Substrate Specificity and Structure-Activity Relationships of Gentamicin Acetyltransferase I

THE DEPENDENCE OF ANTIBIOTIC RESISTANCE UPON SUBSTRATE $V_{\text{max}}/K_m$ VALUES

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Sixteen aminoglycoside antibiotics and derivatives were found to be substrates and nine were identified as competitive inhibitors of purified gentamicin acetyltransferase I. Among the substrates, gentamicin A, kanamycin B, tobramycin, neamine, nebramine (tobramycin), and gentamicin C, were previously questioned on the basis of results obtained from microbiological studies or enzymatic experiments with crude bacterial extracts. Gentamicin C, and sisomicin were the best substrates based on $V_{\text{max}}/K_m$ values ($>10^6$ M$^{-1}$ s$^{-1}$), whereas gentamicin B, is the poorest ($5 \times 10^4$ M$^{-1}$ s$^{-1}$). Gentamicin C, yields the greatest $V_{\text{max}}$ (5 $\mu$mol/min/mg) while tobramycin gives the smallest (0.5 $\mu$mol/min/mg). Neomycin is the best inhibitor ($K_i = 4 \times 10^{-3}$ M) and 2-deoxystreptamine is the poorest ($2 \times 10^{-3}$ M). The kinetic characteristic which correlates with antibiotic resistance mediated by gentamicin acetyltransferase I was found to be the $V_{\text{max}}/K_m$ value of antibiotic substrates.

These kinetic changes are correlated to structural changes by a method of evaluation designed to isolate groups of kinetically independent rate constants under nonrapid and rapid equilibrium conditions. Significant findings are: although the enzyme modifies the 3-N position of the deoxystreptamine ring (II), the minimal requirements for activity include a purpurasamine ring (I); this requirement expresses the binding and catalytic roles of the 2' and 6' (I) amines; methylation about the 6' position reduces binding, which increases reaction velocities by shifting the rate-limiting step; hydroxylation at 3' and 4' reduces catalysis but alters the roles of the 2' and 6' groups; 1-N (II) substitutions also reduce catalysis; the best substrates contain garsamine (III), effecting primarily an increase in catalytic activity solely to the contribution of the 3' (III) amine; the kinetic contribution of ring III, however, is greatly influenced by the identity of ring I; four-ring aminoglycosides bind differently to the enzyme, perhaps inversely. Four enzymatic structure-activity relationships closely correlate with antimicrobial structure-activity requirements, suggesting that attempts to chemically modify existing aminoglycoside antibiotics, in an effort to combat bacterial resistance, are not likely to succeed.

Gentamicin acetyltransferase I catalyzes the inactivation of certain aminoglycoside antibiotics in R factor-containing bacteria. The catalytic features of the enzyme are therefore the limiting characteristics of this mode of antibiotic resistance. Brzezinska et al. (3) and Kobayashi et al. (4) have listed the relative efficiency of some aminoglycosides to accept $[^3]$Clacetate from acetyl-CoA by measuring the amount of radioactivity transferred to antibiotic in a fixed time period in the presence of bacterial extracts containing gentamicin acetyltransferase I. In this paper, the specificity of the purified enzyme is examined in terms of kinetically independent initial velocity constants $V$ and $V/K$, using a new approach which attempts to distinguish between changes in binding and changes in catalysis in order to acquire more quantitative information and to explain why bacteria containing the enzyme will show resistance to some antibiotic substrates and not others. These interpretations are dependent upon knowledge of the kinetic mechanism of gentamicin acetyltransferase I, described in the companion paper (5).

