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# Structure-Activity Relationships Among the Aminoglycoside Antibiotics: Role of Hydroxyl and Amino Groups

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The close structural similarity of several of the deoxystreptamine-containing aminoglycoside antibiotics (gentamicins, neomycins, kanamycins, tobramycin) and the recent isolation of enzymatically *N*-acetylated aminoglycosides have permitted a systematic comparison of the structure-activity relationships in this group of antibiotics. The number and location of amino groups on the hexoses and the site of attachment of the other rings to deoxystreptamine have been shown to exert a profound effect on the ability of these compounds to inhibit or to cause misreading of polypeptide synthesis in vitro. The conclusions allow certain predictions to be made concerning the interaction of these compounds with the bacterial ribosome.

Most studies on structure-activity relationships among antibiotic analogues have been carried out on whole cells. Although this approach is important in finding new antibiotics, it does not always give exact structure-activity information since many compounds may not enter the cell. Studies of antibiotics at their site of action have been somewhat limited, although cell-free systems which carry out the accurate synthesis of macromolecules are available. One such system is protein synthesis, where a variety of antibiotics have been useful in mechanistic studies.

The aminoglycoside-aminocyclitol antibiotics (streptomycin, spectinomycin, neomycins, paromomycin, kanamycins, gentamicins, tobramycin) inhibit protein synthesis through an interaction with the 30*S* ribosomal subunit (12). Structure-activity studies with these antibiotics might provide useful information on the chemistry of the ribosome, as well as suggesting routes for synthetic or semisynthetic production of antibiotics.

Past studies have shown marked differences in activity between different classes of these antibiotics, but recently a number of new deoxystreptamine antibiotics and some enzymatically modified derivatives of gentamicin, kanamycin, and neomycin have become available. A study of the effects of these compounds on protein synthesis in vitro has provided more clues as to the functional groups necessary for maximal biological activity.

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## MATERIALS AND METHODS

**In vitro R17 phage ribonucleic acid (RNA)-directed polypeptide synthesis.** Reaction mixtures (100 μliters) contained 66 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.8 at 5 C), 9 mM magnesium acetate, 50 mM $NH_4Cl$, 2.6 mM adenosine triphosphate, 60 μM guanosine triphosphate, 10 mM phosphoenolpyruvate, 2 μg of pyruvate kinase, 10 mM 2-mercaptoethanol, 3.3 mM dithiothreitol, 0.5 mM of each of the 20 amino acids (excluding valine), 50 μg of transfer RNA, 4 nmol of [$^{14}$C]valine (25 μCi/μmol), and 1.6 $OD_{260}$ (optical density at 260 nm) units of a preincubated S-30 extract (8) of *Escherichia coli* MRE-600 (prototroph, RNase$^-$). Incorporation was started by the addition of 1 $OD_{260}$ unit of R17 phage RNA. Incubation was carried out for 40 min at 37 C; then 1.5 ml of 10% trichloroacetic acid was added and the tubes were heated at 90 C for 20 min, cooled, filtered on glass fiber disks, and counted in a liquid scintillation counter.

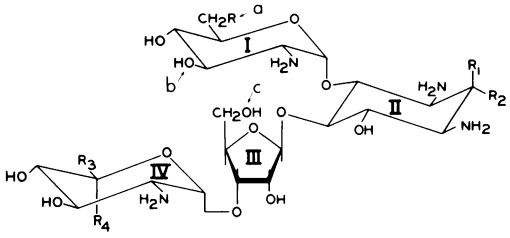
**Misreading of poly U.** Reaction mixtures (100 μliters) were the same as above except 13 mM magnesium acetate was used and 0.53 nmol of [$^{14}$C]-isoleucine (128 μCi/μmol), 0.2 nmol of [$^{14}$C]tyrosine (330 μCi/μmol), and 0.43 nmol of [$^{14}$C]serine (156 μCi/μmol) were added instead of [$^{14}$C]valine. Incorporation was started by the addition of 10 μg of polyuridylic acid (poly U). Incubation was carried out for 15 min at 37 C, then 1.5 ml of 10% trichloroacetic

acid was added and the tubes were heated at 90 C for 20 min, cooled, filtered, and counted.

**Sources of antibiotics.** Gentamicin A, $C_1$, $C_{1a}$, $C_2$, the gentamines, and sisomicin were provided by David Cooper and Peter Daniels of the Schering Corp., tobramycin by Kay Koch of Lilly Research Laboratories, kanamycins A and B by the late A. Gourevitch of Bristol Laboratories, kanamycin C by H. Umezawa, neomycin and neamine by G. B. Whitfield of the Upjohn Co., paromomycin and butirosin by T. H. Haskell of Parke-Davis, methyl neobiosaminide B by Kenneth Rinehart, and ribostamycin by T. Wakazawa. Synthetic substituted deoxystreptamines were provided by Ray Lemieux. 2′-*N*- and 6′-*N*-acetylgentamicin $C_{1a}$ (1), 3-*N*-acetylgentamicin $C_{1a}$ (3), 6′-*N*-acetylkanamycins A and B (1), 6′-*N*-acetylneomycin B (1), and gentamicin $C_{1a}$-adenylylate (2) were prepared by using enzymes obtained from resistant bacterial strains as previously described.

