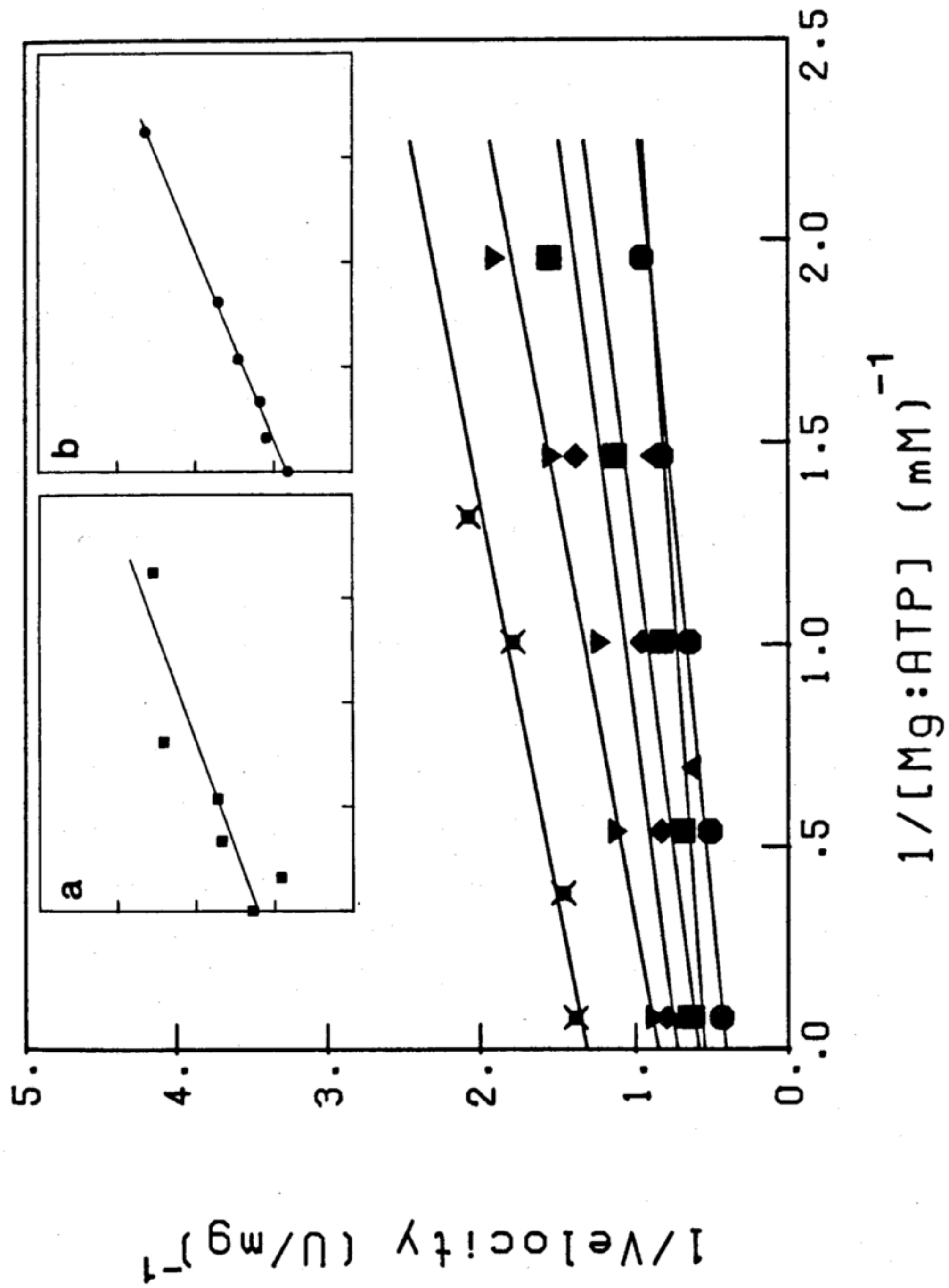
Each solid line represents the data fit to Eq. 1. The concentrations of tobramycin-AMP were 0 mM (●), 0.065 mM (■), 0.135 mM (▲), 0.216 mM (◆), 0.325 mM (▼), and 0.650 mM (●). The concentration ranges of Mg:ATP were 0.51 mM to 12.86 mM for 0.065 mM, 0.135 mM, 0.216 mM, and 0.325 mM tobramycin-AMP and 0.76 mM to 12.86 mM for 0.650 mM tobramycin-AMP. Free Mg ion concentration was fixed at 10 mM for each total nucleotide concentration. Tobramycin concentration was fixed at 0.06 mM. Purified ANT(2")I was from Preparation B.

Inset a: Slope replot of Mg:ATP *versus* tobramycin-AMP

The solid line represents the fit of apparent 1/Vmax values of Mg:ATP to Eq. 12.

Inset b: Intercept replot of Mg:ATP *versus* tobramycin-AMP

The solid line is drawn from the fit of apparent K/V values of Mg:ATP to Eq. 11.



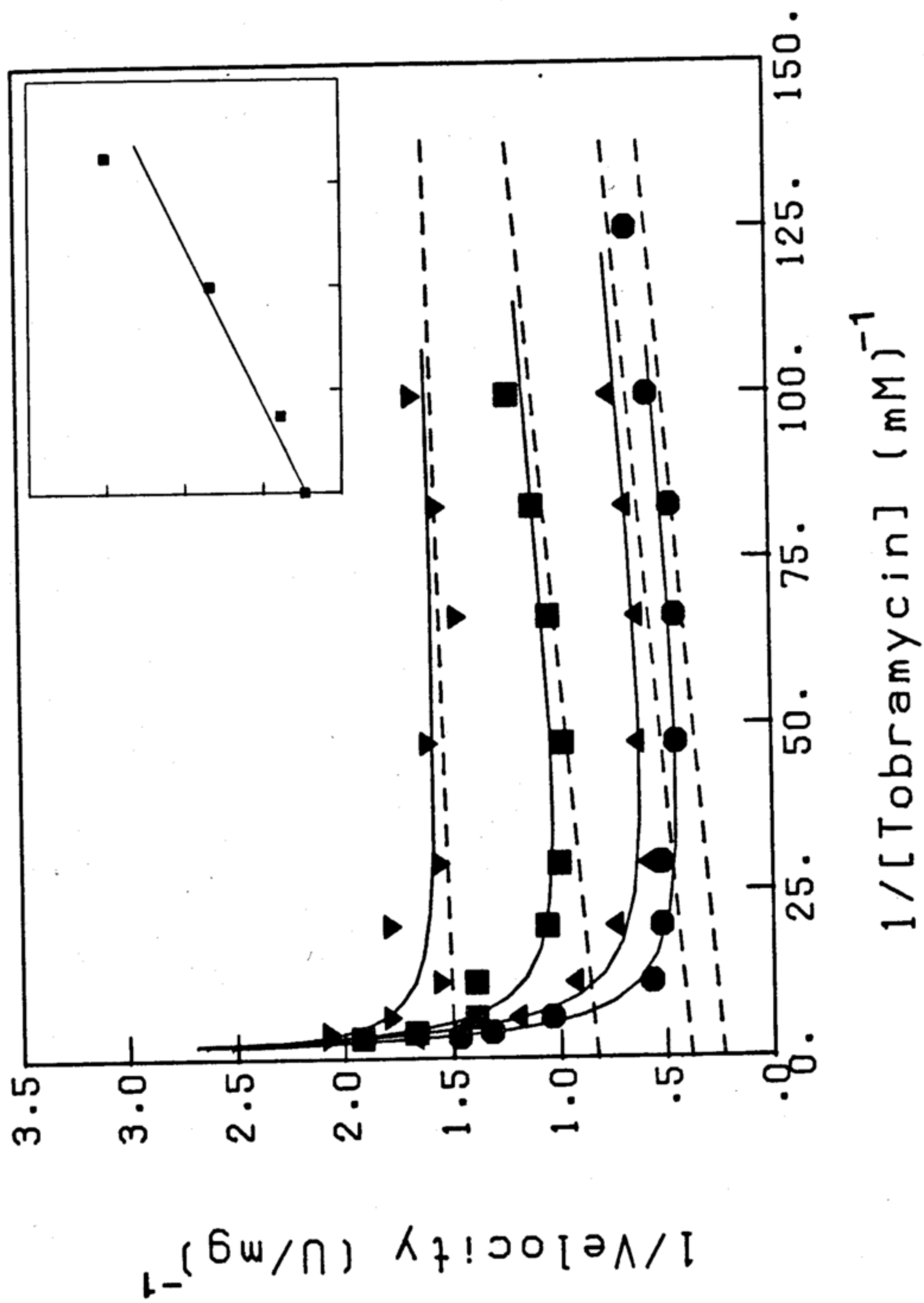
a
b
0.
1.
2.
3.
4.
5.
.0
.5
1.0
1.5
2.0
2.5
1/[Mg:ATP] (mM)-1
1/Velocity (U/mg)-1

Figure 30: Uncompetitive inhibition by tobramycin-AMP with tobramycin as the variable substrate

Each curved, solid line represents the data fit to Eq. 2, and each dashed line notes the position of K/V and 1/V. The concentrations of tobramycin-AMP were 0 mM (●) 0.15 mM (▲), 0.40 mM (■), and 0.65 mM (▼). The concentration range of tobramycin was 0.008 mM to 0.326 mM for 0 mM tobramycin-AMP and 0.01 mM to 0.326 mM for 0.15 mM, 0.40 mM, and 0.65 mM tobramycin-AMP. Mg:ATP and free Mg^{2+} concentrations were fixed at 12.86 mM and 10 mM, respectively. Purfied ANT(2") was from Preparation B.

Inset: Intercept replot of tobramycin <u>versus</u> tobramycin-AMP

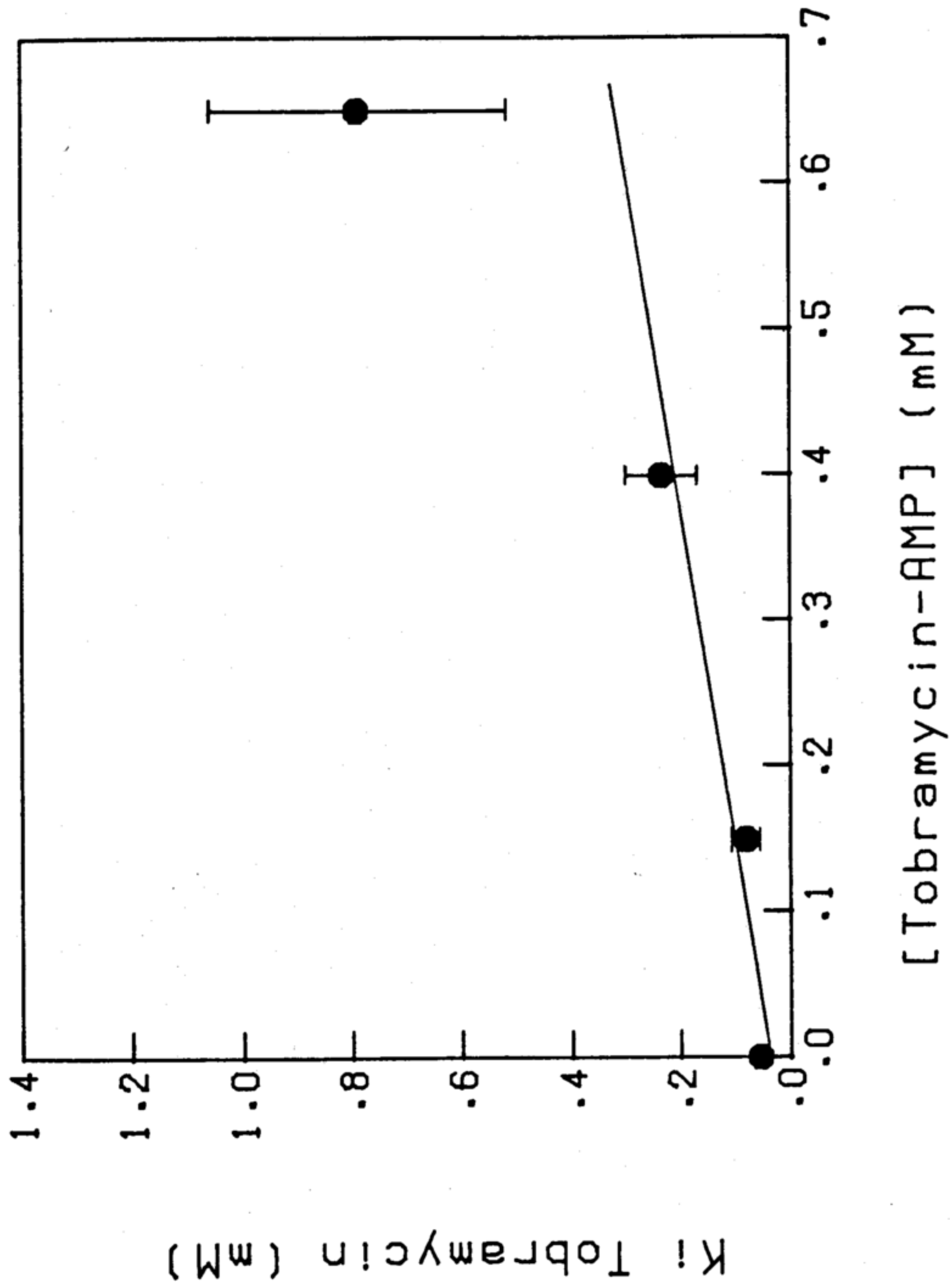
The solid line is drawn from the fit of apparent 1/Vmax values of tobramycin to Eq 11.



1/Velocity (U/mg)$^{-1}$
3.5
3.0
2.5
2.0
1.5
1.0
.5
0.
0.
25.
50.
75.
100.
125.
150.
1/[Tobramycin] (mM)$^{-1}$

Figure 31: Replot of substrate inhibition constant of tobramycin *versus* tobramycin-AMP

The solid line is drawn from the data fit to Eq. 17.



Ki Tobramycin (mM)
1.4
1.2
1.0
.8
.6
.4
.2
.0
.0
.1
.2
.3
.4
.5
.6
.7
[Tobramycin-AMP] (mM)

TABLE 14

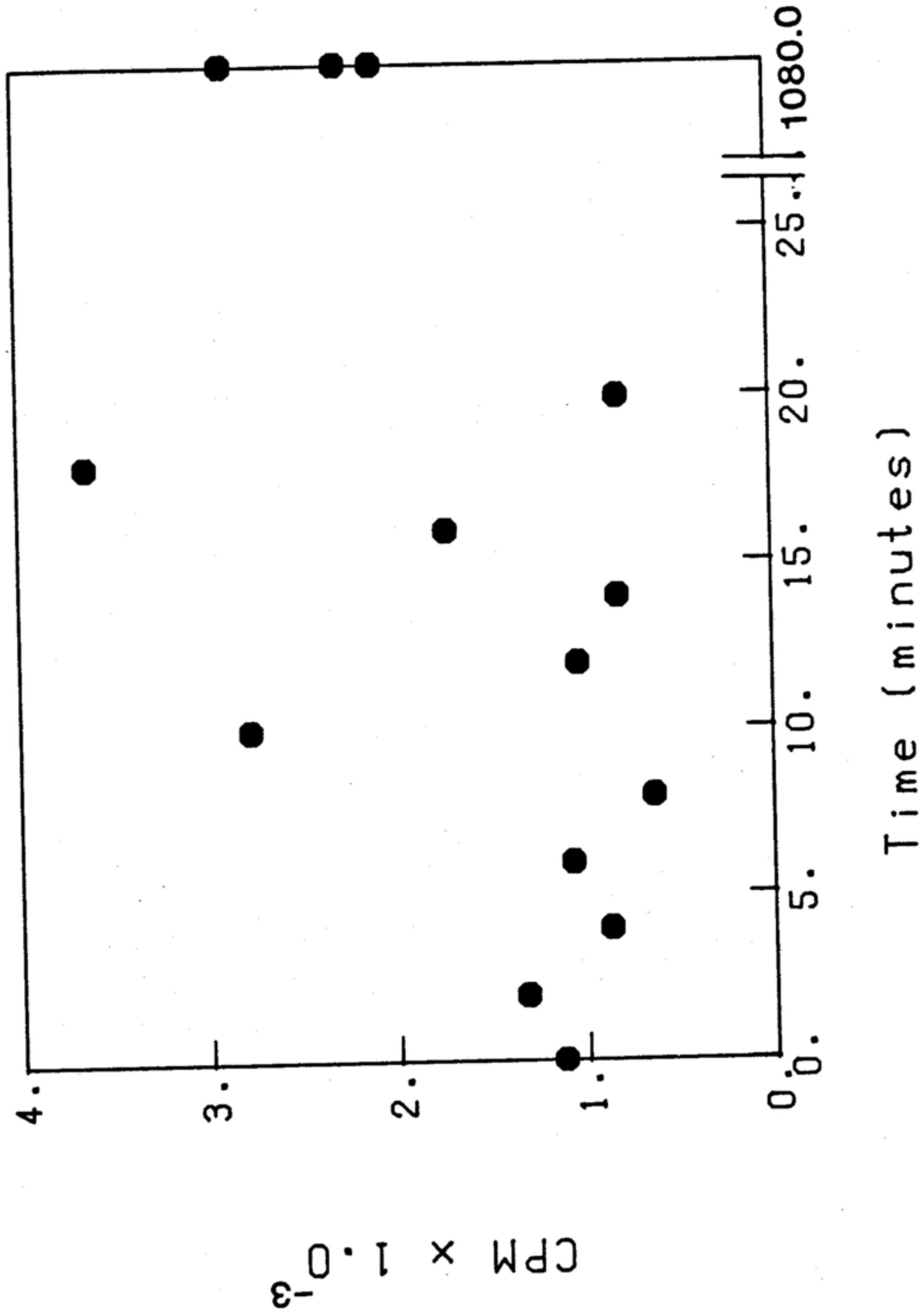
INHIBITION CONSTANTS

Variable Substrate	Inhibitor	Inhibition[a] Type	Slope Ki (mM)	Intercept Ki (mM) Kii	Kid
Mg:ATP	sisomicin	linear UC	-	.65 ± .03[b]	-
Mg:ATP	tobramycin	hyperbolic UC	-	.051 ± .01[c]	.74 ± .17
gentamicin A	neomycin C	S-linear I-hyperbolic NC	.051 ± .004	.10 ± .01[d]	.48 ± .16
Mg:ATP	neomycin C	hyperbolic NC	-	.092 ± .009[c]	.95 ± .30
tobramycin	neomycin C	S-linear C with substrate inhibition	.031 ± .01[e]	-	-
Mg:ATP	AMP-tobramycin	S-linear, I-linear NC	.42 ± .20	.31 ± .04[f]	-
tobra-mycin	AMP-tobramycin	linear UC with substrate inhibition	-	.17 ± .02[g]	

[a] C, competitive; NC, noncompetitive; UC, uncompetitive; S, slope; I, intercept. [b] Obtained by fit to Equation 3. [c] Obtained by fit to Equation 5. [d] Obtained by fit to Equation 6. [e] Obtained by fit to Equation 7. [f] Obtained by fit to Equation 4. [g] Obtained by fit to Equation 9.

Figure 32: Isotope exchange between [^{32}P] pyrophosphate and ATP during catalytic turnover of ANT(2")

The conditions of this experiment are described in METHODS. The amount of ^{32}P is expressed as counts per minute (cpm) as shown on the y axis of this plot. Enzyme was from Preparation A.



0.
5.
10.
15.
20.
25.
1080.0
Time (minutes)
0.
1.
2.
3.
4.
CPM x 1.0^{-3}

F. 4. Effect of pH on the Kinetic Parameters of ANT(2")I

The pH profiles of both V/K and Ki of sisomicin are bell-shaped curves as illustrated in Figures 33 and 34, respectively. The data for V/K fit Equation 14, and the data for Ki fit Equation 15. Both equations assume four ionizable groups, two of which must be ionized and two that must be protonated for activity. The pK values determined by fitting the data to Equations p and o are very similar and are presented in Table 15. Two pK values of 6.8 and two with values of 8.5 appear in V/K. For Ki, two pK values of 6.7 and two values of 8.2 are present.

The pH profile for Vmax of sisomicin, shown in Figure 35, differs markedly from those obtained for V/K and Ki. At first glance, this profile appears to reflect a half-bell shaped profile with decreasing activity at acid pH. However, the data can be fit to Equation 17 which describes a straight line. In contrast, a half-bell profile in which activity is lost at acid pH would have an asymptote with a slope of 1. The pH dependence of Vmax can be adequetely described by a wave function

TABLE 15

IONIZATION CONSTANTS FOR ANT(2")I WITH SISOMICIN AS A SUBSTRATE

Vmax[a]	V/K[b]		Ki[c]	
pK	pKa	pKb	pKa	pKb
8.4 ± 0.1	6.76 ± 0.07	8.45 ± 0.05	6.68 ± 0.08	8.22± 0.06

[a] Obtained by fitting Vmax values to Eq 16; from this fit, V_L = 0.8 + 0.1 and V_H = 11.1 + 1.4. [b] Obtained by fitting V/K values to Eq 14;this resulted in V/K = 1.38 + 0.16 L/(min x mg). [c] Obtained by fitting K_I values to Eq 15 ;from this, K_I = 0.037 + 0.005 mM.

Figure 33: V/K of sisomicin as a function of pH

The solid line represents the data fit of V/K of sisomicin to Eq. 14. The dashed lines designate the asymptotes with slopes of +2, 0, and -2. The breaks in the dashed line mark the positions of the ionization constants determined for this profile. The following buffers were used in this experiment: MES (●), pH 6.0, 6.5, and 6.7; MOPS (▲), pH 6.8, 6.9, and 7.0; HEPES ■), pH 7.0, 7.3, 7.5, 7.7, 7.8, 7.9, and 8.0; TAPS (▼), pH 7.8, 7.9, 8.0, 8.3, 8.5, and 9.0; and CHES (◆), pH 9.0, 9.5, and 10.0. The concentration of Mg:ATP was 8.23 mM for pH 6.0 through pH 7.9 and 14.13 mM for pH 8.0 through pH 10.0. The ratio of Mg:ATP to free ATP was fixed at 390.6:1 at all pH values. Purified ANT(2") was from Preparation B.