MATERIALS AND METHODS

Chemicals—Acetyl-CoA, butyryl-CoA, propionyl-CoA, CoA, and phosphotransacetylase were purchased from P-L Biochemicals, 4,4'-dithiodipyridine (Aldrithiol-4) from Aldrich. Tobramycin, neomycin B, and nebramine were gifts from Dr. Marvin Gorman of Eli Lilly and Co. Kanamycin A, kanamycin B, and neamine were gifts from Dr. Kanrech Price of Bristol Laboratories. Sisomicin, N-ethyl sisomicin, gentamicins A, C1, C1n, B, B, gentamicin C1n, garamine, sisomine, and kanamine were gifts from Dr. Gerald Wagnman and Dr. Peter Danielo of Schering Corp. Gentamicin XK622 was a gift from Dr. A. D. Geizler of Abbott Laboratories. Ribostamycin and lividomycin B were gifts from Dr. Julian Davies (University of Wisconsin). Paromomycin I and 2-deoxystreptamine were gifts from Dr. D. Pearlman (University of Wisconsin). Kanamycin C was a gift from Dr. H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan). Fortimicin A was a gift from Dr. T. Naka (Kyowa Hakko Kogyo Co., Tokyo, Japan). Concentrations of stock solutions of antibiotic and coenzyme substrates were determined by enzymatic assays with gentamicin acetyltransferase I, using the 232 nm extinction coefficient of the thioester bond of acetyl-CoA as a reference (6). Concentrations of inhibitors were calculated on a weight basis, making corrections for purity or potency values (or both) when available. Acetyltobramycin and acetylgentamicin C1 were prepared as described previously (6).

Enzyme and Enzyme Assay—Gentamicin acetyltransferase I was designated AAC(3)-1 according to proposals by the Plasmid Group Nomenclature (2).
prepared by the method of Williams and Northrop (7) and assayed by following the reduction of 4,4'-dithiobispyridine as described in the preceding paper (5).

Data Processing—Kinetic constants were obtained from reaction velocity data by computer fitting to appropriate rate equations using the FORTRAN programs of Cleland (8). Substrate specificity data were fitted to Equation 1 or to Equation 2 if substrate inhibition was observed. Competitive inhibition data were fitted to Equation 3.

\[ V = \frac{VA \cdot k_A}{K + A} \]  
\[ V = \frac{VA \cdot k_A + A^2}{K_x} \]  
\[ V = \frac{VAB \cdot k_A + k_{12} \cdot k_{13} + k_{16}}{K_1 + k_{12} + k_{13} + k_{16}} \]

Theoretical Analysis—Structure-activity relationships of alternate substrates for enzymes have traditionally been evaluated in terms of changes in K and V values. Such evaluations are dependent upon the implied assumptions that K is a measure of binding of the substrate to the enzyme, and V is a measure of the rate of chemical catalysis. However, these assumptions may be true only under rapid equilibrium conditions, and the mechanism studies presented earlier clearly show that rapid equilibrium conditions cannot be assumed for reactions between gentamicin acetyltransferase and the better antibiotic substrates (5). Alternatively, substrate specificity may be evaluated in terms of V/K, the apparent first order rate constant for the combination of substrate with enzyme during catalytic turnover. The concept was first formulated in 1948 by Elkins-Kaufman and Neurath (9) as a measure of evaluating assay data obtained at sub-K concentrations of substrate. Formal usage of V/K values as a substrate specificity constant introduced by Sols and Crane (10) in 1964. This usage was extended in 1965 by Bender and Kozdy (11) who pointed out that under rapid equilibrium conditions, V/K is not affected by nonproductive substrate binding, unlike V and K alone. (This is true under nonrapid equilibrium conditions as well.) More recently, Cleland (13) and later Northrop (14) have shown that for nonrapid equilibrium conditions, V and V/K are the kinetically independent variables. Using these more recent concepts, it is now possible to evaluate V/K together with V data to distinguish between binding and catalytic changes with less uncertainty than the earlier approaches.