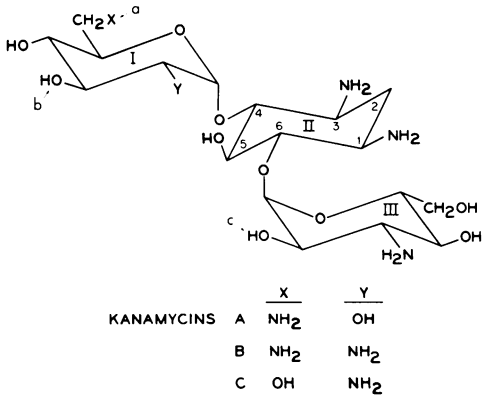
## RESULTS

The deoxystreptamine-containing aminoglycoside antibiotics include the neomycins and paromomycins (Fig. 1), kanamycins (Fig. 2), gentamicins (Fig. 3), and tobramycin (Fig. 4). In order to obtain a clearer picture of the structure-activity relationships in this group of antibiotics, we decided to test these compounds, certain derivatives, and their degradation products for inhibition of phage RNA-directed protein synthesis and for misreading of poly U.



| | R | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|
| NEOMYCIN B | $NH_2$ | H | H | H | $CH_2NH_2$ |
| NEOMYCIN C | $NH_2$ | H | H | $CH_2NH_2$ | H |
| HYBRIMYCIN A1 | $NH_2$ | OH | H | H | $CH_2NH_2$ |
| HYBRIMYCIN A2 | $NH_2$ | OH | H | $CH_2NH_2$ | H |
| HYBRIMYCIN B1 | $NH_2$ | H | OH | H | $CH_2NH_2$ |
| HYBRIMYCIN B2 | $NH_2$ | H | OH | $CH_2NH_2$ | H |
| PAROMOMYCIN | OH | H | H | $CH_2NH_2$ / H | H / $CH_2NH_2$ |

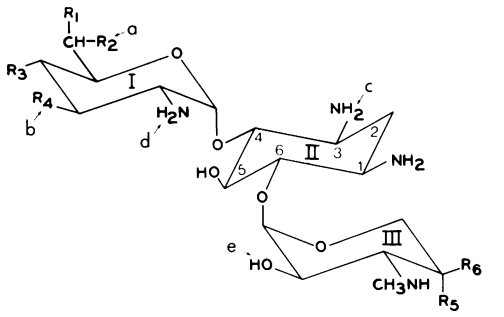
FIG. 1. *Structure of the neomycins, butirosin, ribostamycin, and paromomycin. Neamine (or paromamine) consists of rings 1 + 2, neobiosaminide of rings 3 + 4, and ribostamycin of 1 + 2 + 3. The hybrimycins contain a streptamine ring (hybrimycin $A_1$ and $A_2$) or epistreptamine ring ($B_1$ and $B_2$) instead of 2-deoxystreptamine. Butirosin (1 + 2 + 3) has a 4-amino-2-hydroxybutyryl substituent on N-1 of 2-deoxystreptamine. Arrows indicate where these antibiotics are N-acetylated by kanamycin acetyl transferase to yield 6′-N-acetyl antibiotics (a), and o-phosphorylated by neomycin-kanamycin phosphotransferase (b) and by lividomycin phosphotransferase (c).*



| | | X | Y |
|---|---|---|---|
| KANAMYCINS | A | $NH_2$ | OH |
| | B | $NH_2$ | $NH_2$ |
| | C | OH | $NH_2$ |

FIG. 2. *Structure of kanamycins A, B, and C. Arrows indicate the sites of N-acetylation by kanamycin acetyl transferase to yield 6′-N-acetyl antibiotics (a), the site of o-phosphorylation by neomycin-kanamycin phosphotransferase (b), and the site of o-adenylylation by gentamicin adenylyl transferase (c).*