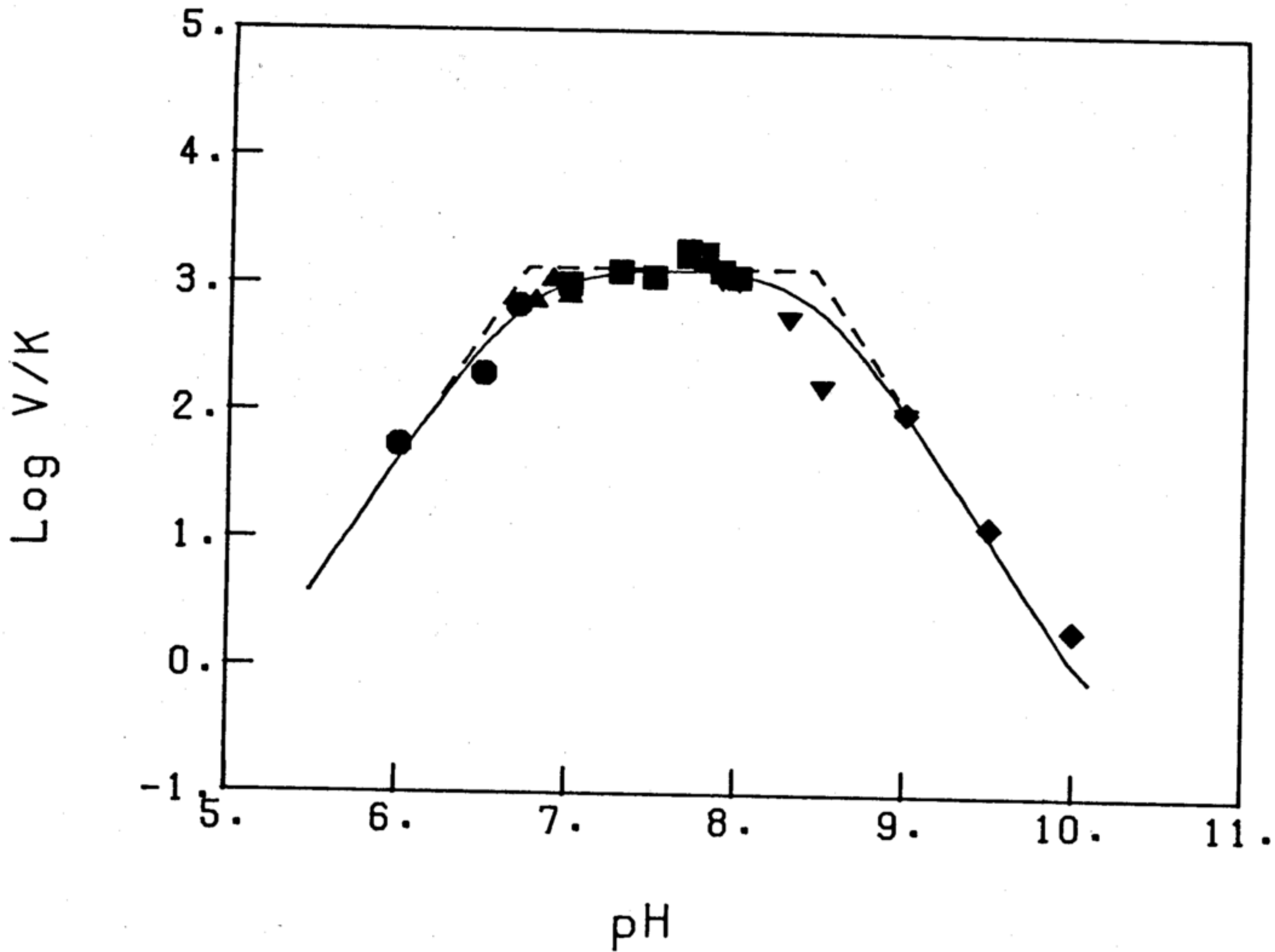

Log V/K
5.
4.
3.
2.
1.
0.
-1.
5.
6.
7.
8.
9.
10.
11.
pH

Figure 34: K_I of sisomicin as a function of pH

The solid line represents the data fit of the K_I of sisomicin to Eq 15. The dashed lines represent asymptotes with slopes of +2, 0, and -2; the breaks inthe dashed lines mark the ionization constants determined from this profile. The buffers, enzyme, and the Mg:ATP concentrations are described in the figure legend 33.

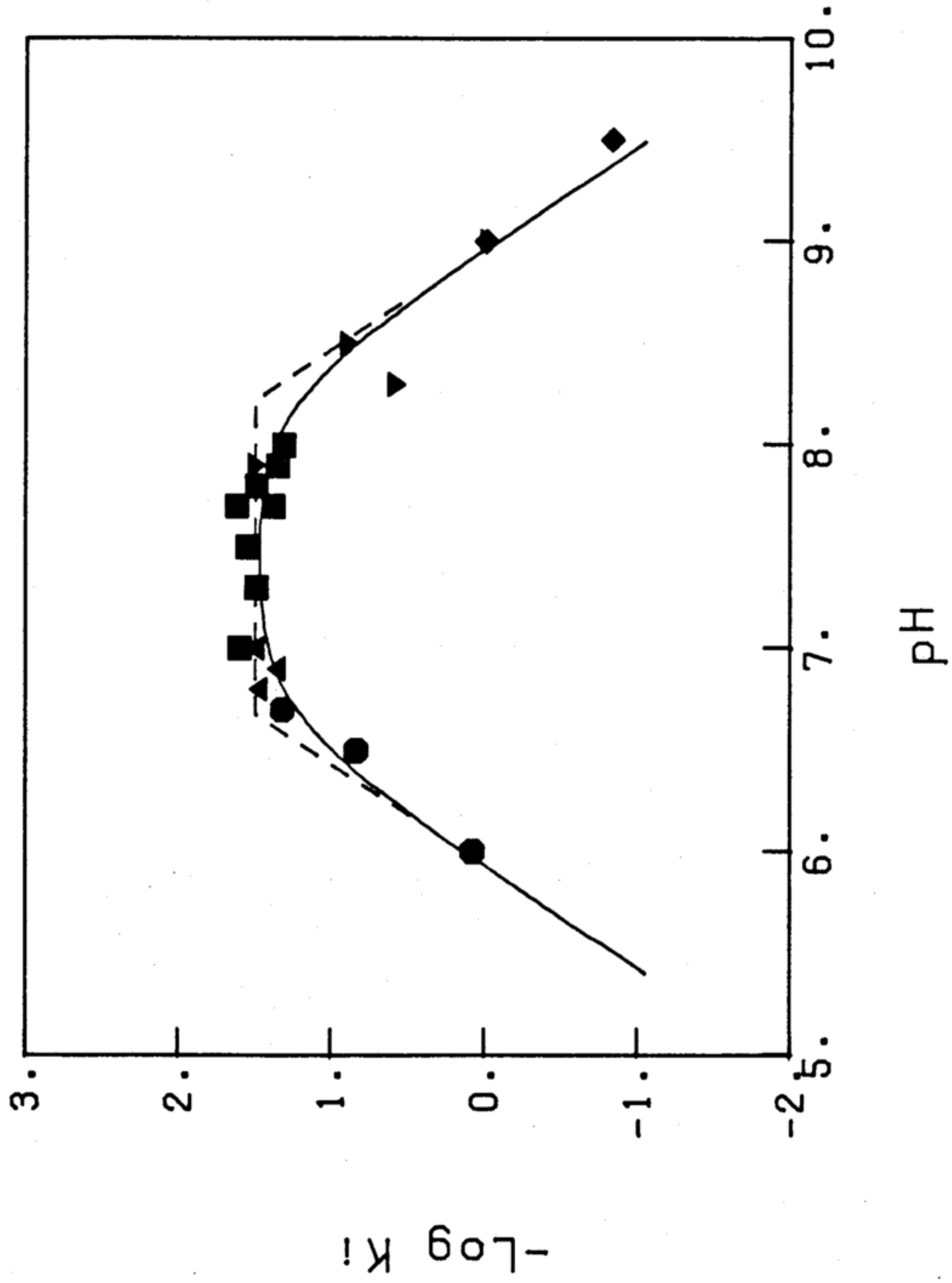
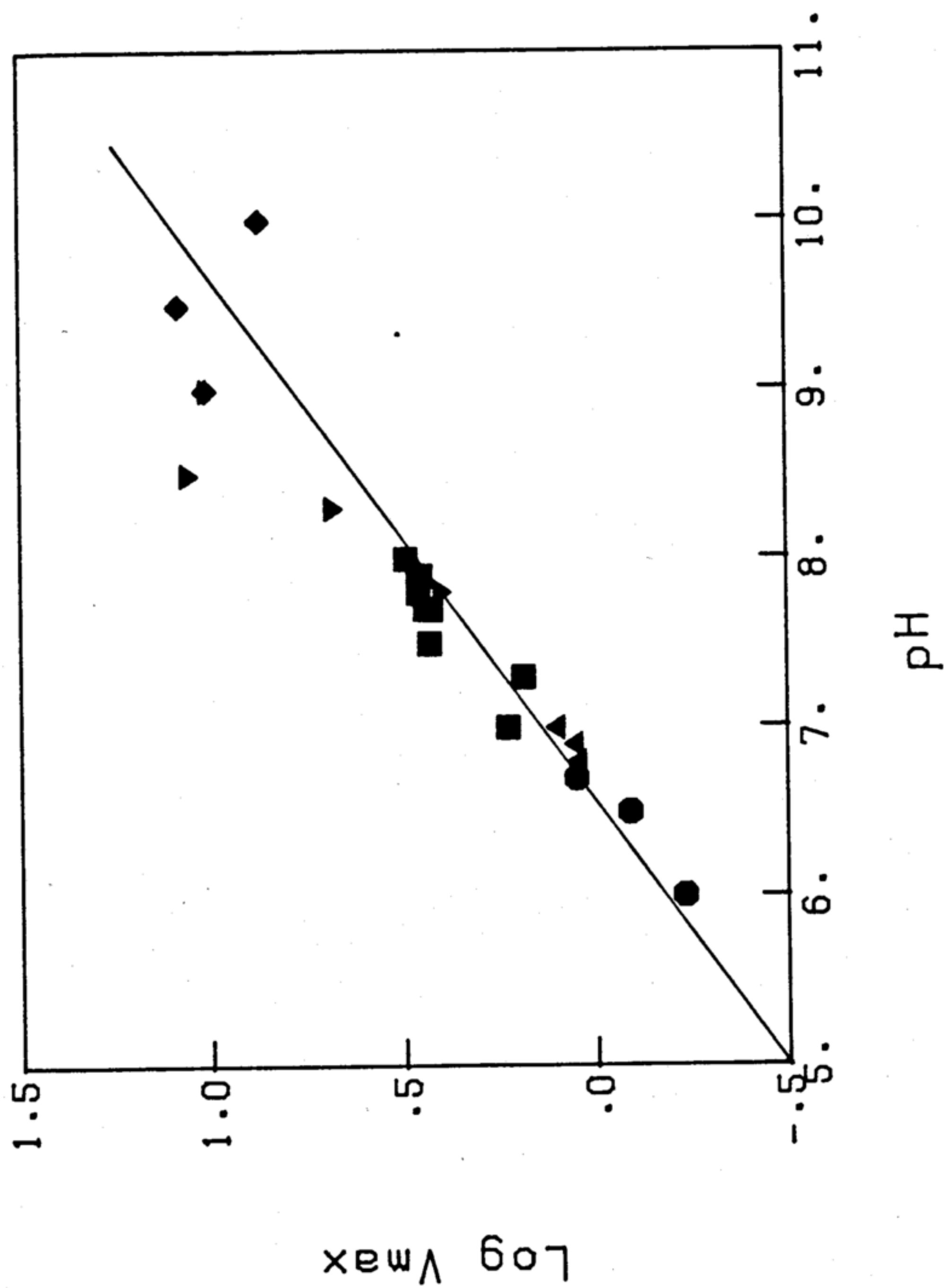

-Log Ki
pH
3.
2.
1.
0.
-1.
-2.
5.
6.
7.
8.
9.
10.

Figure 35: Vmax of sisomicin as a function of pH

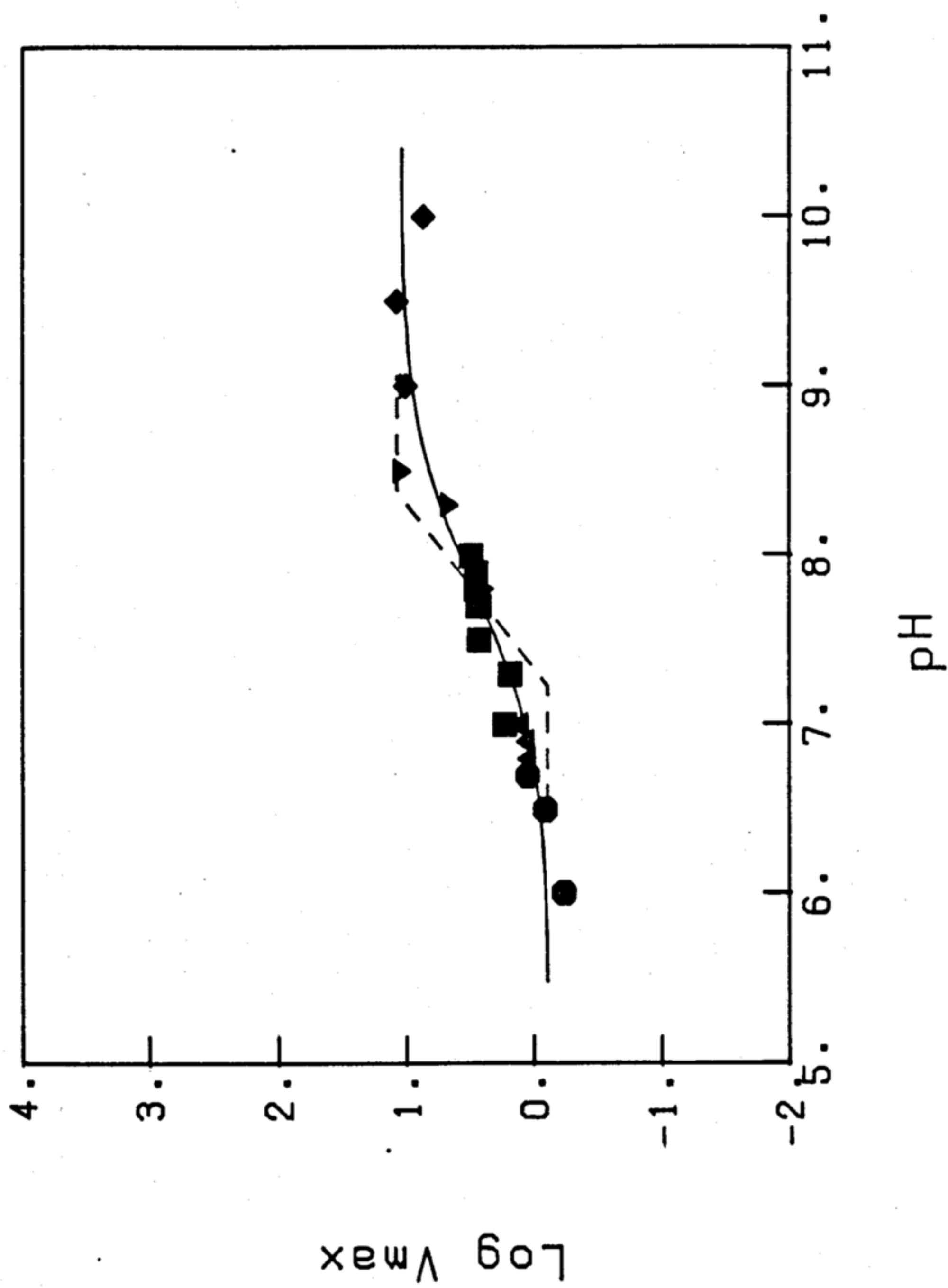
The solid line represents the data fit to Eq 17. The slope of this line is 0.32 , and the intercept is -2.10.



Log Vmax
pH
1.5
1.0
.5
.0
-.5
5.
6.
7.
8.
9.
10.
11.

Figure 36: Vmax of sisomicin as a function of pH

The solid line is drawn from the data fit of Vmax of sisomicin to Eq. 16. The dashed line represents the asymptote with a slope of 1. The buffers and Mg:ATP concentrations used in this experiment are described in the figure legend 33.


Log Vmax
4.
3.
2.
1.
0.
-1.
-2.
5.
6.
7.
8.
9.
10.
11.
pH

(Equation 16) as shown in Figure 36. The resulting pK value is shown in Table 15.

F.5 Effect of Viscosity on the Kinetic Parameters of ANT(2")I

The kinetic constants of three aminoglycoside substrates, sisomicin, gentamicin C1, and netilmicin, were determined in the presence of 0%, 20%, 30%, and 40% glycerol to ascertain the effects of viscosity.

Table 16 presents the kinetic constnats of the three aminoglycosides as a function of estimated relative viscosity. The V/K values of sisomicin and gentamicin C1 decrease 3.8- and 2.5-fold, respectively, with a 3.7-fold increase in viscosity. The effect of viscosity on the V/K of gentamicin C1 is shown in Figure 37a. In constrast, the V/K of netilmicin, a poor substrate of ANT(2")I (see Table 10) is insensitive to increasing viscosity.

The Vmax values of all three aminoglycosides decrease with increasing viscosity. The Vmax of sisomicin and gentamicin C1 are decreased 7- and 10-fold, respectively, whereas Vmax of netilmicin decreases 3-fold with a 3.7-fold increase in viscosity. The effect of

TABLE 16

KINETIC CONSTANTS OF AMINOGLYCOSIDES AS A FUNCTION OF VISCOSITY

SISOMICIN

n/n_o	Vmax (IU/mg)	V/K (L/(mgxmin))	Ki (uM)
1.0	9.68 ± 1.8	.146 ± .014	154 ± 40.7
1.7	3.35 ± 0.39	.123 ± .017	214 ± 39.1
2.6	2.49 ± 0.49	.0532 ± .0076	193 ± 58.2
3.7	1.45 ± 0.17	.0434 ± .0050	183 ± 32.1

GENTAMICIN C_1

n/n_o	Vmax (IU/mg)	V/K (L/(mgxmin))	Ki (uM)
1.0	6.15 ± 0.28	.154 ± .012	1840 ± 352
1.7	2.18 ± 0.079	.119 ± .0086	1680 ± 267
2.6	1.28 ± 0.058	.0850 ± .0074	1740 ± 365
3.7	0.574 ± 0.024	.0582 ± .0061	2633 ± 698

NETILMICIN

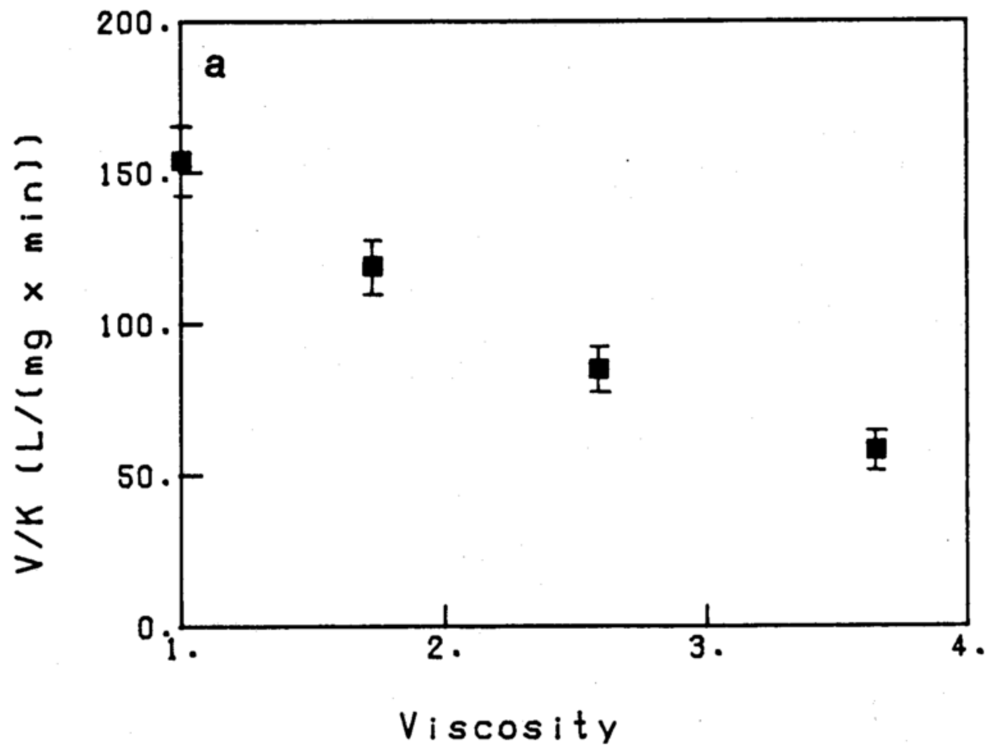
n/n_o	Vmax (IU/mg)	V/K (L/(mgxmin))	Ki (uM)
1.0	5.69 ± 1.2	.00163 ± .00017	5120 ± 153
1.7	3.05 ± 0.45	.00271 ± .00049	8500 ± 238
2.6	2.96 ± 0.63	.00234 ± .00046	5900 ± 216
3.7	1.96 ± 0.25	.00301 ± .00061	5150 ± 121

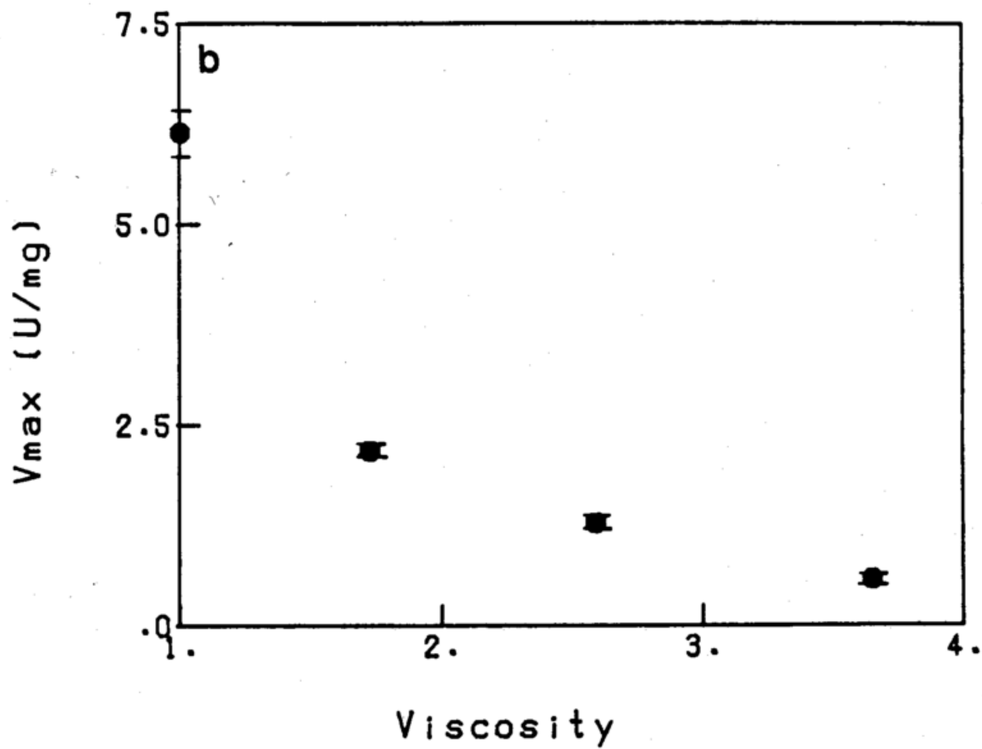
Figure 37a: V/K of gentamicin C_1 as a function of viscosity

The V/K values were obtained by fitting the data to Eq. 2. The concentration range of gentamicin C_1 was from 0.009 mM to 0.913 mM. The concentration of Mg:ATP was fixed at 12.86 mM.