V/K consists of all rate constants up to and including the first irreversible step. V, on the other hand, consists of the rate constants of catalysis and all subsequent steps through product release. For an irreversible reaction such as that catalyzed by gentamicin acetyltransferase I (5), V and V/K will have rate constants of catalysis in common, but otherwise are composed of entirely different groups of rate constants. Consequently, structural changes in substrates which alter catalysis will affect V and V/K similarly, whereas changes which alter binding of substrates and products will affect V and V/K differently. The kinetic expression for apparent values of V and V/K in the present study are obtained as follows. A random mechanism given in Equation 4.

\[ \text{E} \quad \text{A} \quad \text{B} \quad \text{E} \quad \text{A} \quad \text{E} \quad \text{B} \quad \text{E} \quad \text{A} \quad \text{E} \quad \text{B} \quad \text{E} \quad \text{A} \quad \text{E} \quad \text{B} \quad \text{E} \]

reduces to Equation 5 using Cleland’s concept of net rate constants (15) and assuming irreversible catalysis, zero concentration of products (i.e., initial velocity conditions), and saturating concentrations of co-substrate B.

\[ V = \frac{VA \cdot k_A + k_{12} \cdot k_{13} + k_{16}}{K_1 + k_{12} + k_{13} + k_{16}} \]

The expression for apparent V is:

\[ V = \frac{F_1}{k_9 + k_{11} \cdot k_{13}} \]  

where the net rate constant for product release

\[ k_{11} \cdot k_{13} = \frac{1}{k_{12} + k_{13} + k_{12} \cdot k_{13} + k_{16}} \]

approaches the rate of release of the most tightly bound product when products are released at different rates. The expression for apparent V/K is:

\[ \frac{V}{K} = \frac{k_7 \cdot F_1}{k_8 + 1} \]

Subsequent analysis of V and V/K data will depend upon a comparison of Equations 6 and 8. It can be seen that structural modifications which reduce catalysis will reduce both V and V/K, but not necessarily by the same amount. Alternatively, modifications which reduce binding will reduce V/K but increase V, again not necessarily by the same amount. This latter interpretation assumes that binding varies primarily as a function of rates of release (i.e., bimolecular rate constants are diffusion controlled) and that the chemical groups governing the rate of release of products also govern the rate of release of their parent substrate (i.e., “sticky” substrates give rise to “sticky” products). Because all inhibitors in question are competitive, their K values are dissociation constants. Changes in K values will be evaluated assuming such changes are also due to altered rates of release.

RESULTS AND DISCUSSION

Table I lists the apparent kinetic constants for the aminoglycosides found to be substrates with the purified gentamicin acetyltransferase I. The aminoglycosides found not to be substrates are all competitive inhibitors against tobramycin with K values listed in Table II. Product and other dead-end inhibition data are presented in the companion paper (5). In addition to acetyl-CoA, propionyl-CoA was found to be a substrate versus tobramycin, having a 4-fold higher K and 2-fold lower V. Butyryl-CoA and malonyl-CoA did not show substrate activity. Inhibition studies with butyryl-CoA revealed a binding constant equivalent to propionyl-CoA, and independent of the aminoglycoside substrate (5).

Gentamicin acetyltransferase I therefore has a narrow specificity with respect to its coenzyme requirement; only acetyl-CoA is active. However, the expression of V/K is sensitive to the substrate to which it is applied, as shown by the subsequent analysis in Table I.
CoA and propionyl-CoA will serve as acyl donors. Interestingly, free CoA binds more tightly by a factor of two than acetyl-CoA (5), which in turn binds 4-fold better than propionyl-CoA, but propionyl-CoA binds no better than butyryl-CoA. Thus, it appears that the acyl group to be transferred hinders binding, and this hindrance reaches an upper limit at the level of propionyl-CoA, suggesting that the butyryl group is excluded from the transfer site of the enzyme. In addition, the K values for butyryl-CoA and the K values for acetyl-CoA do not change significantly as the aminoglycoside substrate is changed. This lack of binding dependence on the aminoglycoside substrate is an important factor in the following analysis of aminoglycoside specificity.