**Effects of the antibiotics and their enzymatically modified derivatives and degradation products on R17 RNA-directed protein synthesis.** The primary action of aminoglycosides is inhibition of protein synthesis, and R17 RNA-directed synthesis is sensitive to these compounds at low concentrations. In Table 1 are listed typical inhibition data for the compounds tested at various concentrations. It is clear that there is a considerable variation in the activities of these compounds; the greatest levels of inhibition (88 to 96% at the highest concentration tested) are seen with the neomycin-type antibiotics (neomycins B and C, ribostamycin, butirosin, and paromomycin [Fig. 1]). This inhibition has been noted previously (5); neomycin B is the only deoxystreptamine antibiotic which gives close to 100% inhibition at relatively low concentrations. Figures 5 and 6 show representative inhibition curves which indicate that neomycin B inhibits polypeptide synthesis to a greater extent than do the kanamycin or gentamicin antibiotics. It can also be seen that the various acetylated derivatives of gentamicin (Fig. 6), kanamycin (Fig. 5), and neomycin (Table 1) are less potent than their nonacetylated parent compounds. Several of the antibiotics show biphasic curves; the reasons for this are not known. One possibility



| | | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
|---|---|---|---|---|---|---|---|
| GENTAMICINS | A | H | OH | OH | OH | H | OH |
| | $C_{1a}$ | H | $NH_2$ | H | H | OH | $CH_3$ |
| | $C_2$ | $CH_3$ | $NH_2$ | H | H | OH | $CH_3$ |
| | $C_1$ | $CH_3$ | $NHCH_3$ | H | H | OH | $CH_3$ |

FIG. 3. *Structure of the gentamicins. Ring I is purpurosamine, II is 2-deoxystreptamine, and III is gentosamine (gentamicin A) or garosamine (gentamicin C's). Sisomycin is 4,5-dehydrogentamicin $C_{1a}$ (the purpurosamine ring is reduced). In dihydrosisomicin, the carbon #6 group of that ring is inverted (L-sugar). Arrows indicate where this group of antibiotics can be N-acetylated by kanamycin acetyl transferase to yield 6'-N-acetyl antibiotics (a), by gentamicin acetyl transferase II to yield 2'-N-acetyl antibiotic (d), by gentamicin acetyl transferase I to yield 3-N-acetyl antibiotic (c), o-phosphorylated by neomycin-kanamycin phosphotransferase (gentamicin A only) (b); and o-adenylylated by gentamicin adenylyl transferase (e).*

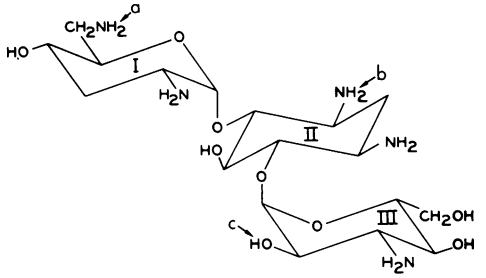


FIG. 4. *Structure of tobramycin (nebramycin factor 6). The arrow indicates where this antibiotic can be enzymatically N-acetylated by kanamycin acetyl transferase to yield 6'-N-acetyl tobramycin (a), by gentamicin acetyl transferase I to yield 3-N-acetyl tobramycin (b), and o-adenylylated by gentamicin adenylyl transferase (c).*

which has been suggested (4, 5) is that the antibiotics can interact with ribosomes at more than one site depending on the drug concentration.

**Effects of the aminoglycosides and their derivatives on poly U-directed misreading.** The activity of the aminoglycoside antibiotics on polypeptide-synthesizing systems from bacteria can be demonstrated in two ways. On one hand the drugs inhibit "natural" messenger RNA-directed synthesis (see above), and on the other the drugs induce translational errors (misreading) in synthetic polymer-directed synthesis. The latter system can be assayed by measuring the extent of incorporation of the amino acids serine, isoleucine, and tyrosine in the presence of poly U and antibiotic. The significance of this effect on the mode of action of the drugs is not clear, but it does provide a very simple and sensitive assay for biological activity (5). Drug-induced misreading often occurs at concentrations of antibiotic which are too low to inhibit protein synthesis, and it is an accurate probe for structure-activity relationships.

All of the aminoglycosides and their deriva-

TABLE 1. *Inhibition of in vitro R17 phage RNA-directed polypeptide synthesis by various aminoglycoside antibiotics and their degradation products*