Figure 37b: Vmax of gentamicin C_1 as a function of viscosity

The Vmax values were obtained by fitting the data to Eq. 2.


a
V/K (L/(mg x min))
200.
150.
100.
50.
0.
1.
2.
3.
4.
Viscosity


b
Vmax (U/mg)
7.5
5.0
2.5
.0
1.
2.
3.
4.
Viscosity

viscosity on Vmax of gentamicin C1 is illustrated in Figure 37b.

In constrast, the substrate inhibition constants are insenstive to viscosity. As shown in Table 16, the Ki values for all three aminoglycosides remain the same (within experimental error) with increasing viscosity.

I. CORRELATION OF MICROBIAL RESISTANCE TO AMINOGLYCOSIDE KINETIC CONSTANTS

The minimum inhibitory concentration (M.I.C) values for *E. coli* W677/pJR76.2 are presented in Table 17. The M.I.C. values of some aminoglycoside antibiotics for *E.Coli* are also presented in this table. These results show that the plasmid JR76.2 confers a high degree of resistance to gentamicin and tobramycin.

Correlations between the kinetic constants of the six aminoglycoside substrates listed in Table 17 and the M.I.C. values were attempted, and the results are presented in Table 18. The best correlation occured for M.I.C. and V/K, and the worst for Vmax and M.I.C. However, at a 90% confidence level for 4 degrees of freedom, the significance limit of correlation is 0.7293. The correlation coefficient of 0.708 falls short of this value, so the correlation between V/K and M.I.C. cannot

be considered statistically significant by this criterion.

TABLE 17

M.I.C. VALUES OF AMINOGLYCOSIDES FOR *E. coli*

	M.I.C. (ug/mL) for *E. coli*	
Antibiotic	285 sensitive strains[a]	W677/JR76.2[b]
Gentamicin C2	1.20[c]	2630
Gentamicin Cla	1.20	1980
Tobramycin	1.30	1650
Sisomicin	0.42	1350
Netilmicin	0.41	40
5-episisomicin	0.30	34

[a] Taken from reference 135; identities of strains were not specified. [b] Each M.I.C. value is a mean from six determinations; M.K. Powers performed these experiments (see Methods). [c] This value is represents the M.I.C. of the gentamicin complex, and is included here for the purpose of comparison.

TABLE 18

LINEAR CORRELATION ANALYSES
BETWEEN AMINOGLYCOSIDE
KINETIC CONSTANTS AND M.I.C. VALUES

Kinetic Constant	Correlation Coeffcient (r)
Vmax	+ 0.070
Km	- 0.594
V/K	+ 0.708
Ki	- 0.570

Each correlation is calculated with 4 degrees of freedom where
(n - 2) = number of degrees of freedom, and
n = the number of x,y pairs in the data set.

CHAPTER IV:

DISCUSSION

A. FLUORESENCE ASSAY OF ANT(2")I

Van Pelt and Northrop reported a Michaelis constant of 2.8 uM for tobramycin in the presence of 0.8 mM ATP (150). Accurate determination of kinetic parameters for aminoglycoside substrates with similarly low Km values requires more sensitivity than that provided by the spectrophotometric assay of Van Pelt and Northrop. Thus, the fluorometric assay for ANT(2")I activity was developed in the present study.

The fluorometric assay of ANT(2") appears to be quantitatively reliable; it is linear both with the amount of NADPH and enzyme as documented in Figures 6 and 8. The assay is conveniently standardized with a known amount of pyrophosphate, the concentration of which is readily determined by a simple colorimetric assay. Although high concentrations of magnesium acetate result in some interference, quenching of fluoresence is not severe. The velocity of the ANT(2") catalyzed reaction as determined by the fluorometric assay compares favorably to that determined by the spectrophotometric assay.

However, there were problems with the fluorometric assay that necessitated a re-evaluation of its usefulness

for the research described in this thesis. A major drawback was the presence of a high amount of background fluoresence in the coupling mixture. Although the PM microphotometer of the Aminco-Bowman fluorometer allowed suppression of this background fluoresence, the change in fluoresence due to the production of NADPH was very small relative to the background emission, and because of the limitations of the instrument, the fluoresence assay appeared to be no more sensitive than the spectrophotometric assay using 5 cm cell in the modified Gilford. For this reason, the spectrophotometric assay was used instead of the fluorometric assay for most of the work described herein. However, accurate determinations of kinetic constants at neutral pH will require greater sensitivity than that provided by the spectrophotometric assay using the 5 cm cuvettes. The Michaelis constant of sisomicin was approximately 1.5 uM from pH 7.0 to pH 8.0. The Km values of some of the better substrates of ANT(2") (e.g. gentamicins C1a and C2) will probably be in the nanomolar range at neutral pH and thus outside the range of sensitivity of the spectrophotometric assay. The use of the fluorometric

assay for ANT(2")I on a more sophisticated instrument than the Aminco-Bowman fluorometer may provide the key to precise evaluation of the kinetic constants of the aminoglycoside substrates at neutral pH.

B. EXTRACTION OF CELLULAR PROTEIN BY SONICATION

Although the osmotic shock of *E. coli* W677/pMY10 is a reliable technique for selective release of ANT(2")I activity from bacteria, sonication appears to have potential as an alternative method. The approach was used earlier, but was rejected after Williams and Northrop reported that sonication of *E. coli* C100/JR 88 cells caused rapid inactivation of AAC(3)I by a first order process with a half-life of 13.7 seconds (161). In contrast, experiments performed late in the present research demonstrated that ANT(2") activity remains stable in even in the crude cellular extract after 4 minutes of sonication with greater than 95% of the activity released in 30 seconds. Approximately 4 units of ANT(2") activity per gram wet weight cells were released by sonication which is comparable with that released by osmotic shock. Osmotic shock has an advantage of affording some selectivity inthe protein releases, but an advantage of sonication is that it may

be accomplished in a matter of minutes rather than the two days required by Van Pelt and Northrop's modified method of osmotic shock (150).

The reason for the contrasting effects of sonication on ANT(2")I and AAC(3)I activity is not clear. It is possible that sonication disrupts the subunits of the tetrameric protein, AAC(3)I. In contrast, electrophoretic and chromatographic evidence shows ANT(2") to be a monomer (150).

C. <u>STABILITY OF PURIFIED ANT(2")I</u>

Purified ANT(2") stability is sensitive to pH as shown in Figure 9 with activity decreasing sharply with prolonged exposure to alkaline pH. When TAPS at pH 8.1 was used as the buffer during purification, the enzyme activity eluted from the gel filtration column was stable for prolonged storage at -20 C. In contrast, ANT(2") eluted from a gel filtration column equilibrated with HEPES at pH 7.8 slowly loses activity when stored at -20 C (152). Freezing and thawing had no effect on activity.

D. EFFECT OF BUFFER ON ANT(2")I ACTIVITY

As shown in Figure 10, TRIS buffer has an inhibitory effect on ANT(2") activity. In contrast, TAPS buffer enhances ANT(2") activity at pH 8.0 versus HEPES buffer (see Figure 9). It is possible that TAPS buffer may somehow specifically interact with the enzyme to stabilize and activate it.

E. INCREASED YIELDS OF ANT(2")

The yield of ANT(2")I activity using the purification described herein represents a 16- to 37-fold increase (see Preparation A and B in Table 7) over the purification described by Van Pelt and Northrop (150). In addition, the specific activities of E_s and E_f represent 7- and 10-fold increases over those reported by the same researchers.

There are at least three factors responsible for the increased activity of ANT(2"). One is optimization of the magnesium ion concentration in the assay which gives a nearly 5-fold increase in enzyme activity over that reported by Van Pelt and Northrop (150). The second factor is the cells used for inoculating the final media were exposed to a higher concentration of kanamycin sulfate, *i.e.*, 0.50 mg/ml *versus* 0.30 mg/ml kanamycin

sulfate. Williams and Northrop reported that the amount of AAC(3)I produced by E. coli/JR88 was increased upon exposure of the cells to high concentrations of gentamicin in the medium; the enzyme was half-maximally inducedat 0.08 mg/ml gentamicin sulfate (161). Although this type of "induction" has not been documented for E. coli/pMY10, it may be that an increase in antibiotic concentration may increase production of ANT(2"). Finally, the ability to harvest the bacterial cells over a short period of time using the continuous flow centrifuge system may contribute to an increased extraction of enzyme. Williams and Northrop demonstrated that a sharp drop in recovered AAC(3) activity occurs as the bacteria enter the stationary phase of the growth cycle. They suggested that this does not reflect a change in the cellular level of enzyme, but rather a change in the bacterial wall or membrane such that the cells were less suseptible to osmotic shock. In order to maximize the yield of enzyme, they found it necessary to harvest the cells within a narrow time period (> 1 hour) of increased activity during the late-log phase of growth (161). With a centrifuge equipped with a

conventional rotor, the harvest of 30 L of bacteria suspended in medium requires at least 2 hours for completion. In contrast, only 40 to 50 minutes are rquired using a continuous flow rotor. Consequently, a higher proportion of cells are sensitive to osmotic shock, and more enzyme may be recovered.

F. SEPARATION OF THE E_f AND E_s FORMS OF ANT(2") USING AFFINITY CHROMATOGRAPHY

ANT(2")I-E_f and -E_s bind differently to kanamycin. Only E_f binds rapidly to the kanamycin-containing resin whereas both E_s and E_f bind rapidly to neomycin-, gentamicin-, and tobramycin Sepharose, which were synthesized by identical procedures. However, lowering the pH of the coupling conditions by 1 pH unit provided a kanamycin-Sepharose resin to which both forms rapidly bind. Thus, the difference in binding behavior may not reside in kanamycin itself, but in the reactions that occur in the synthesis of the resins and the resulting form of the covalently attached ligand.

Synthesis of affinity resins from epoxy-activated Sepharose resins relies on the reactivity of the electrophilic epoxy group and nucleophilic functionalites on the aminoglycoside, i.e., the hydroxyl- and amino-

groups (142). The ionization constants of the amino groups range from 6.5 to 9.8. At a pH value of 10, most are unprotonated and are favored to react with the epoxy group over the less nucleophilic hydroxyl-groups (although certainly some hydroxyl groups will react). At pH 9.0, many amino groups will be protonated and thus rendered ineffective as nucleophiles leaving the hydroxyl groups as the favored reactants. As a result, the binding of kanamycin to the resin will depednd on the pH of the coupling solution. The kanamycin-Sepharose coupled at pH 9.0 may contain kanamycin covalently bound to the resin _via_ a hydroxyl in ring I, whereas at pH 10, it may be linked to a favored amine of ring III. It is thus possible that selective binding could be observed with affinity resins prepared from antibiotics other than kanamycin.

A particularly striking observation was that neither enzyme form binds to amikacin-Sepharose. Amikacin, a semi-synthetic aminoglycoside derived from kanamycin A, is not a substrate of ANT(2") but might be expected to bind as an inhibitor. The significance of the lack of

interaction between this aminoglycoside and ANT(2")I will be discussed in Section H of this chapter.

The differential binding of E_f and E_s was also observed by Van Pelt and Northrop using gentamicin-AffiGel (150). However, the kanamycin-Sepharose resins provide a more efficient separation ofthe two forms and are more stable. Gentamicin AffiGel favored binding of E_f, but also bound some E_s which accounted for 30% of the total protein (150). In contrast, the electropherogram (Fig. 18) of the enzyme that binds rapidly to kanamycin-Sepharose 10 shows that only a single peak corresponding to E_f. Gentamicin-AffiGel loses binding capacity within 6 months (152), but the bisoxirane-linked resins are still functional after 18 months.

G. PROPERTIES OF ANT(2")I-E_f AND -E_s

The results of polyacrylamide gel electrophoresis of native and denatured ANT(2") show two enzyme forms that physically differ from each other. Monomeric molecular weights are approximately 25,000 and 27,000 for E_f and E_s, respectively. Differences in the relative migration values of E_f and E_s were greater in the absence of SDS,

perhaps because of small differences in net charge (see Table 7).

Possible differences in kinetic properties of ANT(2")-E_f and -E_s were consideredby comparing their Vmax, V/K, and Ki values of six aminoglycoside substrates determined for each enzyme form. After correction for the uncertainty of the extinction coefficent of E_s (see section III.), the kinetic constants are nearly identical within experimental error, indicating that the two enzyme forms are not kinetically distinct. ANT(2")-E_f and -E_s are probably the same polypeptide which differ due to some post-translational modification, such as proteolysis or conjugation with sugars or lipids. Although Van Pelt and Northrop note that the exact relationship between the two forms is unclear, they suggest that E_f and E_s have a common ancestor or are the same polypeptide since the amino acid composition and polypeptide chain length are essentially identical (150).

H. SUBSTRATE SPECIFICITY OF ANT(2")I

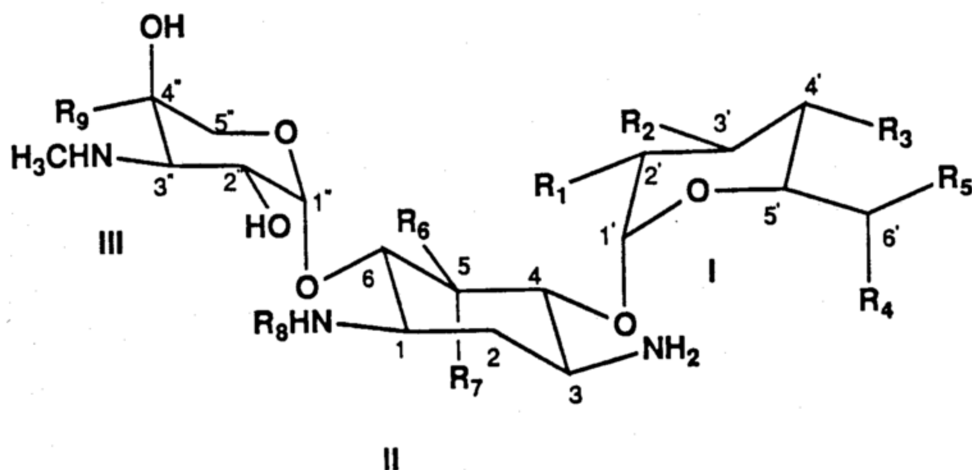
H.1 Aminoglycoside Substrate Specificities and Structure-Activity Relationships

ANT(2")I has been reported to adenylylate a number of gentamicins and kanamycins whose structures are

illustrated in Figures 38 and 39. All of the substrates are 4,6-substituted aminoglycosides that contain the deoxystreptamine ring but differ in the number and positions of amino and hydroxyl groups. The 2"-hydroxyl and the 3-amino groups are common to all substrates as are the 5- and 4"-hydroxyl groups, but the stereochemical orientation differs among the latter.

Although the site of modification of the aminoglycoside substrates is in ring III, structural changes in ring I or II affect substrate specificity. These structural changes and their influence on ANT(2") activity can be evaluated in terms of V/K, the apparent first order rate constant for the combination of the substrate of the enzyme during catalytic turnover. For the kinetic mechanism of ANT(2") (see Section I.1, this chapter) V/K of the aminoglycoside substrate is composed of the steps of binding, catalysis, and release of the first product. Vmax, often the parameter of choice for structure-activity relationships, is here composed only of the steps of release of the final product; it does not contain catalysis. This is illustrated below in Cleland's notation (27):

Figure 38: Structures of gentamicins and related aminoglycosides

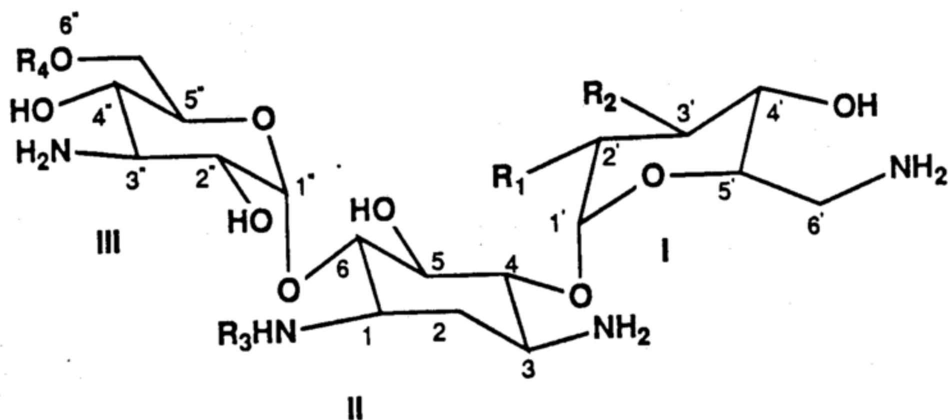


STRUCTURES OF GENTAMICINS AND RELATED AMINOGLYCOSIDES

COMPOUND	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_7	R_8
Gentamicin C1a	NH_2	H	H	H	NH_2	OH	H	H	CH_3
Gentamicin C2	NH_2	H	H	CH_3	NH_2	OH	H	H	CH_3
Gentamicin C_1	NH_2	H	H	CH_3	NH_2CH_3	OH	H	H	CH_3
Sisomicin	NH_2	H	Δ4'	H	NH_2	OH	H	H	CH_3
5-epiSisomicin	NH_2	H	Δ4'	H	NH_2	H	OH	H	CH_3
Netilmicin	NH_2	H	Δ4'	H	NH_2	OH	H	CH_2CH_3	CH_3
Gentamicin A	NH_2	OH	OH	H	OH	OH	H	H	H
Gentamicin B	OH	OH	OH	H	NH_2	OH	H	H	CH_3
Gentamicin B_1	OH	OH	OH	CH_3	NH_2	OH	H	H	CH_3

Figure 39: Structure of kanamycins and related aminoglycosides

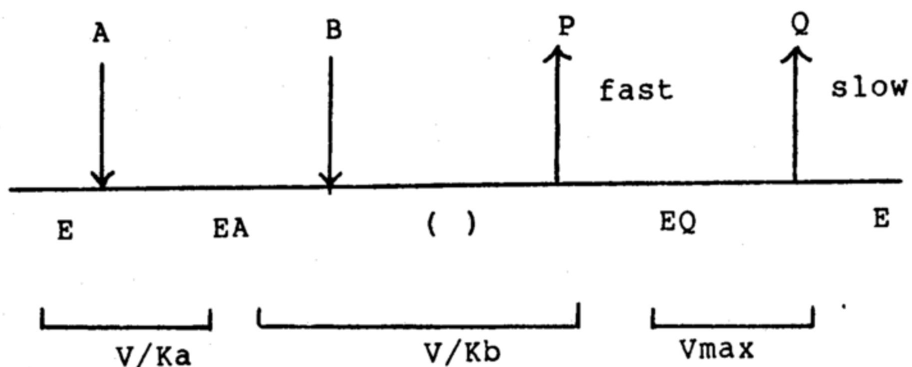
Amikacin is not a substrate of ANT(2")I, but its structure is included in this figure for the purpose of comparison to the kanamycin substrates.