The enzyme has a very broad specificity with respect to aminoglycosides. With the exception of 2-deoxystreptamine, all the aminoglycoside antibiotics and derivatives that were tested displayed significant and specific binding to the enzyme, and most acted as substrates. Notably, gentamicin A, kanamycin B, tobramycin, neamine, nebramine (tobramycin), and gentamicin C1 were previously questioned or thought not to be substrates, based on results obtained from microbiological studies or enzyme experiments using crude bacterial extracts (16, 17). Gentamicin C1 and sisomicin are clearly the best substrates, based on their V/K values, both of which were greater than 10^7 M^-1 s^-1. Gentamicin C1 shows the greatest V, which will be explained below, while tobramycin shows the lowest V; hence tobramycin was chosen for kinetic mechanism studies to ensure rapid equilibrium conditions (5). Neomycin B is the best inhibitor, with a submicromolar inhibition constant.

The kinetic specificity correlates well with the resistance spectra of bacteria possessing the enzyme. Biddlecome et al. (18) have reported minimal inhibitory concentrations of various aminoglycosides against *Escherichia coli*-containing gentamicin acetyltransferase I. The bacteria displayed resistance against gentamicin C1, gentamicin C16, and sisomicin, all good substrates for the enzyme (Table I). The aminoglycosides for which no resistance was expressed were either inhibitors or poor substrates for the enzyme. Notable among the inhibitors was neomycin, which binds tightly to the enzyme (5). Hence, an expression of catalysis, and not simple binding, is necessary to convey resistance. The poor substrates were tobramycin and kanamycin B. Comparing K values, tobramycin differs from gentamicin C1 by less than a factor of two; comparing V values, kanamycin B differs from gentamicin C1 by only a factor of three; comparing V/K values, each poor substrate differs from each good substrate by a factor greater than 10 (Table I). Since the minimal inhibitory concentrations of resistant and nonresistant substrates differed by a factor greater than 10 (18), these data correlate the increase in resistance with V/K values. In order to conclude that resistance is dependent upon V/K, it is necessary to assume that any differences in the access of various antibiotics to the enzyme are proportional to the differences in access to the bacterial ribosomes, the site of antimicrobial action. The magnitude of differences should be greater with respect to the ribosomes than the enzyme because the ribosomes are intracellular, while the enzyme is periplasmic (7). Similarities of minimal inhibitory concentrations of the clinically useful antibiotics from tests against sensitive *E. coli* (17) indicate that these greater differences are themselves small. Consequently, the dependence of resistance on V/K can be considered to hold for the clinically useful antibiotics. In addition, dependence on V/K suggests that the concentration of antibiotic in the periplasmic space is maintained at less than apparent K values.

Structure-activity relationships may be deduced by evaluating kinetic differences within each aminoglycoside structural series according to the kinetic theory presented above. Beginning with the C series of the gentamicins, structural differences are limited to differences in the degree of methylation of the purpurosamine ring (I) at the 6’ position and its amino substituent (Fig. 1). As illustrated in Table III, methylation at the 6’ position (C6) gives the amino substituent (XK62-2), or both (C1), progressively lowers V/K values but nearly equivalently increases values of V. The simplest interpretation of progressive but inverse effects on V and V/K is that methylation increases the rate of release of these antibiotics and their acetylated products from the enzyme (k1 and k11,13 of Equation 6 and 8). Nearly equivalent expression of these changes on V and V/K requires that rates of release (k1 and k11,13) and catalysis (k0) be of similar magnitude (giving further support for a nonrapid equilibrium mechanism (5)). Furthermore,

### Table II

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>K&lt;sub&gt;&lt;i&gt;s&lt;/i&gt;&lt;/sub&gt; μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>Paromomycin I</td>
<td>8.3 ± 0.7</td>
</tr>
<tr>
<td>Lividomycin B</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>91 ± 18</td>
</tr>
<tr>
<td>Kanamycin C</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Amikacin</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>2-Deoxystreptamine</td>
<td>1900 ± 400</td>
</tr>
<tr>
<td>Garamine</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Kanamidine</td>
<td>208 ± 16</td>
</tr>
</tbody>
</table>

* Tobramycin was the varied substrate, acetyl-CoA held constant at 60 μM, all patterns were competitive.