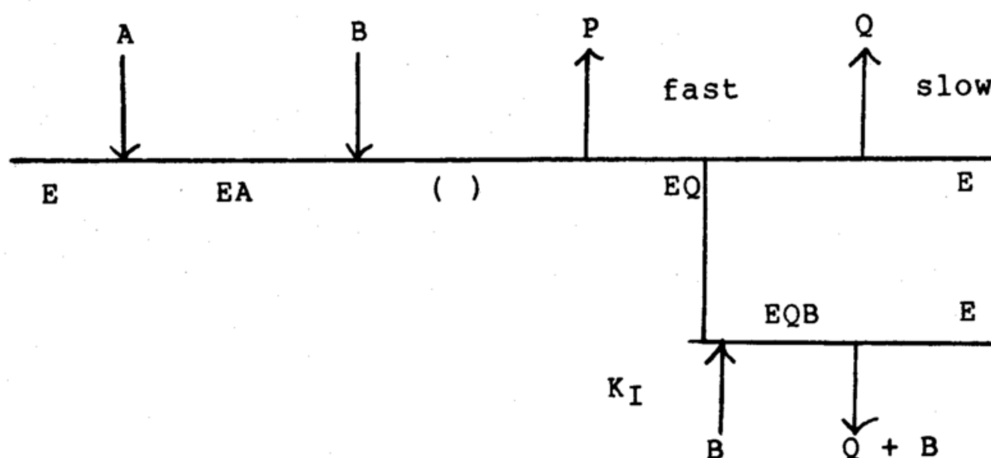
STRUCTURES OF KANAMYCINS AND RELATED AMINOGLYCOSIDES

COMPOUND	R_1	R_2	R_3	R_4
Kanamycin A	OH	OH	H	H
Amikacin	OH	OH	L-AHBA	H
Kanamycin B	NH_2	OH	H	H
Tobramycin	NH_2	H	H	H
Nebramycin 4	NH_2	OH	H	$CONH_2$

AHBA = 4-amino-l-hydroxylbutyryl



For ANT(2")I, A is Mg:ATP, B is aminoglycoside, P is pyrophosphate, and Q is AMP-aminoglycoside. In addition, the aminoglycoside substrate can bind to EQ which results in substrate inhibition:



The substrate inhibition constants, K_I ,shown in Table 10, section III, are true equilibrium constants since nearly all enzyme is present as EQ due to the slow release of Q. Therefore, the K_I values reflect only binding, whereas the V/K values reflect binding and catalysis. Since several pairs of the aminoglycoside substrates differ from each other by a single structural feature, a comparison of their V/K and K_I values should distinguish between contributions to catalysis and binding. This assumes that the aminoglycoside (B) binds to the enzyme-AMP-aminoglcoside complex (EQ) and the enzyme-Mg:ATP complex (EA) in the same manner; the pH-dependence of V/K and Ki indicates that this assumption is valid (Section I.4). Ratios of V/K and K_I were calculated from the data shown in Table 10 and are presented in Table 19.

In case A, the presence of the methyl group on the 6'-C results in an increase in K_I which indicates decreased binding of the methylated aminoglycoside. The negative change in V/K is of similar magnitude. Therefore, the presence of the methyl substituent affects only binding. Similarly, case B shows an increase in K_I and a comparable decrease in V/K, supporting an effect of

TABLE 19

KINETIC EFFECTS OF STRUCTURAL DIFFERENCES BETWEEN AMINOGLYCOSIDES

case[a]	antibiotic	position	group	V/K (x-fold)	K_I (x-fold)
A	gentamicin C1a	6'-C	H		
	gentamicin C2		CH_3	-1.6	+2.8
B	gentamicin B	6'-C	H		
	gentamicin B1		CH_3	-2.8	+1.8
C	kanamycin B	2'-C	NH_2		
	kanamycin A		OH	-5.8	+5.6
D	kanamycin B	3'-C	OH		
	tobramycin		H	1.0	1.0
E	gentamicin C1a	4'-5'	C-C		
	sisomicin		C=C	-11.1	+9.6
F	gentamicin C2	6'-N	H		
	gentamicin C1		$NHCH_3$	-10.3	+22.7
G	sisomicin	1-N	H		
	netilmicin		CH_2CH_3	-270.0	+26.5
H	sisomicin	5-OH	equat.[b]		
	5-episisomicin		axial	-18.0	-
I	kanamycin B	6"-C	OH		
	nebramycin 4		$CONH_2$	-1.6	+2.1

[a] Data derived from Table 10. [b] equat., equatorial

the methyl subtituent at 6'-C on binding alone. Likewise, the presence of an amino group at the 2'-C is clearly favored over a hydroxyl group for substrate binding as illustrated in case C. Substitution of a hydrogen with a hydroxyl group at the 3'-carbon has no effect on V/K or K_I as shown in case D. Case E shows that the presence of a single bond at the 4'-position is favored over that of a double bond for substrate binding.

In case F, the presence of a secondary amino group at the 6'-N position significantly decreases binding as indicated by the 23-fold increase in K_I. However, the change in V/K is less than that observed for K_I. This situation could arise if catalysis still proceeds efficiently despite decreased binding at the 6'-N position. Indeed, the V/K value of gentamicin C_2, one of the pair in case F, is the second highest of any aminoglycoside substrate surveyed and approaches that of diffusion control (see Section I.5). Hence, the secondary amino group of gentamicin C_1 affects binding, but a relatively high rate of catalysis prevents this decreased binding effect from being fully expressed in V/K. It is of note that gentamicin A possesses a

hydroxyl group at the 6' position, and its V/K value is low compared to other substrates. It might be expected that the secondary amino group would result in increased binding since the basicity of the 6'-amino group should be enhanced by the inductive effect of the methyl substituent; the more basic secondary amine would then give more positive charge at pH 9.1 and thus, tighter binding. In this case, the decreased binding is probably due to stearic hindrance on the part of the methyl group.

The presence of an ethyl substituent at the 1-N position of ring II has a large effect on Ki, but a much greater effect on V/K as illustrated in case G. This large negative effect on V/K is very likely due to a reduction in catalysis, consistent with the close proximity of the 1-ethyl group and the site of modification. Carbon-13 NMR spectral analysis of tobramycin and related aminoglycosides has shown that the 2"-OH group and the 1-amino group are spatially close to one another in the preferred, overall equilibrium conformation in solution (73). As the enzyme "sees" it, these two groups are on the same "side" of the substrate in this conformation. Reduction in catalysis may result from stearic hindrance at the 1-N position and is

supported by the fact that amikacin, an aminoglycoside with a bulky 4-amino-1-hydroxybutyryl substituent at the 1-amino position, is not even a substrate for ANT(2"). Moreover, purified ANT(2") does not bind to an affinity column with amikacin as the covalently attached ligand (see Section IV.F).

Another structural alteration in ring II that reduces V/K is an axial *versus* an equatorial hydroxyl at the 5-carbonas illustrated in case H. An 18-fold decrease in V/K occurs when the 5-hydroxyl group is in an axial position. Substrate inhibition was not observed with 5-episisomcin, even at a concentration of 9.4 mM which is 50-fold greater than the K_I of sisomicin. Substrate inhibition may be absent for 5-episisomicin for the following reasons: (1) the kinetic mechanism is changed with this substrate, and as a consequence, the proportion of EQ significantly decreases, thus eliminating substrate inhibition;(2) the axial 5-hydroxyl group prevents binding of 5-episisomicin to EQ, but not to EA; or (3) substrate inhibition exists, but the K_I is higher than 10.0 mM. The first reason seems unlikely, especially in view of the fact that netilmicin, a

substrate whose V/K is 14-fold less than that of 5-episisomicin, still exhibits substrate inhibition which is in turn dependent on the kinetic mechanism of ANT(2"). Therefore, the axial hydroxyl group at the 5-carbon probably affects binding alone, but the magnitude in the change in V/K is much less than that of Ki because catalysis is not drastically affected (see case F).

In case I, esterification of the 6"-OH results in a decrease in V/K and an increase in Ki with changes of comparable magnitude. Although this structural feature is on the same ring as the site of modification, it appears that only binding is affected. By inference from the preferred, equilibrium conformation, the amide substituent at the 6"-position is on the opposite "side" of the substrate that the enyzme likely sees. Therefore, the lack of effect on catalysis is not unexpected.

Although ANT(2") is capable of modifying aminoglycosides of considerable structural variation, it does not accept all aminoglycosides as substrates. Consequently, it is important to consider what structural features are required for activity. The 5-hydroxyl group is common to all the substrates in Tables 14 and 15, and it appears that it must be in an equatorial position for

optimum activity. Other than this, the enzyme does not seem to require a hydroxyl group at any particular position on its substrates (other than the one that is nucleotidylated), although all substrates contain some number of hydroxyl substituents. Among the amino groups, the three at positions 1, 3, and with the lone exception of gentamicin A, 6' are common to all the substrates in Table 10. As noted above, modifications of the 6' carbon or nitrogen affect binding and reduce but do not abolish activity; The result of modification of the amino group at the 3 position is unknown, but modification of the 1-amino group drastically reduces catalytic activity.

One means of combatting enzyme-mediated bacterial resistance involves the production of semisynthetic antibiotics derived from chemical modification of existing drugs (147). Semi-synthetic aminoglycosides have been produced with the hopes of designing a drug that is immune to enzymatic attack. This strategy presumes that the structural modification which makes the antibiotic invulnerable to enzymatic modification will allow the chemically modified drug to retain its antimicrobial activities. However, certain structure-

activity relationships of ANT(2") parallel the antibiotic activity of its substrates. For example, the gentamicins are generally better antibiotics than the kanamycins (90). ANT(2")I modifies both types of aminoglycosides but has a higher V/K for more of the gentamicin substrates than for the kanamycins, and thus, greater resistance. Similarly, gentamicins containing the 6'- and 2'-amino groups are better substrates of ANT(2")I (see Table 10). The amino groups on aminoglycoside antibiotics also play an important role in antibiotic activity with compounds possessing both the 2'- and 6'-amino groups acting as the best antibiotics as shown below (11):

2',6'-diamino > 6'-amino > 2'-amino > no amino

This order is observed when the amino groups are replaced by hydroxyl groups or are acetylated. Antibiotics with maximal antimicrobial activity contain amino groups at the 6', 2', 1, 3, and 3" positions. Acylation of the 3-amino group and dimethylation of the 3"-amino substituent abolishes antimicrobial activity. However, antimicrobial activity is retained in the gentamicin C series where the 6' position is N- and C-methylated. Another important site of modification that leads to the retention of

antibiotic activity is the 1-amino group in ring II. Like the enzyme, antibiotic activity appears to have little, if any, dependence on hydroxyl groups.

Studies of AAC(3)I (160) and AAC(6')IV (118) also indicated similarities between enzymatic and antimicrobial structure-activity relationships. Resistant bacteria containing aminoglycoside acetyltransferases decrease antibiotic activity by acylation of the amino groups required for efficient binding of the antibiotic to the ribosome. Similarly, alkylation of the 6'-amino group appears to be the most promising type of modification that would reduce acetyltransferase activity and still retain some antibiotic potency. In the case of AAC(3)I, hydroxylation of the 4'-carbon also reduces enzyme activity (160), but hydroxyl groups are not important to antibiotic activity. However, the nucleotidylating enzymes, such as ANT(2")I, modify these non-essential hydroxyl groups. Although the amino groups necessary for antibiotic-ribosome binding are intact, the introduction of the bulky nucleotide group probably decreases binding of the modified aminoglycoside to the ribosome by stearic

hindrance. The present study shows that modifications at 5-OH and 1-N in ring II are very effective for reducing ANT(2")I activity, and, at least for the 1-amino group modifications, decreased enzyme activity is probably a result of stearic hindrance.

Because neither the 1-amino nor the 5-hydroxyl group is critical to antibiotic activity, modification of these postions with the goal of designing drugs resistant to enzymatic attack has been a strategy of medicinal chemists in recent years. The end result has been the development of potent, semi-synthetic antibiotics that retain the antimicrobial activity of their parent compounds (94). Moreover, these modifed aminoglycosides are not only effective against suseptible bacteria, but also against resistant strains containing ANT(2")I. However, chemical modifications of aminoglycosides have been considered in the context of activity as measured in the presence of excess antibiotic. Consequently, the researchers who designed these drugs did not target a specific kinetic parameter of this enzyme. The data in Table 19 show why netilmicin, amikacin, and the 5-epi-aminoglycosides act as better antimicrobial agents against ANT(2")-containing resistant strains.

Modification at the 5-OH position results in a substantial decrease in enzyme activity as shown by the low V/K of 5-episisomicin. An even more dramatic effect on enzyme activity occurs with modification of the 1-amino group as illustrated in the case of netilmicin. However, these structural modifications result in little, if any, change in Vmax values for ANT(2")I (see Table 10).

Radika and Northrop suggest that because the kinetic property that correlates with microbial resistance is V/K, structural modifications that reduce this kinetic constant are the most promising for combatting enzymatically-mediated resistance to aminoglycosides (118). Using the suspect radioactive assay and unstable enzyme, Bongaerts and Molendijk observed a correlation between V/K and antimicrobial activity for ANT(2")I produced by *E. coli* L58058.1; these researchers also note that there is no correlation between the substrate profile of ANT(2") tested with an excess of aminoglycoside and *in vitro* antibiotic activity (12). Data obtained in the present study also support the correlation of V/K with antimicrobial activity. The

chemical modifications resulting in netilmicin and 5-episisomicin affect V/K and are thus effective antibiotics against those resistant bacteria that contain ANT(2")I.

2. Nucleotide Substrate Specificity

All of the nucleotides listed in Table 9 are substrates of ANT(2"). The higher V/K values of the pyrimidine nucleotides show that these are better substrates than the purines with the pyrimidine deoxyribonucleotides being the best substrates of all. By assuming an average molecular weight of 27,000 and one active site per enzyme molecule, the V/K values range from 4.0×10^5 to 6.0×10^6 M^{-1} sec^{-1}.

I. KINETIC MECHANISM OF ANT(2")I

I.1 Alternative Substrate Kinetics

Because ANT(2") accepts such a large number of aminoglycoside and nucleotide substrates, it was an ideal candidate for application of the alternative substrate diagnostic developed by Radika and Northrop (115) (see Chapter I, section E.4e for a general description of the diagnostic). The aminoglycosides and nucleotides chosen as alternative substrates for ANT(2")I had significant differences in V/K to ensure that the kinetic differences

of the alternative substrate had the potential to be expressed in the kinetic constants of the varied substrate.

When the concentration of gentamicin C_1 was varied in the presence of saturating concentrations of Mg:ATP, Mg:dATP, or Mg:dGTP, intersecting lines were obtained (see Figure 20). Furthermore, the changes seen in the V/K of gentamicin C_1 are of the same magnitude as the differences among the V/K values of the alternative nucleotide substrates used in the diagnostic (see Table 9). These results are consistent with a sequential kinetic mechanism for ANT(2").

Variation of Mg:ATP in the presence of gentamicin A, C_1, or C_{1a} resulted in a pattern of two coincident and one intersecting lines (see Fig. 21). However, the differences among the Vmax and V/K values of Mg:ATP determined with gentamicin A, C_1, or Cla (see Table 10) are small relative to the differences in the V/K values of the alternative aminoglycosides used in the diagnostic, <u>i.e.,</u> 7- to 120-fold differences among the antibiotic V/K values are not expressed in the small change (2-fold at most) in the V/K of Mg:ATP. Therefore,

the pattern obtained when Mg:ATP is varied in the presence of alternative aminoglycosides are nearly coincident lines. These results, together with the pattern obtained with gentamicin C_1 as the varied substrate, are consistent with a Theorell-Chance kinetic mechanism for ANT(2")I in which Mg:ATP binds to the enzyme first, followed by the aminoglycoside; pyrophosphate and the nucleotidylated antibiotic are the first and second products released from the enzyme, respectively. Although central complexes must exist in the ANT(2")I-catalyzed reaction, catalysis is kinetically insignificant in the forward reaction, and the turnover rate is instead controlled by the slow release of the nucleotidylated aminoglycoside product. The proposed mechanism is illustrated in Fig. 40. The V/Ka of Mg:ATP is composed of the binding step of the nucleotide; V/Kb of the aminoglycoside consists of the steps of binding of the antibiotic, catalysis, and the release of pyrophosphate; and Vmax is composed solely of the release of the nucleotidylated aminoglycoside product; the steps that comprise V/Ka, V/Kb, and Vmax in the proposed mechanism are also illustrated in Figure 41. Additional data using alternative substrates were

Figure 40: Kinetic Mechanism of Aminoglycoside Nucleotidyltransferase 2"-I

MECHANISM OF AMINOCYCLITOL
NUCLEOTIDYLTRANSFERASE

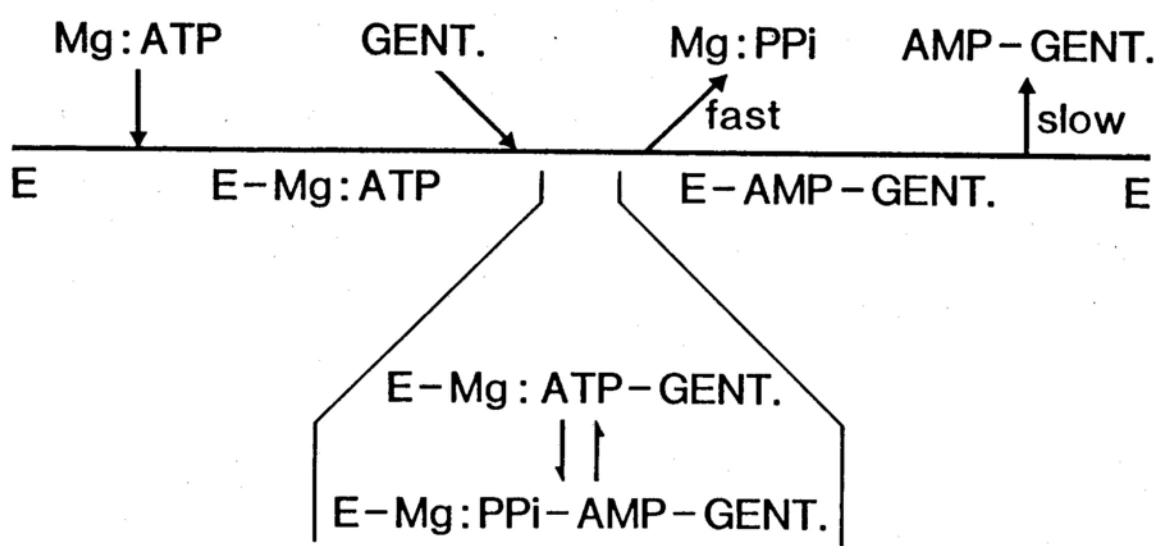
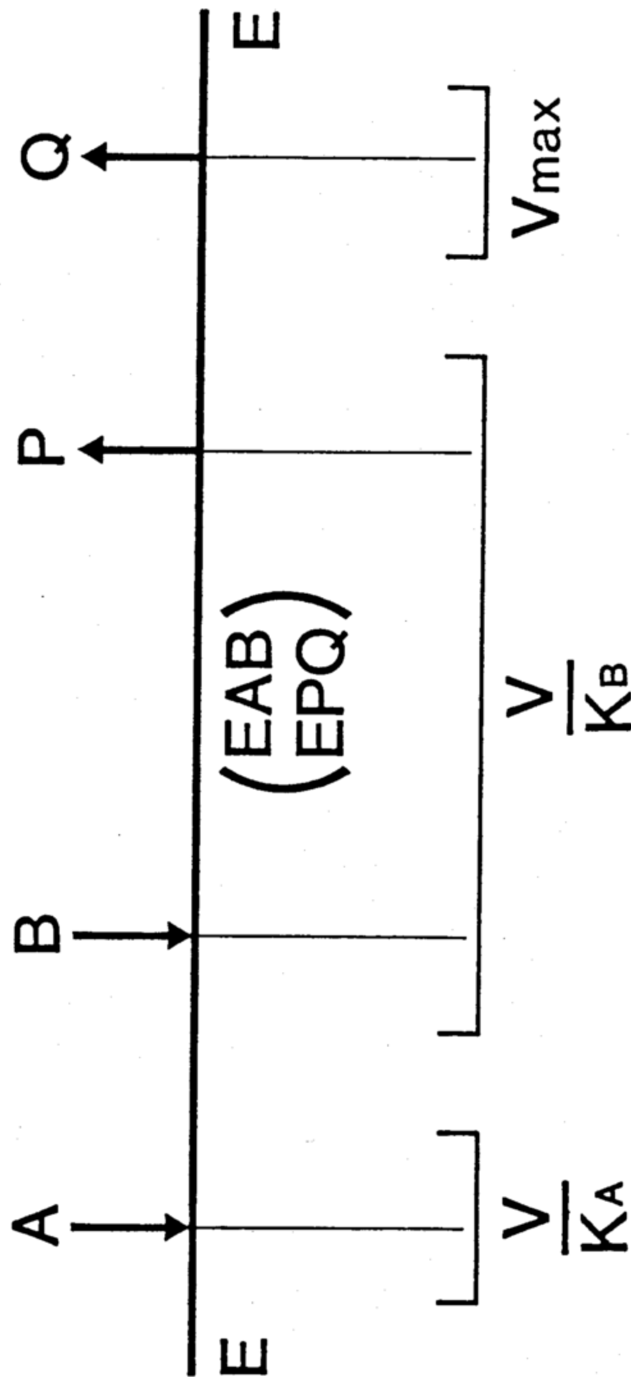


Figure 41: Definition of kinetic constants of a Theorell-Chance mechanism.



A
B
P
Q
E
E
(EAB)
(EPQ)
V/KA
V/KB
Vmax

obtained with the fluorometric assay and support this proposed mechanism (see Appendix A).