### Table III

<table>
<thead>
<tr>
<th>Data extracted from Table I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin 6’-R</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>C1a</td>
</tr>
<tr>
<td>C2</td>
</tr>
<tr>
<td>XK62-2</td>
</tr>
<tr>
<td>C1</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>B₁</td>
</tr>
</tbody>
</table>

### Fig. 1.

Structure of the gentamicins. Ring I is purpurosamine, II is 2-deoxystreptamine, and III is garosamine (gentamicin C compounds) or gentosamine (gentamicin A).

### Table III

<table>
<thead>
<tr>
<th>Effect of methylation about the 6’ position</th>
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<tbody>
<tr>
<td>Data extracted from Table I.</td>
</tr>
<tr>
<td>Gentamicin 6’-R</td>
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<tr>
<td>C1</td>
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<tr>
<td>B</td>
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<tr>
<td>B₁</td>
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</tbody>
</table>
gentamicin C₁₄ substrate inhibition requires that release of CoA is partially rate-determining (5), hence the release of CoA and acetylgentamicin C₁₄ must be of similar magnitude sufficient for both to contribute to \( k_{11,12} \). Expression of increased rates of release of one product as changes in \( V \) are then suppressed by Equation 7. From the percentage change observed on \( V \), it follows that acetylated product release is primarily rate-determining with gentamicin C₁, and less so with C₂, XK62-2, and C₁ in that order, which explains why gentamicin C₁ yields the greatest maximal velocity. The effect of methylation at the 6' position can also be seen in the B series of gentamicins, which differ from the C series by hydroxylations at the 2', 3', and 4' positions (Fig. 1). Methylation (Bₙ) again lowers \( V/K \) relative to its unmethylated homologue (B), but the effect is much greater than observed in the C series, and is accompanied by a small decrease in \( V \). These results indicate that the 6'-amino group makes a greater contribution to binding in the B series than in the C series, which is consistent with the absence of a 2'-amino group in the B series (which is also important for binding, see below). Thus, product release is not partially rate-determining in this series.

The kanamycin series differ only in the replacement of amine groups by hydroxyl groups at the 2' and 6' position (Fig. 2). As illustrated in Group A of Table IV, either replacement abolishes catalytic activity and greatly reduces binding. Similarly, with the two-ring degradation products, neamine and kanamine shown in Group B of Table IV, replacement at the 2' position also abolishes catalytic activity and reduces binding, but to a much greater extent than in the kanamycin series. To a first approximation, it would appear that the difference in effect between groups A and B may be due to a binding contribution of the kanosamine ring (III') of kanamycin, but later experiments indicate that the kanosamine ring contributes little to the binding of tobramycin (Table VII). Group C contains antibiotics consisting of four-ring structures (Fig. 3), all of which are inhibitors. Again, replacement of the 6'-amine group with a hydroxyl group reduces binding, to an extent between that observed in Groups A and B.

Gentamine C₁₀, nebramine (tobramine), and neamine consist of the first two rings (I and II) of gentamicin C₁₄, and differ only in their degree of hydroxylation at positions 3' and 4'. As illustrated in Group A of Table V, the presence of 3'- and 4'-hydroxyl groups lowers both \( V \) and \( V/K \). The simplest explanation of these progressive and similar effects is that hydroxylation lowers the rate of catalysis (\( k_o \) of Equations 6 and 8).

![Fig. 2. Structure of the kanamycins. Ring III' is kanosamine.](image)

**TABLE IV**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>2'-R</th>
<th>6'-R</th>
<th>( K )</th>
<th>( K_i )</th>
<th>( \Delta K_i^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>-NH₂</td>
<td>-NH₂</td>
<td>2.9</td>
<td>90</td>
<td>+9x</td>
</tr>
<tr>
<td>Kanamycin C</td>
<td>-NH₂</td>
<td>-OH</td>
<td>26</td>
<td>208</td>
<td>+32x</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>-OH</td>
<td>-NH₂</td>
<td>90</td>
<td>208</td>
<td>+32x</td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neamine</td>
<td>-NH₂</td>
<td>-NH₂</td>
<td>2.2</td>
<td>208</td>
<td>+32x</td>
</tr>
<tr>
<td>Kanamine</td>
<td>-OH</td>
<td>-NH₂</td>
<td>90</td>
<td>208</td>
<td>+32x</td>
</tr>
<tr>
<td>C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neomycin B</td>
<td>-NH₂</td>
<td>-NH₂</td>
<td>0.37</td>
<td>8.3</td>
<td>+22x</td>
</tr>
<tr>
<td>Paromomycin I</td>
<td>-NH₂</td>
<td>-OH</td>
<td>8.3</td>
<td>-OH</td>
<td>+22x</td>
</tr>
</tbody>
</table>

*a* Because substrates displayed low \( V/K \) values and inhibitors were all linear competitive, \( K \) and \( K_i \) values were considered equivalent to dissociation constants, \( K_D \).

Furthermore, since the low \( V/K \) values in this series indicate that the denominator of Equation 8 is dominated by \( h_9/k_o \), and since the changes in \( V \) and \( V/K \) are similar, the expression of \( V \) must be dominated by \( 1/k_o \) of Equation 6. Thus, catalysis...
is rate-determining for the substrates in Group A. In contrast, the antibiotics in Group B differ from Group A by the presence of the kanosamine ring (III'), where the effect of 3'-hydroxylation is an increase, rather than decrease in both $V$ and $V/K$. Consequently, these data show that the effect of modification on the purpurosamine ring (I) is significantly modulated by the presence of the kanosamine ring (III'). Because $V$ and $V/K$ are both increased, the effect of hydroxylation is expressed primarily on the rate of catalysis, which again must be largely rate-determining. As a further contrast, Group C of Table V contains antibiotics consisting of four-ring structures, where 3'-hydroxylation causes a significant decrease in $K_v$ values. Comparison with the results with members of Groups A and B suggests that binding of the four-ring series of antibiotics is significantly different from the three-ring series.

Substitutions at the 1-N position of the deoxystreptamine ring are illustrated in Table VI. Acylation of kanamycin with 1-n-aminohydroxybutyric acid to yield amikacin shows no significant change in binding as revealed by $K_v$ values. Ethyl substitution of sisomicin (Fig. 4) or replacement of the amino group by a methoxy group in fortimicin A (as compared to gentamicin C18, its closest homologue, Fig. 5) give near parallel reductions in $V$ and $V/K$. These reductions are consistent with a reduction in the rates of catalysis.

Also shown in Table VI, Group D, is the effect of modification of the 3-N position by gentamicin acetyltransferase I itself. Comparing the $K_v$ of tobramycin with the $K_v$ of acetyl-tobramycin from product inhibition studies (6), reveals a 6-fold decrease, indicating that the 3-amino group participates in binding to the enzyme.

The aminoglycosides also vary in the number and identity of the various rings comprising their structure. Table VII illustrates the relative kinetic contribution of many of these rings, beginning with the composition of gentamicin C18 in Group A. Although the 2-deoxystreptamine ring (I) contains the site of modification by the enzyme, it binds poorly to the enzyme and is not a substrate. The addition of either garosamine (III) or purpurosamine (I) rings to 2-deoxystreptamine greatly enhances binding, although only the latter structure will serve as a substrate. The addition of both rings has an additive effect on binding, and increases catalysis as both $V$ and $V/K$ are increased by the addition of garosamine (III) to gentamicin C18, or sisomine.

The effects of the addition of kanosamine (III') is illustrated in Groups C, D, and F, which differ in the identity of the first ring. The variety of changes show that the kinetic contribution of the third ring is greatly influenced by the identity of the first.

The major contributions of the third ring appears to be made by the 3"-amine group. This can be seen by comparing fortimicin A (Group F), which terminates in a glucyl amide instead of a glucosamine ring (Fig. 5), to the other two- and three-ring aminoglycoside substrates of Table VII. Fortimicin A is a much better substrate than any of the other two-ring aminoglycosides, and kinetically resembles the three-ring series. As pointed out earlier (Table VI), it appears to bind
equally well to the enzyme as gentamicin C₈, its closest homologue. Molecular model studies show that the α-amino group of glycyl amide can be superimposed on the 3"-amino position of the series, neomycin B. These results support the earlier postulation that the four-ring aminoglycosides bind differently to the enzyme surface (Table V, Group C).