The diagnostic pattern of the Theorell-Chance mechanism is one of coincident lines when A is the varied substrate as a function of alternatives of B. Radika and Northrop note that this assumes that the structural change provided by B is carried into product P, and thus, the Vmax cannot be affected by structural change due to B, B', or B" (108). However, the antibiotic (B) is carried over into the structure of the nucleotidylated aminoglycoside, and the resulting products are Q, Q', or Q", depending on the identity of alternative substrate B. The release of Q may then vary due to different binding properties of the aminoglycoside moiety, and thus Vmax has the potential to change as a function of substrate B. This caveat explains why the Vmax value of Mg:ATP with gentamicin C1a as an alternative substrate is not exactly the same as the Vmax value when gentamicin A or C1 is the alternative substrate. Moreover, the large changes in V/K of the alternative antibiotics are not reflected in the Vmax values of Mg:ATP (see Table 13). The slight variation of the V/K values of the nucleotide as a

function of the identity of the aminoglycoside may arise from a degree of randomness in the reaction, but the reaction must be predominantly ordered since these small V/K variations represent only about 1 to 2% of the change seen in the V/K values of the alternative antibiotics.

I.2 Inhibition Kinetics

Because the Theorell-Chance kinetic mechanism is relatively uncommon, and the alternative substrate diagnostic of Radika and Northrop is newly developed, inhibition kinetics of ANT(2")I were examined with the intent of confirming the kinetic mechanism.

Uncompetitive substrate inhibition *versus* Mg:ATP is consistent with the aminoglycoside binding to the enzyme:AMP-antibiotic complex (EQ) since EQ is the only enzyme form isolated from the binding of Mg:ATP by irreversible steps. Moreover, inhibition by the aminoglycoside substrate suggests that the AMP-aminoglycoside product is released in an "ordered" fashion consisting of three steps: (1) the aminoglycoside portion of the product swings out of the active site; (2) a conformational change occurs which allows step (3), the release of AMP. The first step exposes the aminoglycoside binding site which allows the antibiotic

substrate to bind to EQ and trap the AMP-aminoglycoside product. The rate constants of this ordered release of Q must be such that the enzyme form following step 1 is the dominant one during turnover.

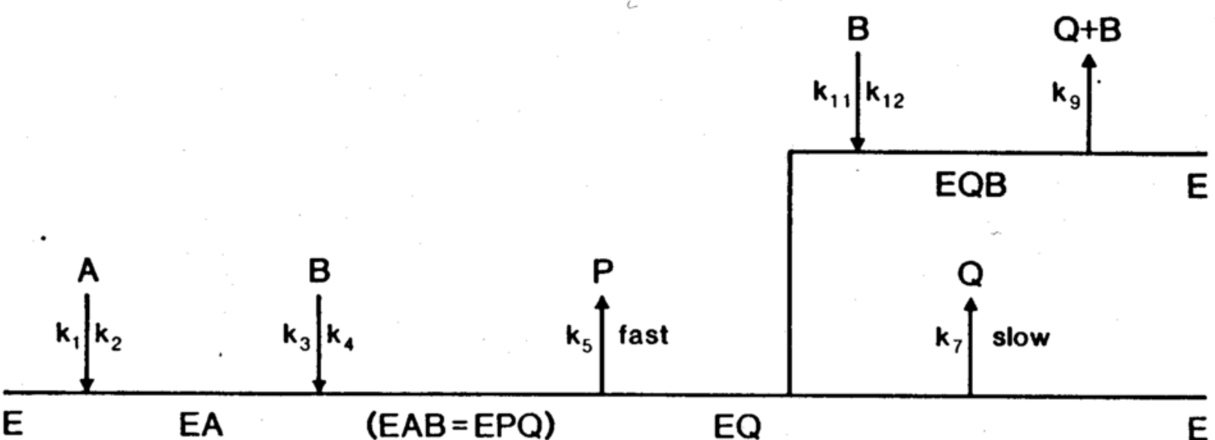
Partial inhibition requires a slower release of Q from EQB than from EQ. The mechanism of partial inhibition by the aminoglycoside substrates is illustrated in Figure 42 and is described by the rate equation below:

$$\frac{v}{E_t} = \frac{V\,AB}{KiaK_b + K_bA + K_aB + AB\,((1 + B/Kii)/(1 + B/Kid))} \qquad (18)$$

where A is Mg:ATP, B is the antibiotic, K_a and K_b are the Michaelis constants for the nucleotide and the aminoglycoside, respectively (see Appendix B for the derivations of this and the following rate equations). The binding of B to EQ was assumed to be in rapid equilibrium. That this is a valid assumption is supported by the effect of viscosity on the kinetic parameters of ANT(2")I (see Section I.5). Kii is a true dissociation constant of B from EQ and is defined as k_{12}/k_{11}, and the value of Kid, the inhibition constant that describes the alternative pathway of release of Q

Figure 42: Mechanism of substrate inhibition by aminoglycoside

SUBSTRATE INHIBITION



from EQB is k_7k_{12}/k_9k_{11}. The values of the Michaelis constants, K_a and K_b, Kia, V/K_a and V/K_b are the same as those of an Ordered BiBi mechanism (see Appendix B). For the latter kinetic mechanism, Vmax would be evaluated as $k_5k_7'/(k_5 + k_7'$ but for the Theorell-Chance mechanism of ANT(2")I, the ordered release of Q from the enzyme is solely rate-limiting, and Vmax reduces to k_7'. The constant k_7' is a net rate constant consisting of the three steps of the ordered release of Q (see above). The expression for the partial inhibition on the intercept can be extracted from equation 18:

$$1/V = 1/V\ ((1 + B/Kii)\ /\ (1 + B/Kid)) \qquad (19)$$

In contrast to tobramycin, the inhibitory effect of sisomicin on the intercepts appears to be linear. This may result from either the formation of an abortive EQB complexfrom which Q cannot escape or a value of Kid much greater than the concentrations of sisomicin used in the experiment. If sisomicin acts as a true dead end inhibitor or if the Kid is greater than the highest concentration of B, equation 19 becomes

$$1/V = 1/V + 1/V\ (B/Ki) \qquad (20)$$

The noncompetitive and uncompetitive inhibition patterns in Figures 25 and 26 show that neomycin C binds to two enzyme forms. The linear inhibition on the slope when gentamicin A is the variable substrate together with uncompetitive inhibition against Mg:ATP suggest that neomycin C binds to the enzyme:Mg:ATP (EA) complex. This supports the results of the alternative substrate diagnostic which predict that Mg:ATP binds to ANT(2") prior to the aminoglycoside. But, the presence of an intercept effect when gentamicin A was the varied substrate indicates that neomycin C is also binding to the enzyme:AMP-aminoglycoside complex (EQ). Moreover, partial (hyperbolic) inhibition by neomycin C (I) on Vmax indicates that Q escapes from the EQI complex, but again at a slower rate than from EQ. The mechanism of

dead-end inhibition is illustrated in Figure 43 and described by the following rate equation:

$$\frac{v}{Et} = \frac{V\,AB}{KiaK_b + K_bA(1+I/Kis) + K_aB + AB\,\frac{(1+I/Kii')}{(1+I/Kid')}} \qquad (20)$$

where the value of Kis is k_{14}/k_{13}, Kii' is k_{18}/k_{17}, and Kid' is equal to $k_7k_{18}/k_{15}k_{17}$. This equation describes the observed experimental results of linear slope and hyperbolic intercepts of non-competitive inhibition. The equation for the intercept effect is

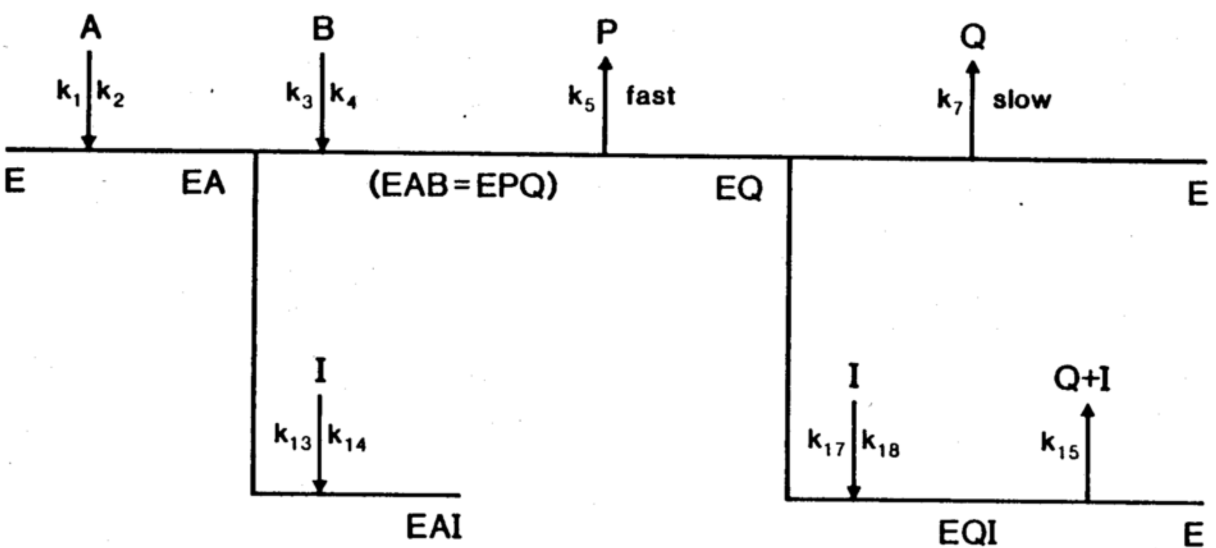
$$1/V = 1/V((1 + I/Kii')/(1 + I/Kid')) \qquad (21)$$

Like equation 19, a hyperbolic effect on the intercept is predicted by this equation, and this was observed for both uncompetitive and noncompetitive inhibition by neomycin C.

From the proposed mode of binding of the aminoglycoside inhibitor to EQ, it might be expected that neomycin C and tobramycin compete for the same binding site on the enzyme:AMP-aminoglycoside complex. To test for this potential competition, tobramycin was varied in

Figure 43: Mechanism of dead-end inhibition by neomycin C

DEAD END INHIBITION



the presence of neomycin C in an experimemt of multiple inhibition.

The pattern in Figure 27 is competitive but it is complicated by the presence of substrate inhibition. The linear slope effect (see inset, Fig. 27) shows that neomycin is competing with tobramycin for the EA complex. Moreover, slope effects of neomycin C determined with either tobramycin or gentamicin A as the variable substrate are not significantly different (see Kis values in Table 14). There appears to be little inhibitory effect by neomycin C on the intercept, but the presence of substrate inhibition precludes an accurate evaluation of intercept effects. However, the increase in the K_I values of tobramycin with increasing concentrations of neomycin C suggests that neomycin C and tobramycin are competing for binding to EQ. The K_I of tobramycin is dependent on the distribution of the EQ complex. Binding of neomycin C changes this distribution by drawing of EQ into another pathway, and thus the value of Ki of tobramycin becomes greater as there is less EQ available. Binding of the two inhibitors is mutually exclusive as

indicated by the fit to Eq. 7, i.e., either neomycin C or tobramycin can bind to EQ, but not both.

The mechanism for multiple inhibition by tobramycin and neomycin C is illustrated in Figure 44. Although a hyperbolic intercept effect was not observed in the multiple inhibition pattern, partial inhibition was inferred from the patterns of the inhibitors acting alone (e.g. Figures 23, 25, and 26). At high tobramycin concentrations, neomycin C behaves as an activator instead of an inhibitor as illustrated in Figure 45. "Activation" by an inhibitor can only occur if inhibition is partial and diverts the enzyme from a slower to a faster pathway; activation by neomycin arises because the rate release of Q from EQI is faster than from EQB. If both inhibitions were not partial, multiple inhibition would result in the pattern illustrated in Figure 46 in which the ascending lines in never cross the y-axis nor each other. In contrast to this, Figure 47 illustrates the enlarged intercept region of the multiple inhibition pattern and shows that the curved lines cross over one another; these cross overs may arise from the superimposition of the two partial inhibition pathways.

Figure 44: Mechanism of multiple inhibition by neomycin C and tobramycin

COMBINED SUBSTRATE AND DEAD END INHIBITION

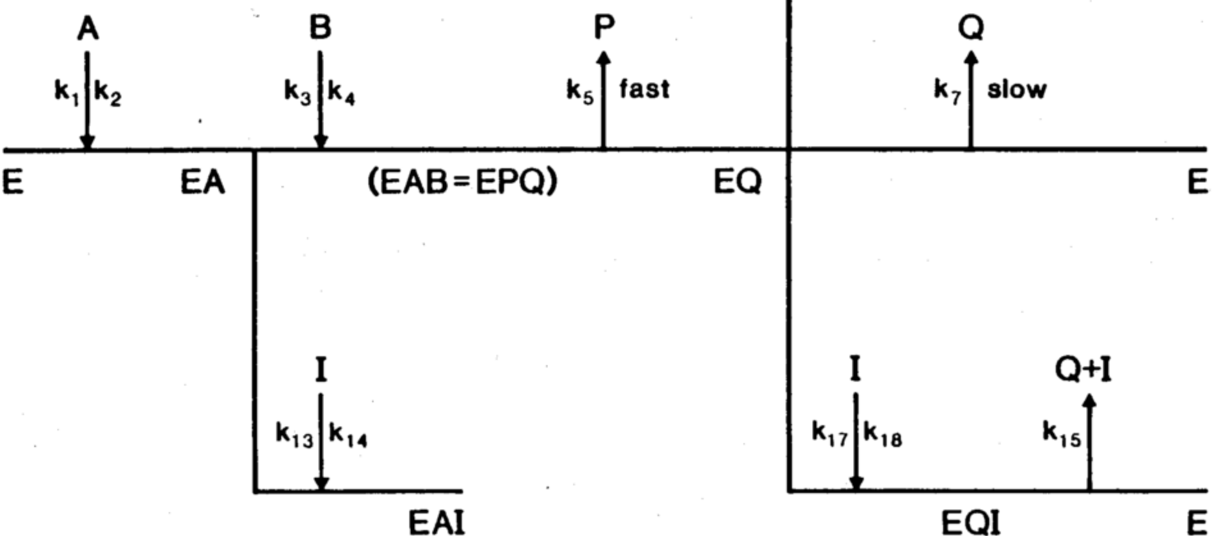


Figure 45: Apparent activation of ANT(2")I activity by neomycin C in the presence of high concentrations of tobramycin.

Each solid line represents the data fit to Equation 10. The concentrations of tobramycin were 0.61 mM (■), 0.47 mM (●), and 0.33 mM (▲).

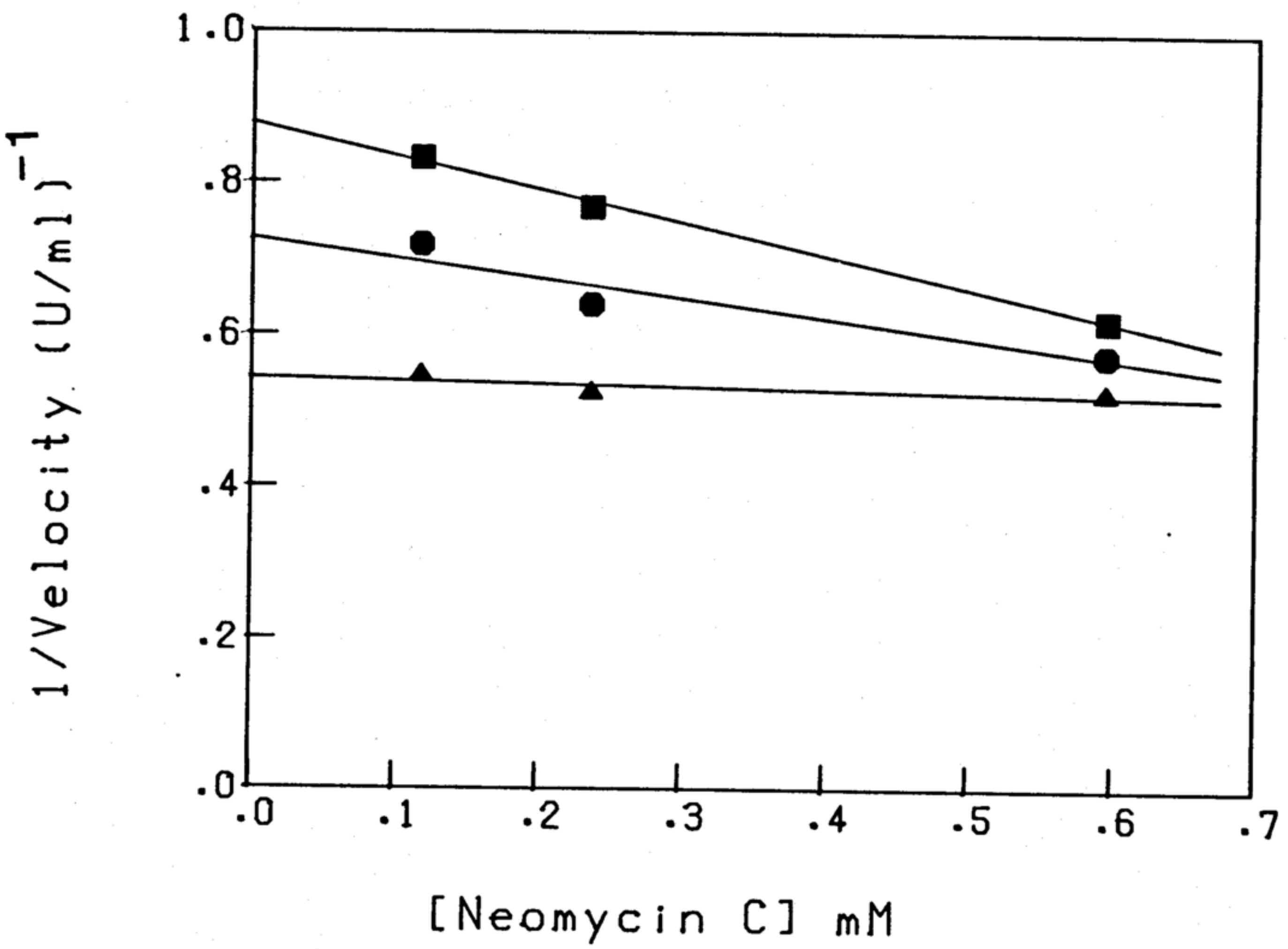
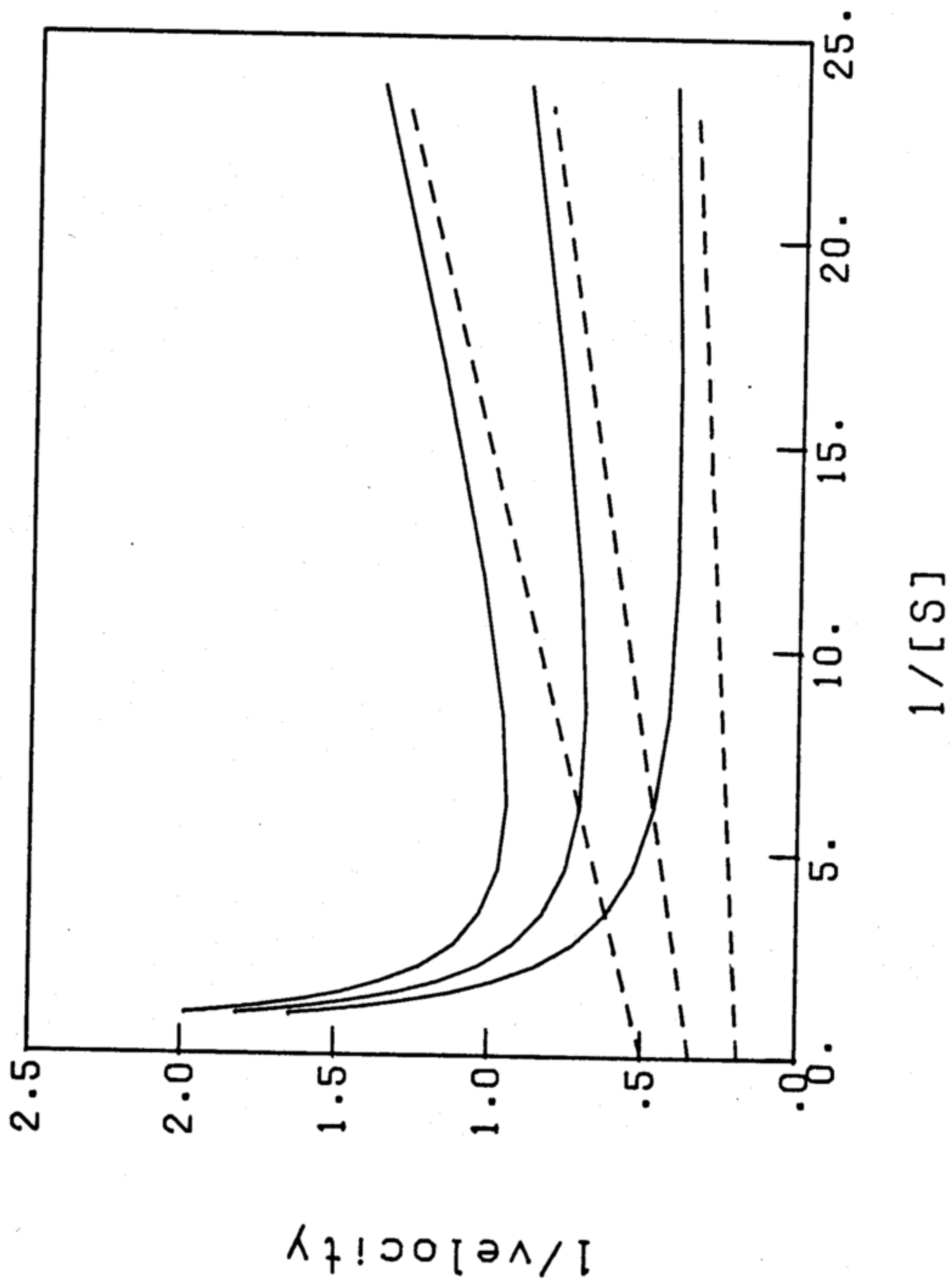
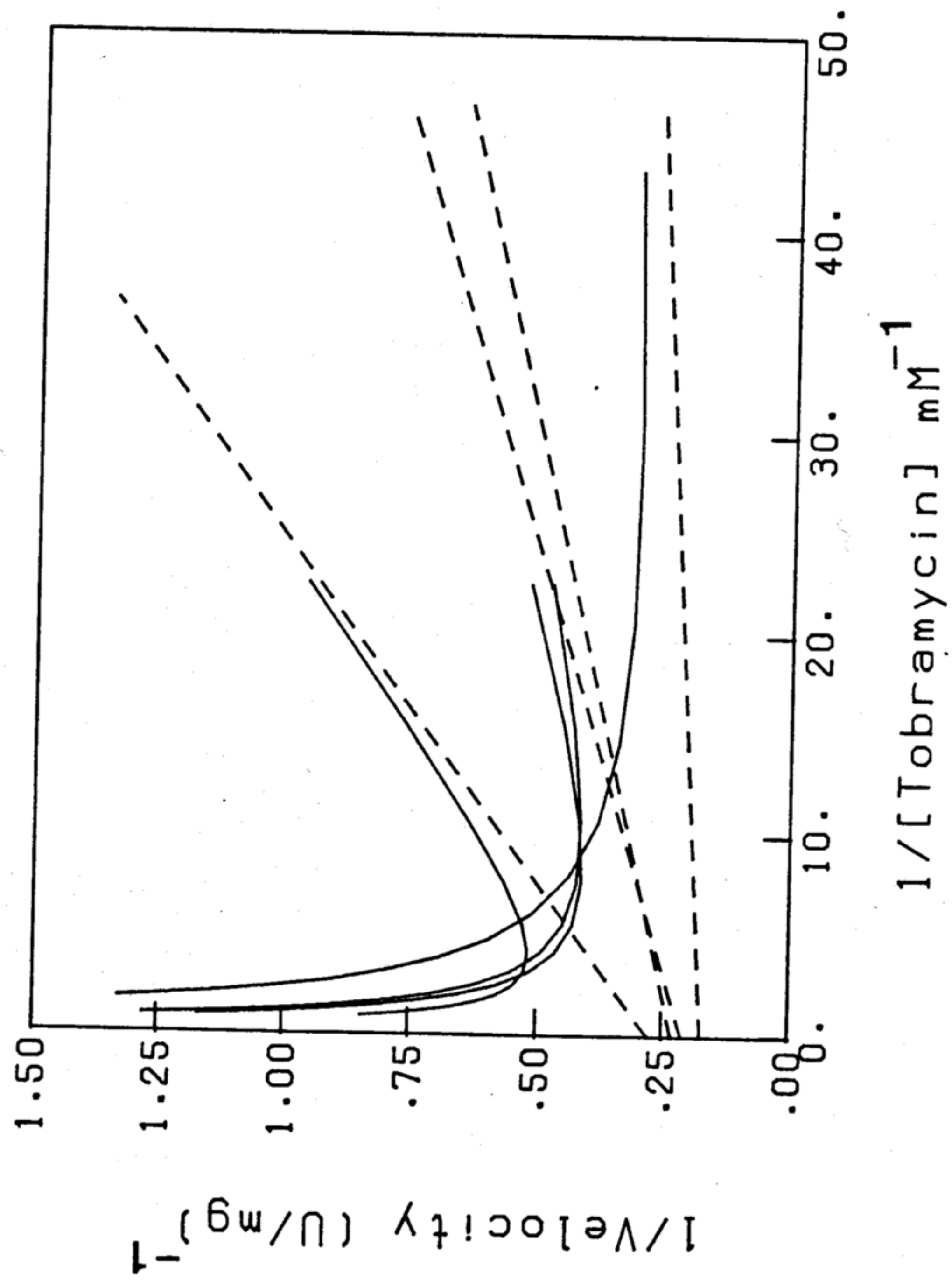

1.0
.8
.6
.4
.2
.0
.0
.1
.2
.3
.4
.5
.6
.7
1/Velocity (U/ml)$^{-1}$
[Neomycin C] mM

Figure 46: Inhibition pattern for linear, mutually exclusive, multiple inhibition.



1/velocity
1/[S]
.0
.5
1.0
1.5
2.0
2.5
0.
5.
10.
15.
20.
25.

Figure 47: Intercept region of multiple inhibition pattern in Figure 27.


1/Velocity (U/mg)$^{-1}$
1.50
1.25
1.00
.75
.50
.25
.00
0.
10.
20.
30.
40.
50.
1/[Tobramycin] mM^{-1}

Equation 23 describes the partial multiple inhibition mechanism:

$$\frac{v}{Et} = \frac{V\,AB}{K_{ia}K_b + K_bA(1+I/K_{is}) + K_aB + AB\dfrac{(1+B/K_{ii}+I/K_{ii}')}{(1+B/K_{id}+I/K_{id}')}} \qquad (23)$$

where Kis, Kii, Kii', Kid, and Kid' have the same values described above.

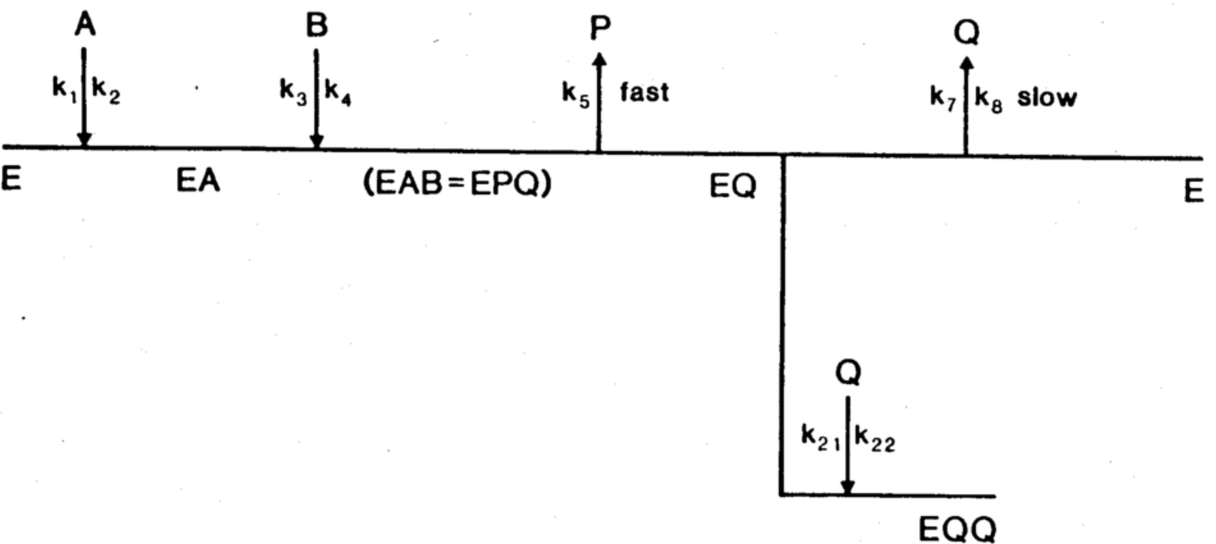
For Ordered BiBi and Theorell-Chance kinetic mechanisms, product inhibition by Q is competitive when A is the variable substrate since Q and A are competing for free enzyme (E). When B is the variable substrate, Q is a non-competitive inhibitor since B and Q bind to different enzyme forms (25). However, for ANT(2")I, noncompetitive inhibition by Q against A shows that AMP-tobramycin (Q) is not only competing with Mg:ATP (A) for free enzyme, but engages in secondary binding to another enzyme complex. The slight inhibition on the slope (Figure 29, inset a) suggests that only a small proportion of free enzyme is available for binding of Q, even at concentrations of Mg:ATP equal to its Km. The uncompetitive inhibition by AMP-tobramycin against tobramycin, B, is consistent with Q binding to the EQ

complex which is isolated by irreversible steps from the addition of B. Moreover, the increase in the Ki of tobramycin inhibition with increasing AMP-tobramycin concentration indicates AMP-tobramycin is competing with tobramycin for secondary binding. This mode of inhibition probably results from binding of the antibiotic portion of the AMP-tobramycin molecule to the exposed aminoglycoside binding site of the EQ complex. Compared to the inhibition constant of free tobramycin, the high Kii value of AMP-tobramycin suggests that the nucleotide portion of the product hinders secondary binding of Q to the EQ complex (see Table 14). Furthermore, the lack of partial inhibition indicates that the nucleotidylated antibiotic prevents escape of Q from the EQQ complex, unlike free aminoglycoside inhibitors. The mechanism of mixed product and dead-end inhibition by AMP-tobramycin is illustrated in Figure 48. The rate equation that describes this inhibition is given below:

$$\frac{v}{Et} = \frac{V\ AB}{KiaK_b + K_aB(1 + Q/Kiq) + K_bA + AB(1 + Q/Kiq')} \qquad (24)$$

Figure 48: Mechanism of combined product and dead-end inhibition by AMP-tobramycin

MIXED PRODUCT AND DEAD END INHIBITION



where Kiq is k_8/k_7 and Kiq' is k_{22}/k_{21}.

The foregoing discussion of the inhibition kinetics of ANT(2")I has presumed that the inhibitory effects on Vmax arise from the inhibitor binding to the EQ complex. The classic test for this type of binding is enhancement of inhibition by the addition of Q which increases the amount of EQ available to the inhibitor. For example, addition of $NADP^+$ enhances substrate inhibition by alpha-ketoglutarate by increasing the steady-state level of the enzyme:$NADP^+$ (EQ) complex for the reaction catalyzed by glutamate dehydrogenase (122). However, in the case of isocitrate dehydrogenase, the addition of $NADP^+$ resulted in an absence of enhancement of inhibition by alpha-ketoglutarate, indicating that alpha-ketoglutarate was actually binding to the central complex and not to EQ (102).

For ANT(2")I, addition of Q does not result in enhancement of substrate inhibition by B. This might argue that B, I, or Q binds to the central complex rather than to EQ and that the irreversible step isolating the complex occurs in catalysis. However, most nucleotidyltransferases catalyze reversible reactions

with equilibrium constants of 0.25 to 2.0 for the production of pyrophosphate and the nucleotidylated product (9, 155). Therefore, it is unlikely that catalysis is irreversible for ANT(2"), but the reverse reaction has never been demonstrated. Instead, the irreversible step is the release of pyrophosphate, and EQ is only the enzyme complex isolated from the rest of the enzyme forms by this irreversible step.

But, how can binding of the inhibitor to the EQ complex be accomodated with the lack of enhancement of inhibition by addition of AMP-tobramycin? This situation could result if ANT(2")I exists predominantly as the enzyme:AMP-tobramycin complex during steady-state turnover. Thus, the presence of excess Q does not affect the distribution of the EQ complex. If AMP-tobramycin did increase the steady-state concentration of EQ, a parabolic intercept effect for mixed product and dead-end inhibition should have resulted as more of the latter enzyme complex became available for Q to bind and form the abortive complex (25). However, the linear intercepts indicate that a very high proportion of ANT(2")I exists as the enzyme:AMP-aminoglycoside complex during steady-state turnover because of the rate-limiting

release of the nucleotidylated antibiotic.

Because the distribution of the enzyme:AMP-aminoglycoside complex changes very little, if at all, during turnover, the values of Kii for B, I, and Q are true dissociation constants, and the inhibitor-EQ binding is therefore a rapid equilibrium segment. This was the basis for the V/K_b and Ki comparisons of the structure-activity relationships discussed previously.

In summary, the inhibition data are highly supportive of the Theorell-Chance kinetic mechanism in which the turnover rate is controlled by the release of the second product, and Vmax is isolated from V/K_a and V/K_b by irreversible steps. The results of the inhibition experiments also show that an ordered release of the nucleotidylated aminoglycoside product occurs with the antibiotic portion of the molecule being released prior to the nucleotide.

I.3 Non-Equilibrium Isotope Exchange Between Pyrophosphate and Mg:ATP

Most isotope exchange studies of sequential mechanisms are performed at chemical equilibrium in the presence of all substrates and products of the enzyme-

catalyzed reaction. An alternative approach measures exchange from a product back into a substrate while catalytic turnover occurs in the forward direction. If a significant amount of the EQ complex is present, this reverse exchange is fast relative to the rate of the reaction being catalyzed in the forward direction (25). Since ANT(2")I exists mostly as EQ during steady-state turnover, rapid exchange from pyrophosphate back into ATP was expected. However, no exchange between pyrophosphate and nucleotide was observed, even after prolonged incubation with the enzyme and aminoglycoside.

One possibility for the lack of exchange is that the net rate constnat for catalysis in the reverse reaction is quite low. However, without kinetic data on the reverse reaction and evaluation of the Haldane relationships, equilibria between the central complexes or between EA and EQ remain unknown.

I.4 Effect of pH-Variation on Kinetic Parameters

Enzyme activity as a function of pH has been reported for a few aminoglycoside-modifying enzymes and then only in the presence of excess substrates. The activity of purified AAC(3)I shows a broad pH optimum from pH 5.5 to 8.5 with a peak of activity at pH 8.0

(161). Another acetylating enzyme, AAC(6')IV, displays a sharp peak of activity at pH 5.5. On the acid side, no activity is detected at pH 4.0, but, on the alkaline side ,there is a broad plateau between pH 6.5 and 8, and then a gradual decrease in activity as the pH becomes more alkaline (117). Van Pelt and Northrop reported that the activity profile of ANT(2")I has a sharp peak with an unusually high optimum of pH 9.5. They postulated that the high pH optimum was reflective of the tight binding of the AMP-aminoglycoside product and its subsequent slow release from the enzyme. They suggested that as the amino groups of the AMP-aminoglycoside product became deprotonated at higher pH, the product is released more rapidly from the enzyme (150). The present study separates the pH-activity profile of ANT(2") into its kinetic components, namely Vmax, V/K, and Ki of substrate inhibition. This is the first report of specific pH effects on the kinetic parameters of an aminoglycoside-modifying enzyme.

From the bell-shaped curve of Ki of sisomicin as a function of pH, it is apparent that at least four ionizable groups are required for binding as shown by the

asymptotes of slopes +2 and -2 (Fig. 34). The very similar profile and values of pK_a and pK_b for V/K indicate that only the ionizable groups required for binding of sisomicin to the enzyme are expressed in the pH dependence of catalysis. In addition, the similarlity of the two profiles indicates that sisomicin is not a particularly sticky[1] substrate since the pK values of the V/K profile are not displaced relative to those of the pKi profile (24). The probable identity of the ionizable groups expressed in V/K and Ki can be deduced by the values of the ionization constants of the substrate, sisomicin.

The structure-activity relationships for ANT(2")I (see section IV.H) clearly demonstrate that amino groups are crucial for binding of the aminoglycoside to the enzyme. It follows that the ionization states of the primary and secondary amino groups of sisomicin should be reflected in the pH profiles of the kinetic constants. Indeed, the two pK_b values of 8.2 to 8.4 found in each profile (Table 15) fall within the range of the

[1] A sticky substrate is one that reacts more rapidly than it dissociates from the enzyme.

ionization constants of the amino groups of sisomicin shown in Table 20. This suggests that two ionized amino groups on the aminoglycoside substrate are required for binding to the enzyme, but the identity of the two is uncertain. The amino groups required for binding might be identified by examining the effect of pH on the kinetic paramters of substrates lacking the 6'-, 2'-, or 3"-amino groups.

The two pK_a values of 6.7 calculated from both profiles clearly are lower than any amino group pK of the substrate. It is therefore likely that these two groups belong to the enzyme. Although the identity of the ligands on enzyme must be confirmed by other types of experiments, such as solvent perturbation or temperature dependence of the pK_a values, they are probably carboxyl groups. The pK values of carboxyl groups generally range from 3 to 5, but values as high as 7 are attained if the carboxyl is buried in a hydrophobic part of the active site (23).

As noted above, the pKi profile reflects the pH dependence of binding of the aminoglycoside to the enzyme:AMP-aminoglycoside (EQ) complex. The similarity

TABLE 20

IONIZATION CONSTANTS OF AMINO GROUPS FOR SISOMICIN[a]

Position of amino group	Position of beta-carbon[b]	Calculated pK[c]
6'	5'	9.80
	4'	9.83
2'	3'	8.37
	1'	8.94
1	6	7.80
	2	7.88
3	2	7.88
	4	8.80
3"	4"	8.82
	2"	8.88
	7"	8.79

[a] Raw data in the form of ^{13}C NMR chemical shifts as a function of pH were obtained from Dr. T.L. Nagabhushan, Schering Corp. (see reference 97). [b] Protonation of amino groups in amino sugars causes upfield shifts for carbons in a beta position relative to the methine bearing the amino moiety (see ref. 98) Therefore, the positions of the carbons beta to the designated amino groups are reported here. [c] The values in this column were derived from fitting the titration data of each beta carbon's chemical shift to Eq.16 ; the true ionization constant of each amino group falls within the range of the values.

of this profile with the V/K profile suggests that the antibiotic binds to EQ and the enzyme:Mg:ATP (EA) complex in the same manner, i.e, the same ionized and protonated groups are required for binding to either enzyme form.

In contrast to the V/K and pKi profiles, the Vmax versus pH plot was a very shallow line with a slope of 0.32 or possibly a shallow wave. Vmax clearly has different ionization requirements than V/K or Ki, but the nature of the ionizable groups that contribute to Vmax, and thus to the release of the nucleotidylated antibiotic, is less apparent. It might be argued that the shallow wave of the Vmax profile (Fig. 36) is generated by a series of wave functions arising from multiple amino groups on the nucleotidylated aminoglycoside. Profiles of waves result when protonation or deprotonation decreases but does not eliminate activity (24). If the protonated amino groups of the product bind more tightly to the enzyme, but deprotonation does not abolish binding, the resulting profile will be a wave that increases on the alkaline side of the pK. Alternatively, the pH dependence of Vmax may be expressive of the release of the nucleotide portion of the AMP-aminoglycoside product. The pK of 8.4 is much higher than

the ionization constant (3.7) for the primary amino group of AMP or the secondary phosphate group (6.1) profile. It is possible that the pK of 8.4 arises from a a lysine amino group on the enzyme required to bind the nucleotide phosphate.

The expression of the release of nucleotide rather than the aminoglycoside portion of the product in the pH dependence of Vmax is consistent with the results of the substrate inhibition experiments (see section IV.I.2) which suggest an ordered release of the AMP-aminoglycoside in which with the aminoglycoside portion of the product is released prior to the nucleotide. However, if turnover was controlled solely by the release of the AMP part of the product, then the Vmax values of different aminoglycosides should remain the same. The fact that Vmax does change (see Table 10) suggests that the antibiotic portion of Q must make a contribution to the net rate constant for the release of the nucleotidylated antibiotic.

It is also possible that the Vmax profile arises from a pH-dependent conformational change of the enzyme. This may be the result of titration of groups remote from

the active site. Although the pK values of aminoglycoside do not appear to be expressed in the pH dependence of Vmax, the identity of the antibiotic may influence this putative conformational change. The presence of such a conformational change in Vmax may be detected by examining the pH-dependence of this kinetic constant as a function of tempertaure. The temperature coefficients of the ionizations for conformational changes are much greater than those of simple titrations, and thus, the two processes may be distinguished.

The Vmax, V/K and pKi profiles are unusual, but they are consistent with the proposed Theorell-Chance mechanism for ANT(2")I illustrated in Figure 40. The lack of expression of ionization requirements of V/Kb within the Vmax profile demonstrates that Vmax is independent from catalysis. The similarities of the pH-dependence of V/K and Ki indicate that most of the enzyme exists as EQ during turnover, <u>i.e.</u>, Ki is a true equilibrium constant.

One problem that sometimes occurs with pH-variation studies is a change in the kinetic mechanism of the enzyme as a result of pH-variation. However, this can be ruled out for ANT(2")I. The data supporting the

Theorell-Chance mechanism were obtained at pH 9.1. The mechanism probably becomesmore extreme at lower pH values where the release of AMP-aminoglycoside is even more rate-limiting and where catalysis proceeds at higher rates than at alkaline pH. The presence of substrate inhibition throughout the pH range also supports the constance of the mechanism since substrate inhibition will only occur if there is a sufficient amount of EQ available for the binding of the aminoglycoside.

I.5 Variation of Kinetic Parameters of ANT(2") with Viscosity

The extent of diffusion control in an enzymatic or nonenzymatic reaction can be measured directly by investigation of the observed reaction rate with increasing concentrations of a viscogenic substance (19, 20, 99). Kirsch and co-workers evaluated the diffusion-controlled components of chymotrypsin and beta-lactamase I by investigating the effect of viscosity on the kinetic parameters of these enzymes (14, 59). They found that the substrates with V/K values approaching diffusion-controlled limits ($> 10^7$ M^{-1} sec^{-1}) were more sensitive to changes in solution viscosity than poor substrates of

the enzymes. They were able to evaluate the rate constant of association of the substrate with the enzyme using this technique. By adapting Kirsch's procedure to the variation of isotope effects with viscosity for the reaction catalyzed by $NADP^+$ malic enzyme, Grissom and Cleland were able to dissect the forward commitment to catalysis into its internal and external components; the latter includes the partition ratios for substrate dissociation and thus is affected by changes in viscosity (56).

For the proposed Theorell-Chance mechanism, the release of the nucleotidylated aminoglycoside product is expected to be limited by diffusion. In contrast, the Vmax for a kinetic mechanism in which the the rate is not limited by product release should be relatively insensitive to the effects of viscosity. However, a caveat of this procedure is the possibility of general effects of a viscogenic reagent on enzyme activity. A substrate whose V/K value is very low and thus far below diffusion-controlled limits should be insensitive to viscosity and can be used as a control for gross changes induced in the protein structure by the viscosogen (14, 59). In the present study, netilmicin, the substrate

with the lowest V/K of any of the aminoglycosides (see Table 10), served as a control for non-specific effects on ANT(2")I due to glycerol. The lack of effect on V/K of netilmicin (see Table 16) suggests that glycerol does not result in non-specific effects on ANT(2") activity.