Finally, although kinetic effects of one modification being altered by a second structural feature have been presented, the most dramatic examples of this phenomenon are found in the kinetics of gentamcins A and B. These are the only compounds tested having a hydroxy group at position 2' or 6'. The above kinetic data were compared to antimicrobial structural requirements of aminoglycosides. Antimicrobial data were drawn from the cell-free studies of Benveniste and Davies (20), and the review of antimicrobial activity by Price et al. (17). From a correlation of the aminoglycoside structure with antimicrobial activity, it can be seen that many structural requirements for antimicrobial activity closely parallel those necessary for aminoglycoside acetyltransferase I substrate activity. First, the removal or blockage of the 2'- and 6'-amino group (or both) of purpurosamine (ring I) greatly reduces or eliminates acetylation by the enzyme (Tables III and IV). Benveniste and Davies (20) have concluded that the number and identity of amino groups are important determinants of antibiotic activity, and have identified the 2' and 6' as being the most important. Price et al. (17) have shown that there is an absolute requirement that there be an amino function in ring I for antimicrobial activity. Second, the addition of the amino hexose (ring III or III') to gentamicin C₈, sisomine, and kanamycin increases V/K 5- to 20-fold (Table VII), which compares to similar increases in antimicrobial activity of 2'-to 8-fold (17). The apparent satisfaction of the third ring requirement by the glycol moiety of fortimicin A suggests that the 3'-amine is responsible for these enzymatic activity increases. The increases in antimicrobial activity have also been attributed to the 3'-amine group, as the latter is eliminated by N,N'-dimethylation (21). Third, the amine requirements are additive in both enzymatic and antimicrobial activities. The highest enzymatic activity is found in aminoglycosides with amine groups in the 1, 3', 2', 6', and 3" positions. A full complement of these amino groups is also required for maximum antimicrobial potency, and acylation in any of these positions except position 1 eliminates antimicrobial activity. That this same amine is also the least important to enzymatic activity is evidenced by the relative kinetic standings of 1-N-ethyl sisomicin, amikacin, and fortimicin A (Table VI). Fourth, the activity series produced by degradation of gentamicin C₈ correlates very well. Gentamicin C₉, is a better substrate than gentamicin C₈, garamine is not a substrate but a much better inhibitor than 2-deoxystreptamine (Table VII). The antimicrobial activity reported for this series is gentamicin C₉ > gentamicin C₈ > garamine, while 2-deoxystreptamine has no activity.

Differences between enzymatic and antimicrobial activity do exist, however. Methylation at the 6' position significantly increases V and decreases V/K of the gentamicins (Table III) but causes very little of any effect on the antimicrobial activity (20). Hydroxylation at the 3' or 4' position (or both) decreases V and V/K (Table V), but the presence or absence of hydroxyl groups at these positions does not have a marked effect on the biological activity. This is shown by the similar antimicrobial activity of kanamycin B, tobramycin, gentamicin C₉, and the semisynthetic aminoglycoside 3',4'-dideoxykanamycin B (20). Degradation products of the neomycins show a different activity profile for antimicrobial versus enzymatic activity. All four rings yield maximal antimicrobial activity, followed by three rings (ribostamycin) and then two (neamine) (17). In the binding order of these degradation products to the enzyme (Table VII), ribostamycin is clearly out of sequence, binding less well than either neamine or neomycin.
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provide a guide in these efforts, and suggest that alkylation at the 6' and hydroxylation at the 4' position would be the most promising modifications.  

REFERENCES


4 Hydroxylation at the 3' position renders aminoglycosides susceptible to phosphorylation by other inactivating enzymes and is therefore not as advantageous as hydroxylation at 4' position (16).
Substrate specificity and structure-activity relationships of gentamicin acetyltransferase I. The dependence of antibiotic resistance upon substrate $V_{\text{max}}/K_{\text{m}}$ values.

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