The proportional decreases in V/K of sisomicin and gentamicin C_1 with a three-fold increase in viscosity (see Table 16) demonstrates the presence of diffusion-controlled components. The absolute magnitudes of the V/Kb values are unknown due to the uncertainty of the true molecular weight of ANT(2")I and the number of active sites per molecule, and thus the value for the association rate constant cannot be accurately evaluated. However, the V/K for sisomicin is estimated[2] to be 2.5 x 10^5 M^{-1} sec^{-1} and for gentamicin C_1, 1.1 x 10^5 M^{-1} sec^{-1} (calculated from values in Table 10). These are not particularly high rates and are well below the proposed 10^7 M^{-1} sec^{-1} limit (141). These values suggest that although catalysis is not rate-limiting, neither does it appear to be extraordinarily fast in comparison with

[2] Estimated assuming a molecular weight for ANT(2") of 27,000 and one active site per molecule.

some other enzymes. However, the upper limit of V/K is $2.7 \times 10^6\ M^{-1}\ sec^{-1}$ for gentamicin Cla, a value that approaches diffusion control. In addition, all the V/K measurements were performed at pH 9.1 and may be as much as an order of magnitude higher at neutral pH.

The large decrease in Vmax of gentamicin C_1 and sisomicin is consistent with diffusion-limited release of the nucleotidylated product.However, Vmax decreases in excess of the inverse proportionality predicted by the Stokes-Einstein equation (44).

In contrast to gentamicin C_1 and sisomicin, Vmax of netilmicin decreased proportionately with viscosity. That there was any effect at all on Vmax was surprising since netilmicin has a very low V/K value which might be expected to shift the rate-limiting segment of the reaction from release of product to catalysis. The observed result suggests that although catalysis is drastically reduced, the Theorell-Chance mechanism of ANT(2") is still maintained. The insensitivity of the substrate inhibition constants of all three substrates to changes in viscosity provides additional evidence that ANT(2")I exists predominantly as the EQ complex during turnover. Because increased viscosity decreases the

rate of release of the nucleotidylated product, it would be expected that an increased distribution of the EQ complex would occur if the mechanism were simply Ordered BiBi, and enhanced substrate inhibition would be observed. The absence of any change on Ki also shows that glycerol is not binding in an inhibitory fashion to the EQ complex since competition between glycerol and the aminoglycoside would result in increasing the value of Ki. The results are also consistent with the Ki of the substrate as a true equilibrium binding constant since such an equilibrium process should be unaffected by changes in viscosity. Finally, changes in viscosity may perturb the ionization constants of ligands on the substrate and enzyme (59). If viscosity did perturb the pK values of the antibiotic amino groups and the enzyme ligands, these would also be expressed as changes in Ki.

In summary, the data obtained from investigating the effects of viscosity on the kinetic parameters are highly supportive of the proposed Theorell-Chance kinetic mechanism of ANT(2")I and demonstrate that variation of kinetic constants with viscosity may serve as a useful diagnostic tool for kinetic mechanism.

J. SIGNIFICANCE OF THE THEORELL-CHANCE KINETIC MECHANISM OF ANT(2")I

In 1951, Theorell and Chance proposed the Theorell-Chance mechanism to explain the kinetics of horse liver alcohol dehydrogenase (144). At this time, the reaction was perceived to be so extreme that hydride transfer was thought to occur independently of any central complex. However, it has since been demonstrated that central complexes exist in the alcohol dehydrogenase-catalyzed reaction and that although the reaction is predominantly ordered, there is some degree of randomness of binding (165, 166). The release of NADH is rate-limiting with primary alcohols which are good substrates of the enzyme, but catlysis becomes rate-limiting with poorer substrates such as secondary alcohols. In contrast to primary alcohols, secondary alcohols do not display substrate inhibition because of insufficient levels of EQ during the steady-state turnover (33).

Other enzymes have been reported to possess kinetic mechanisms that follow or closely approximate the Theorell-Chance mechanism. Rabbit muscle lactate dehydrogenase exhibits a kinetic mechanism similar to that of alcohol dehydrogenase (169). Purine nucleoside

phosphorylase follows a Theorell-Chance mechanism (83, 84). A detailed analysis of choline acetyltransferase using dead-end inhibition kinetics and equilibrium isotope exchange revealed that although this enzyme follows a predominantly ordered, Theorell-Chance mechanism, random binding of substrates and products can occur in an alternative pathway (62, 63). Huang and co-workers reported a complex hybrid mechanism consisting of a two-site ping pong segment and a Theorell-Chance sequential segment for asparagine synthetase (91). When viologen dyes are the reducing substrate, nitrate reductase follows a Theorell-Chance mechanism (95). However, many claims of the Theorell-Chance mechanism have been based solely on product inhibition which fails to distinguish between a Theorell-Chance mechanism and a random sequential mechanism with two dead end complexes (26).

The kinetic mechanism of ANT(2")I is not without precendent, but it has its own unique features that distinguish it from other enzymes. Uncompetitive substrate inhibition suggests that the mechanism of ANT(2") is more ordered than liver alcohol dehydrogenase.

For the latter enzyme, inhibition by primary alcohols is noncompetitive with hyperbolic intercepts and slopes. The hyperbolic intercept effect arises from the slower rate of NADH (Q) release from the EQB complex as opposed to the EQ complex. The hyperbolic slope effect results from release of NADH from the EQB complex, leaving enzyme:alcohol to which NAD^+ can now bind and form a catalytically competent complex (EAB). The partial slope effect arises because NAD^+ binds with free enzyme more readily than with E:alcohol (33). In contrast, no slope effect is observed for substrate inhibition of the ANT(2")I-catalyzed reaction even though release of the nucleotidylated aminoglycoside (Q) also may result in an EB complex. For ANT(2")I, the presence of the nucleotide triphosphate on the enzyme prior to the aminoglycoside appears to be required for formation of a catalytically competent species.

The estimated turnover rates of ANT(2") range from 2 to 6 turnovers per second. This is more than an order of magnitude less than the estimated turnover rates of purine nucleoside phosphorylase (83) and as much as 80-fold less than the turnover numbers reported for nitrate reductase (95), both of which are claimed to follow the

Therell-Chance mechanism. The turnover rate of a Theorell-Chance mechanism is represented by the rate-limiting release of the second product. Therefore, it appears that the mechanism of ANT(2")I may be more extreme.

Product inhibition is frequently used as a diagnostic for the Theorell-Chance mechanism. However, this is not a viable technique for ANT(2")I in the presence of the UDP-glucose pyrophosphorlase coupling enzyme. Another diagnostic for a Theorell-Chance mechanism evaluates the ratio of the sum of the vertical coordinates of the crossover points of the initial velocity patterns in the forward and reverse reactions to the sum of the reciprocals of Vmax in both directions. If an enzyme possesses a Theorell-Chance mechanism, this ratio will be equal to 0 (68). However, in addition to kinetic data from the reverse reaction, this technique requires extremely accurate kinetic data. The presence of substrate inhibition by the majority of the aminoglycoside substrates in the forward reaction precludes accurate evaluation of the crossover coordinate from an initial velocity pattern.

The alternative substrate diagnostic provides a powerful alternative to the techniques discussed above for determination of the kinetic mechanism of ANT(2") and avoids the ambiguity of product inhibition kinetics and the complication of initial velocity patterns by substrate inhibition. Although Morpeth and Boxer did not apply the alternative substrate diagnostic of Radika and Northrop *per se*, they used alternative substrates for both reductant and electron acceptor in their study of nitrate reductase. By recognizing the significance of lack of change in Vmax and V/K of the electron acceptor as a function of alternative electron donator, they concluded that nitrate reductase follows a Theorell-Chance mechanism in which there is an ordered addition of substrates, nitrate followed by the reduced viologen, and turnover is controlled by the release of nitrite (95). Moreover, this was the sole basis for their conclusion that nitrate reductase follows a Theorell-Chance mechanism with viologen dyes as the reducing substrate.

The kinetic mechanism of ANT(2")I has fundamental significance to understanding antibiotic resistance. Specifically, the Theorell-Chance raises the question as to how the enzyme continues to confer resistance during

multiple turnovers since the nucleotidylated aminoglycoside appears to be a self-generated, tight binding inhibitor of ANT(2")I. However, inhibition by the AMP-aminoglycoside affects Vmax alone. Therefore, resistance to aminoglycosides should continue only if resistance due to ANT(2")I correlates to another kinetic parameter of the enzyme. From the correlation coefficients presented in Table 18, it is clear that antimicrobial resistance does not correlate to Vmax or to Ki but does correlate with V/K. The apparent correlation between Km and M.I.C. may be fortuitous due to the kinetic mechanism of ANT(2")I. The Michaelis constant for the aminoglycoside substrate is the ratio of the totally separate kinetic parameters, Vmax and V/K. This ratio, Km, will reflect changes in the V/K values alone since the Vmax values show little variation. These data agree with the correlation of V/K and antimicrobial resistance conferred by AAC(3)I (160), AAC(6')IV (116) , and ANT(2")I (12). It is likely that a more significant correlation of V/K and resistance due to ANT(2")I would be obtained using V/K values determined at a pH value around neutrality which is thought to be the

physiological pH of the bacterial cell.

K. CONCLUSIONS AND DIRECTIONS FOR THE FUTURE

Optimization of assay conditions, pH, buffer, and steps in the early stages of purification have resulted in improved yields, greater stability, and higher specific activity of the enzyme. The separation of the two forms of ANT(2")I has been accomplished through the use of bisoxirane-linked aminoglycoside Sepharose resins. The two forms can be distinguished by electrophoretic properties, but they do not appear to be kinetically distinct from each other. This suggests that post-translational modification of a common protein occurs. The nature of this modification should be investigated to see if the differences of the two forms are due to the presence of a conjugated group, such as a sugar or lipid; alternatively, a small peptide may be cleaved from one form. The molecular weight of the two forms should be established by analytical gel filtration and sedimentation analysis. An accurate molecular weight is crucial for accurate evaluation of the turnover number and V/K values of the substrates.

The enzyme uses a large number of nucleotide triphosphates and aminoglycosides as substrates. Optimal

activity occurs with gentamicins containing primary amino groups at the 6' and 2' postitions. Modifications in ring I affect binding, whereas alkylation at the 1-amino group in ring II results in drastic reductions in catalysis. The structural requirements of the aminoglycoside antibiotics are similar for enzyme and antimicrobial activity with the exception of 1-amino and 5-hydroxyl modifications which decrease enzyme activity but not antibiotic efficacy.

Alternative substrate kinetics show that ANT(2") follows a Theorell-Chance kinetic mechanism. Data from inhibition, pH-dependent, and viscosity-dependent kinetics are all highly supportive of this mechanism. The mechanism contrasts with the random sequential mechanisms of AAC(6')IV, AAC(3)I, and the bifunctional acetylating-phosphorylating enzyme of *Staphylococcus*. The results of the present study show that ANT(2")I follows a more extreme kinetic mechanism than the Ordered BiBi mechanism suggested by Lombardini and Chen-Chu (85). The release of the two parts of nucleotidylated antibiotic appears to be ordered with the antibiotic being released first, followed by a conformational change of the enzyme,

then release of the nucleotide.

There are many future kinetic experiments that could be performed with ANT(2")I, but only a few will be discussed here. The significance of the central complexes of the the ANT(2")-catalyzed reaction should be evaluated using the cross-over point analysis of Janson and Cleland (68). This requires that the reverse reaction is studied with an aminoglycoside substrate having reduced levels of substrate inhibition. Alternatively, equilibrium isotope exchange may be investigated. The pH-dependent kinetics of several aminoglycoside substrates needs to be performed to further evaluate the role of the amino groups in binding. The pH-dependence of Vmax should be determined as a function of temperature to confirm the presence of a conformational change of the enzyme. The association rate constants and their contribution to V/K of the aminoglycoside may be evaluated using the viscosity variation technique when ANT(2")I's molecular weight and the number of active sites per molecule are determined.

The AMP-aminoglycoside product appears to act as a self-generated, tight-binding inhibitor of the enzyme. It is actually a "collected product" inhibitor since it

contains nearly all of the ligands of both nucleotide and aminoglycoside substrates. The combination of the millimolar and micromolar binding ranges of the nucleotide and aminoglycoside, respectively, should result in a collected product with a binding constant in the nanomolar range. The inhibition constant for binding of this product to the free enzyme is unknown and could not be determined by product inhibition studies. The binding constant of the nucleotidylated antibiotic will have to be determined by an alternative technique.

The self-generated inhibition of ANT(2")I raises the question as to how the enzyme continues to confer resistance to the bacteria. The reason for this is that the slow release of the product is associated with Vmax alone, and it is V/K of the aminoglycoside that appears to be associated with resistance. Thus, resistance is dependent upon how quickly the enzyme encounters the antibiotic in a committed fashion, not how quickly it carries out the modification. These observations restrict the type of inhibitor that would be useful as a potentiator of antimicrobial activity. A collected product or substrate analogue would probably affect the

enzyme in much the same way as the natural product, and resistance would still continue. A better inhibitor is one that specifically affects V/K. This might be a mechanism-based inhibitor.

Fifteen years have passed since the discovery of aminoglycoside nucleotidyltransferase 2"-I by Benveniste and Davies (10). During this time, a minimum of kinetic data was accrued. The author of this thesis hopes that the research described herein contributes both to understanding of enzyme mechanisms in general and to a better understanding of enzyme-mediated microbial resistance to antibiotics. Finally, with regard to this tome in general,

> "If you cannot - in the long run - tell everyone what you have been doing, your doing has been worthless."
>
> \- Erwin Schrodinger

APPENDIX A:

KINETIC DATA OBTAINED WITH THE FLUOROMETRIC ASSAY OF ANT(2")I

The apparent kinetic constnats of eight aminoglycoside substrates were determined with the fluorometric assay. The results of these experiments are presented in Table A-1. The Mg:ATP concentration used was approximately four times the value of Km of ATP reported by Van Pelt and Northrop (142). The ratio of Mg:ATP to ATP was fixed at 500:1. The Vmax values are very similar, but the V/K values differ by as much as 200-fold. Most of the aminoglycosides in Table A-1 exhibited substrate inhibition.

Alternative Substrate Kinetics

Alternative nucleotide kinetics were determined using gentamicin C1 as the variable substrate with Mg:ATP, Mg:dGTP, or Mg:dCTP as the alternative nucleotides. The Vmax values of the antibiotic show a 1.4-fold change, and the V/K values vary by a factor of 2 as shown in Table A-2; the change in the V/K values of gentamicin C1 are reflective of the change in V/K of the alternative nucleotide (footnote of Table A-2). The resulting pattern of intersecting lines is illustrated in Figure A-1.

Next, the kinetic constants of Mg:ATP were determined as a function of gentamicin A, B, or C1 in two

TABLE A-1

ANTIBIOTIC SPECIFICITY OF GENTAMICIN NUCLEOTIDYL TRANSFERASE

SUBSTRATE	Vmax (units/mg)	Km (uM)	V/K -	Ki (uM)
Tobramycin	1.9 ± 0.3	7.7 ± 1.7	0.247	48 ± 12
Sisomicin	3.1 ± .05	9.0 ± 2.5	0.344	112 ± 45
Gentamicin A	2.3 ± .02	209 ± 38	0.011	-
Gentamicin C_1	2.0 ± .05	13.8 ± 1	0.145	2600 ± 600
Gentamicin B	2.2 ± .12	3.34 ± .62	0.646	567 ± 99
Kanamycin A	1.7 ± .19	109.4 ± 22	0.016	1470 ± 356
Kanamycin B	1.2 ± .18	1.87 ± 1.3	0.65	332 ± 144
Netilmicin	1.52 ± .07	587 ± 71	0.0026	-

Assayed at pH 9.1 with 1.6 mM Mg:ATP

TABLE A-2

ANTIBIOTIC KINETIC CONSTANTS USING ALTERNATIVE NUCLEOTIDES

Gentamicin C_1		Nucleotide	Conc.
Vmax (IU/mg)	V/K (L/min x mg)		(mM)
3.8 ± .1	.17 ± .008	Mg:ATP	10.5
1.5 ± .1	.08 ± .008	Mg:dGTP	1.0
1.8 ± .1	.07 ± .006	Mg:dCTP	4.1

Relative values of Vmax and V/K of the alternative nucleotides are 4.7:1:1.3 and 1:2.6:1.2, respectively (from ref. 150).

Figure A1: Alternative substrate diagnostic with gentamicin C_1 as the variable substrate - fluorometric assay

The alternative nucleotide substrates were Mg:ATP (●) at 10.5 mM, Mg:dCTP (▲) at 4.1 mM, or Mg:dGTP (■) at 1.0 mM. The solid lines are drawn from the data fit to Eq. 1. The concentration range of gentamicin C_1 was from 7.7 uM to 100 uM with Mg:ATP as the alternative nucleotide and from 15.3 uM to 200.0 uM with Mg:dGTP or Mg:dCTP as the alternative nucleotide substrates. Free Mg^{2+} concentration was fixed at 10.0 mM. Enzyme from a purification similar to that of preparation A was used; the specific activity was 0.6 IU/mg.

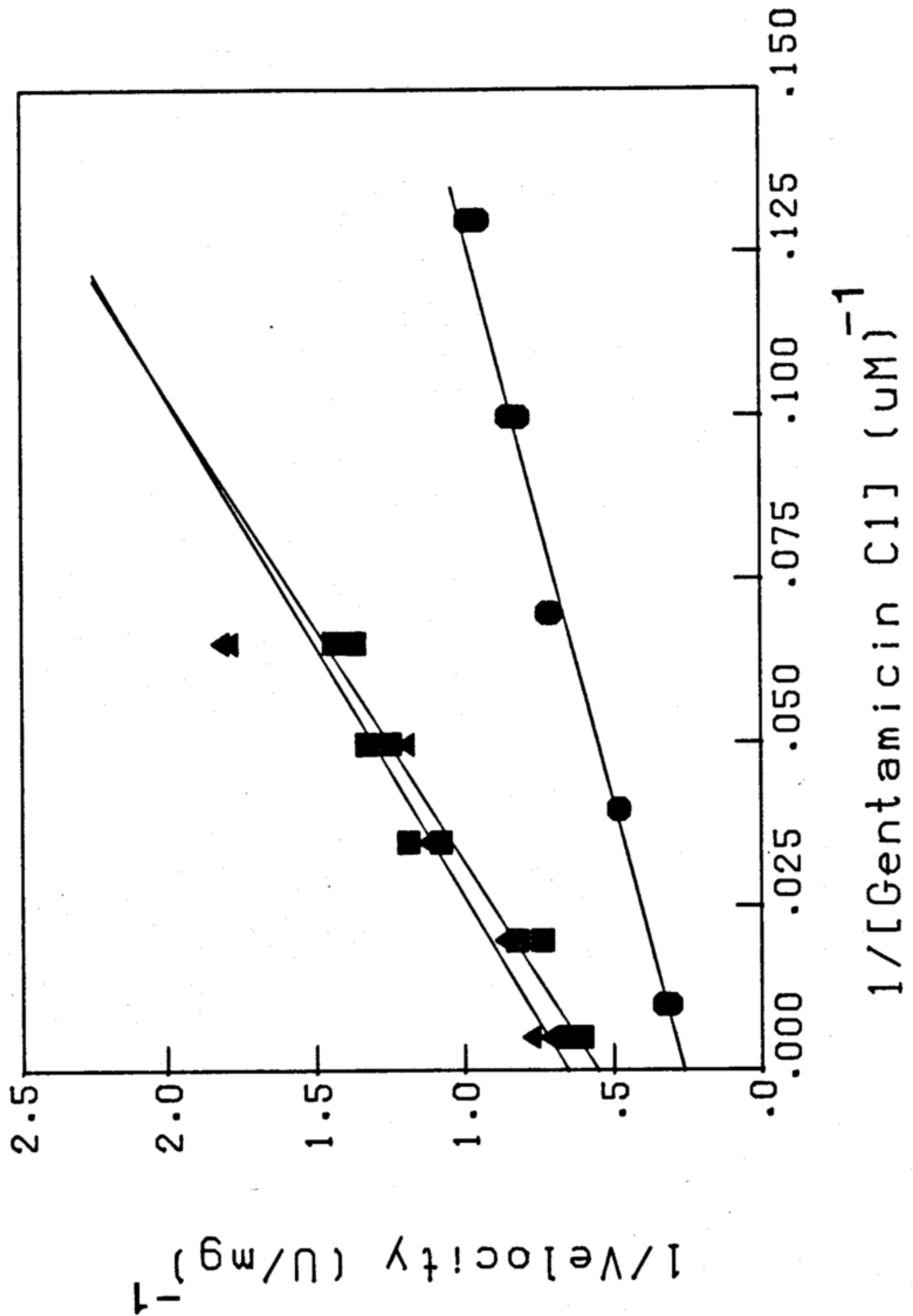

1/Velocity (U/mg)$^{-1}$
.0
.5
1.0
1.5
2.0
2.5
.000
.025
.050
.075
.100
.125
.150
1/[Gentamicin C1] (uM)$^{-1}$

TABLE A-3

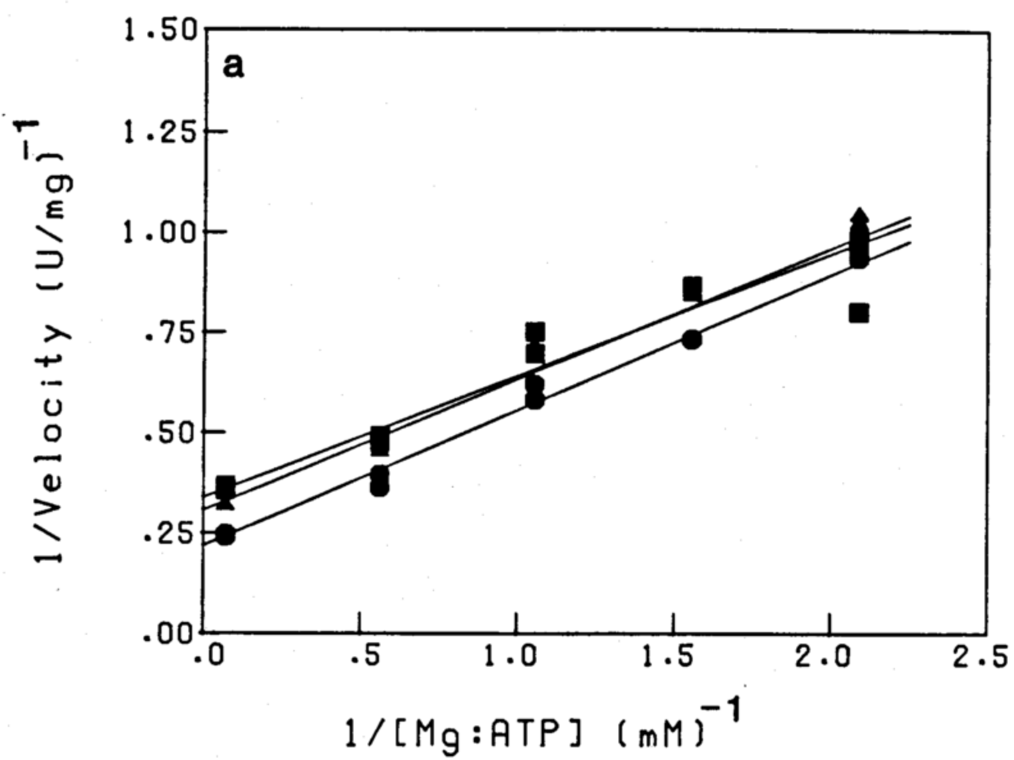
Mg:ATP KINETIC CONSTANTS USING ALTERNATIVE ANTIBIOTICS

Mg:ATP		Antibiotic	Conc.
Vmax (IU/mg)	V/K (L/(mg x min))		(uM)
A.			
4.6 ± .1	3.0 ± .2	Gentamicin A	3430.0
3.0 ± .1	3.3 ± .2	Gentamicin B	40.0
3.2 ± .1	3.0 ± .2	Gentamicin C1	140.0
B.			
4.0 ± .3	2.0 ± .1	Gentamicin A	3430.0
3.1 ± .2	1.8 ± .1	Gentamicin B	40.0
4.2 ± .2	1.6 ± .04	Gentamicin C1	140.0

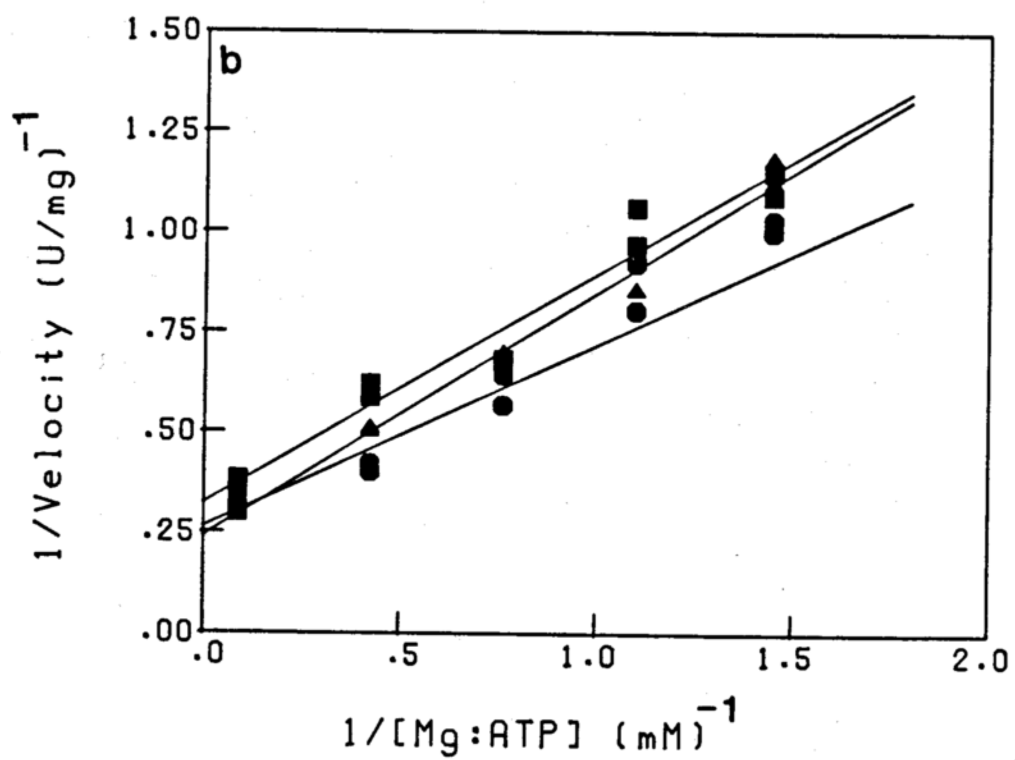
Relative values of V/K of alternative antibiotics are 1:14:8, respectively.(see Table A-1).

Figures A2a and A2b: Alternative substrate diagnostic with Mg:ATP as the variable substrate - fluorometric assay

The alternative aminoglycoside substrates were gentamicin A (▲) at 3430 uM, gentamicin B (■) at 40 uM, and gentamicin C_1 (●) at 140 uM. The solid lines represent the data fit to Eq. 1. The kinetic constants for A2a are shown in the upper part of Table A3,and the constants for A2b are shown in the lower part of the same table. The concentration range of Mg:ATP was from 0.49 mM to 14.16 mM for 23a, and the Mg:ATP concentration range for the data shown in 23b was 0.69 mM to 11.8 mM. with all alternative aminoglycoside substrates. The Mg:ATP to ATP ratio was maintained at 500:1 for each total nucleotide concentration.



a
1/Velocity (U/mg)$^{-1}$
1.50
1.25
1.00
.75
.50
.25
.00
.0
.5
1.0
1.5
2.0
2.5
1/[Mg:ATP] (mM)$^{-1}$



b
1/Velocity (U/mg)$^{-1}$
1.50
1.25
1.00
.75
.50
.25
.00
.0
.5
1.0
1.5
2.0
1/[Mg:ATP] (mM)$^{-1}$

separate experiments. The resulting patterns are shown in Figures A-2a and A-2b. The Vmax and V/K values of Mg:ATP vary over a 1.5-fold and 1.1-fold range, respectively, as a function of alternative antibiotic, shown in Table A-3. The 65-fold difference in V/K of the aminoglycosides is not reflected in the V/K or Vmax values of the nucleotide. Therefore, the patterns appear to be those of coincident lines.

APPENDIX B:

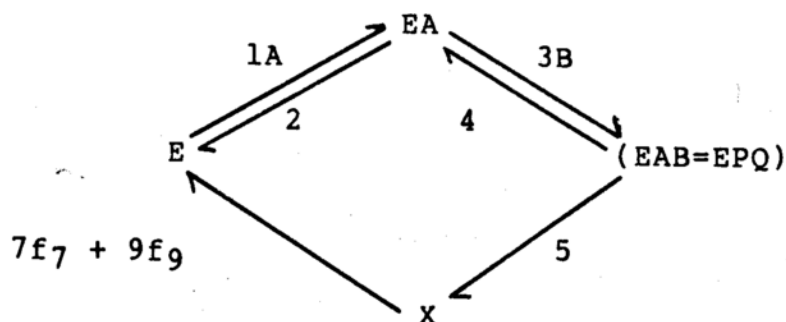
DERIVATION OF RATE EQUATIONS

DERIVATION OF RATE EQUATIONS

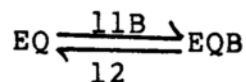
The rate equations were derived using the method of Cha (21). The strategy for derivation was to initially treat the mechanism of ANT(2") as Ordered BiBi, but the kinetic constants are defined using the restriction of $k_5 \gg k_7$. Binding of the substrate or inhibitor to EQ is assumed to be in rapid equilibrium. The kinetic constants are expressed as whole numbers; integers represent real numbers, e.g., $1 = k_1$ and 1. = 1.0

1. Substrate Inhibition

Scheme 1



where X is defined as:



and f_7 and f_9 are given by

$$f_7 = \frac{12}{11B + 12} \qquad f_9 = \frac{11B}{11B + 12}$$

The distribution equations are

$$\frac{E(denom)}{Et} = 3*5(7f_7 + 9f_9)B + 2*5(7f_7 + 9f_9) + 2*4(7f_7 + 9f_9) \qquad (B1)$$

$$\frac{EA(denom)}{Et} = 1*5(7f_7 + 9f_9)A + 1*4(7f_7 + 9f_9)A \qquad (B2)$$

$$\frac{(EAB=EPQ)(denom)}{Et} = 1*3(7f_7 + 9f_9)AB \qquad (B3)$$

$$\frac{X(denom)}{Et} = 1*3*5AB \qquad (B4)$$

and (denom) is the denominator which is the sum of all terms in the distribution equation. The rate equation is given as:

$$\frac{v}{Et} = \frac{5(EAB=EPQ)}{denom} \qquad (B5)$$

After subtituting equation B3 into B5 and multiplying the numerator and denominator by 1/(11B + 12), the numerator of equation B5 is

$$1*3*5*7*12\ AB + 1*3*5*9*11\ AB^2$$

and factors into

$$1*3*5*7*12AB\ (1. + \frac{9*11B}{7*12})$$

The terms in the denominator are

2*7*12(4+5) + 1*7*12(4 5)A + 2*9*11(4+5)B + 3*5*7*12B + 3*5*9*11B^2 + 1*9*11(4+5)AB + 1*3*12(5+7)AB + 1*3*11(5+9)AB^2

The $(1. + \frac{9*11B}{7*12})$ term may now be factored out of matching terms of the denominator. For example, the coeffcients of A and one of the AB coefficients may be factored as shown below:

1*7*12(4+5)A

1*9*11(4+5)AB become $1(4+5)7*12A\ (1. + \frac{9*11B}{7*12})$

This is now the coefficient of the A term; similar expressions can be derived for the constant, coefficient of B, and coefficient of AB. However, one AB coefficient has a different factor in it that arises from matching one AB and an AB^2 coefficient:

1*3*5*12AB

1*3*9*11AB^2 become $1*3*5*12(1. + \frac{11B}{12})$

After division of each term in the numerator and denominator by

$$\frac{1.}{12}\left(1.+\frac{9*11B}{7*12}\right)$$

and collecting terms, the equation becomes

$$\frac{v}{Et} = \frac{1*3*5*7AB}{2*7(4+5) + 1*7(4+5)A + 3*5*7B + 1*3*7AB + 1*3*5AB\frac{(1.+11B/12)}{(1.+9*11B/7*12)}} \quad (B6)$$

The AB terms may be factored into the following expression:

$$1*3(5+7)AB - 1*3*5AB + 1*3*5AB\frac{(1.+ 11B/12)}{(1.+9*11B/7*12)}$$

The 1*3(5+7) term is the coefficient of AB for an Ordered BiBi mechanism in the absence of inhibition. The definitions of the kinetic constants for the Ordered BiBi mechanism of Scheme 1 are shown in the left column, and the definitions for the Theorell-Chance mechanism are shown in the right column:

	Ordered BiBi	ANT(2")I 5 >> 7
$Vmax = \frac{numerator}{coeff\ AB}$	$\frac{5*7}{(5+7)}$	7
$Ka = \frac{coeff\ B}{coeff\ AB}$	$\frac{5*7}{1(5+7)}$	$\frac{7}{1}$
$Kb = \frac{coeff\ A}{coeff\ AB}$	$\frac{7(4+5)}{3(5+7)}$	$\frac{7(4+5)}{3(5+7)}$
$Kia = \frac{constant}{coeff\ A}$	$\frac{2}{1}$	$\frac{2}{1}$
V/Ka =	1	1
V/Kb =	$\frac{3*5}{(4+5)}$	$\frac{3*5}{(4+5)}$
Kii =	-	$\frac{12}{11}$
Kid =	-	$\frac{7*12}{9*11}$

The coefficients of A, B, and AB in rate equation (B5) are converted into kinetic constants by dividing each term in the numerator and denominator by 1*3(5+7):

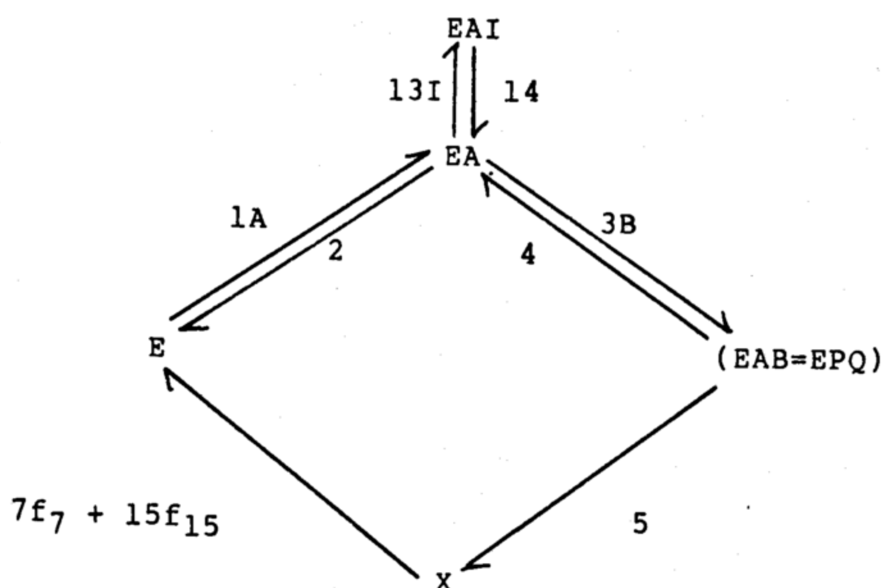
$$\frac{v}{Et} = \frac{Vmax\ AB}{KiaKb + KbA + KaB + AB - \frac{5(AB)}{(5+7)} + \frac{5AB}{(5+7)}\frac{(1.+ B/Kii)}{(1.+ B/Kid)}}$$

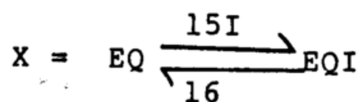
But for 5 >> 7, the "excess" collection of rate constants, 5/(5+7), reduces to 1.0 and the <u>final form of the equation</u> is

$$\frac{v}{Et} = \frac{Vmax\ AB}{KiaKb + KbA + KaB + AB\ ((1.+ B/Kii)/(1.+B/Kid))} \tag{B6}$$

2. Dead-End Inhibition

The same strategy described above was used for the following mechanism:





$$f_7 = \frac{EQ}{EQ + EQI} = \frac{18}{17I + 18} \quad ; \; f_{15} = \frac{17I}{17I + 18}$$

The distribution equations are the same as those above except that $(7f_7 + 15f_{15})$ is substituted for $(7f_9 + 9f_9)$, and the EA(denom) equation is multipled by (1.+13I/14). After substituting equation B(3) into B(5) and

multiplication of the numerator and denominator by 1/(17I+18), the numerator of the rate equation (see B5) is

    1*3*5*7*18AB + 1*3*5*15*17ABI

which factors into

    1*3*5*7*18AB(1. + 15*17I/7*18)

This factor is used in the same way to collect terms in the denominator to give A, B, and AB coefficients (see above); the A term includes (1.+13I/14). The definitions of the kinetic constants are the same as those for substrate inhibition. The definitions of the inhibition constants are

Kis = 14/13

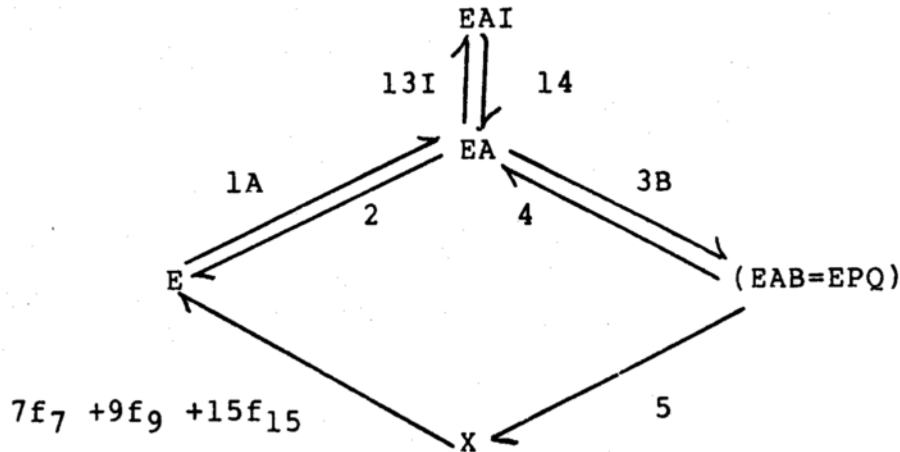
Kii'= 18/17

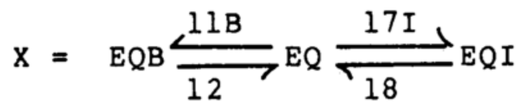
Kid'= 7*18/15*17

The final form of the equation is

$$\frac{v}{Et} = \frac{Vmax\ AB}{KiaKb + KbA + KaB(1.+I/Kis) + AB\frac{(1.+I/Kii')}{(1.+I/Kid')}}$$

3. Multiple Inhibition





$$f_7 = \frac{EQ}{EQ + EQI + EQB} = \frac{12*18}{12*18 + 11*18B + 12*17I}$$

$$f_9 = \frac{EQB}{EQ + EQI + EQB} = \frac{11*18B}{12*18 + 11*18B + 12*17I}$$

$$f_{15} = \frac{EQI}{EQ + EQI + EQB} = \frac{12*17I}{12*18 + 11*18B + 12*17I}$$

The distribution equations are the same as B(1) through B(2) except that $(7f_7 + 9f_9 + 15f_{15})$ is substituted for $(7f_7 + 9f_9)$. Equation B(2) is multipled by (1.+13I/14).

Substitution of B(3) into B(5) and multiplication of the numerator and denominator by 1/(12*18 + 11*18 + 12*17I) gives a numerator which can be factored into

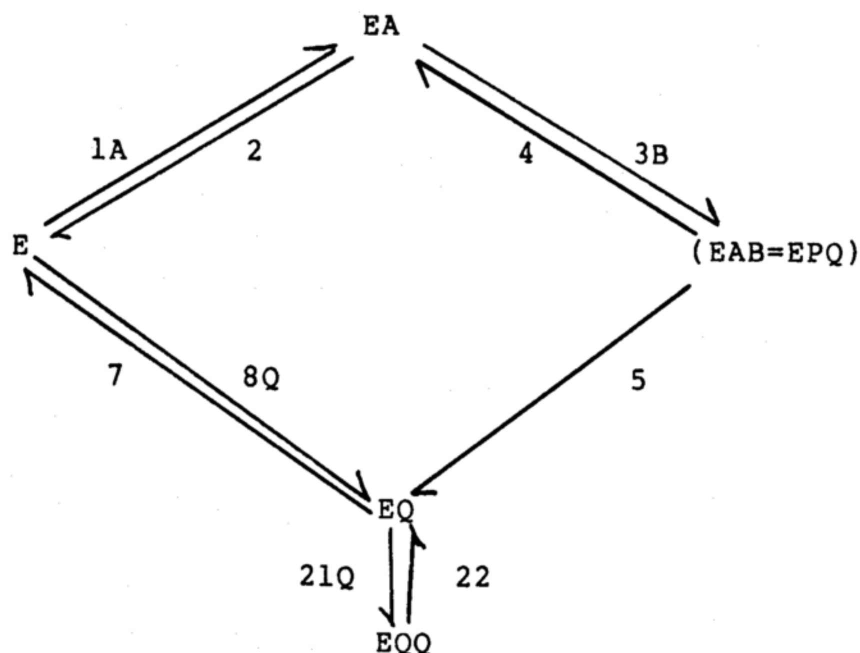
$$1*3*5*7*12*18AB\left(1. + \frac{9*11B}{7*12} + \frac{15*17I}{7*18}\right)$$

Collection of terms follows the same procedure described above, and the final form of the rate equation is

$$\frac{v}{Et} = \frac{V\ AB}{KiaKb + KaB + KbA(1.+I/Kis) + AB\dfrac{(1.+ B/Kii + I/Kii')}{(1.+ B/Kid + I/Kid')}}$$

The definitions of the kinetic and inhibition constants are the same as given above.

4. Mixed Product and Dead-End Inhibition



The distribution equations for this mechanism are the same as B(1) through B(3), except B(4) becomes

```
EQ    (1*3*5AB + 3*5*8BQ + 2*5*8Q + 2*4*8Q)(1. + 21Q/22)
-- = --------------------------------------------------
Et                      denom.
```

The denominator is 2*7(4+5) +1*7(4+5)A + 3*5*7B +

3*5*8(1.+ 21Q/22)BQ + (1*3*7 + 1*3*5(1.+ 21Q/22))AB

The AB term may be factored as

1*3(5+7)AB + 1*3*5(21Q/22)AB

Equation B(3) is substituted into B(5). The definitions of the kinetic constants are the same as those for substrate inhibition, and the 1*3(5+7) coefficient is used to convert the collections of rate constants into kinetic constants. This yields the following equation:

$$\frac{v}{Et} = \frac{Vmax\ AB}{KiaKb + KaB + KbA + KaBQ/Kiq + AB + \frac{5}{(5+7)}(21Q/22)AB}$$

As before, the "extra" collection of rate constants, 5/(5+7) is equal to 1. when 5 >> 7. The final form of the equation is

$$\frac{v}{Et} = \frac{Vmax\ AB}{KiaKb + KaB + KbA + \frac{KaBQ}{Kiq} + AB(1. + \frac{Q}{Kiq'})}$$

in which Kiq = 8/7 and Kiq' = 22/21.

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