| Antibiotic | Drug concn (μg/ml)[a] | | | |
|---|---|---|---|---|
| | 0.1 | 1.0 | 10 | 50 |
| Gentamicin $C_{1a}$ | 26 | 45 | 55 | 78 |
| 2'-*N*-acetyl gentamicin $C_{1a}$ | 0 | 7 | 40 | 55 |
| 6'-*N*-acetyl gentamicin $C_{1a}$ | 0 | 0 | 22 | 41 |
| 3-*N*-acetyl gentamicin $C_{1a}$ | 0 | 0 | 9 | 27 |
| Gentamicin $C_{1a}$-adenylylate | 0 | 0 | 0 | 10 |
| Gentamicin $C_1$ | 28 | 37 | 37 | 60 |
| Gentamicin A | 8 | 8 | 30 | 50 |
| Sisomicin | 23 | 41 | 56 | 75 |
| Dihydrosisomicin | 0 | 0 | 0 | 2 |
| Tobramycin | 22 | 45 | 55 | 80 |
| Kanamycin A | 11 | 21 | 50 | 55 |
| B | 22 | 48 | 58 | 82 |
| C | 0 | 9 | 30 | 50 |
| 6'-*N*-acetyl kanamycin A | 0 | 0 | 0 | 4 |
| 6'-*N*-acetyl kanamycin B | 0 | 5 | 14 | 35 |
| 4,6-di-*O*-(α-D-glucopyranosyl)-deoxystreptamine | 0 | 0 | 0 | 0 |
| 4,6-di-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-deoxystreptamine | 0 | 0 | 10 | 20 |
| Neomycin B | 28 | 55 | 80 | 96 |
| C | 10 | 35 | 72 | 88 |
| 6'-*N*-acetyl neomycin B | 0 | 6 | 52 | 76 |
| Ribostamycin | 5 | 45 | 65 | 92 |
| Butirosin | 30 | 60 | 72 | 88 |
| Methyl neobiosaminide B | 0 | 0 | 0 | 10 |
| Paromomycin | 18 | 40 | 65 | 80 |
| Gentamine A[b] | 0 | 9 | 9 | 32 |
| Neamine | 2 | 6 | 37 | 75 |
| Gentamine $C_{1a}$ | 0 | 0 | 32 | 72 |
| Tobramine | 0 | 3 | 30 | 65 |

[a] Expressed as percent inhibition of polypeptide synthesis at four concentrations.

[b] Same as paromamine (Fig. 1).

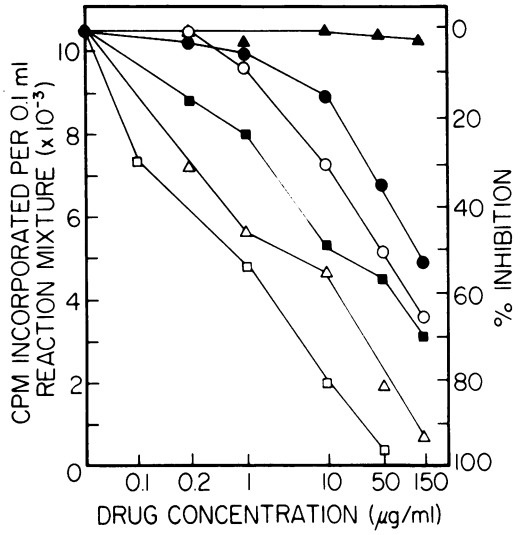



FIG. 5. *Inhibition of in vitro R17 phage RNA-directed polypeptide synthesis by acetylated and unacetylated antibiotics. The incubation conditions are described in Materials and Methods. Symbols:* ▲, *6'-N-acetyl kanamycin A;* ●, *6'-N-acetyl kanamycin B;* ○, *kanamycin C;* ■, *kanamycin A;* △, *kanamycin B;* □, *neomycin B.*

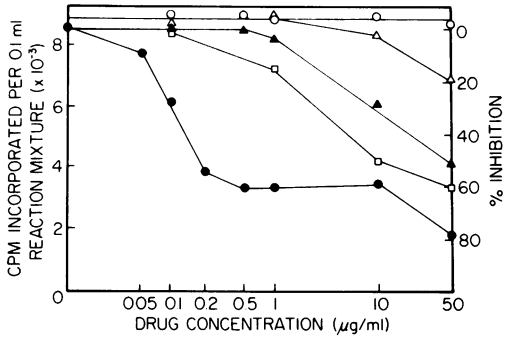



FIG. 6. *Inhibition of in vitro R17 phage RNA-directed polypeptide synthesis by gentamicin $C_{1a}$ and its enzymatically modified derivatives. Symbols:* ○, *gentamicin $C_{1a}$-adenylylate;* △, *3-N-acetylgentamicin $C_{1a}$;* ▲, *6'-N-acetylgentamicin $C_{1a}$;* □, *2'-N-acetylgentamicin $C_{1a}$;* ●, *gentamicin $C_{1a}$.*

tives were tested in this system. Some representative results are shown in Fig. 7 through 9. Again, it is clear that there is a wide variation in drug activity.

## DISCUSSION

Rinehart (10) has noted that the deoxystreptamine-containing aminoglycoside antibiotics can be divided into two classes based on the positions of substitutions of sugar moieties on the deoxystreptamine ring. These two classes are the 1,2 substituted compounds which include the neomycins, paromomycins, ribostamycin, neamine, butirosins, and lividomycins. The 1,3 substituted group includes the kanamycins, gentamicins, and tobramycin. It is

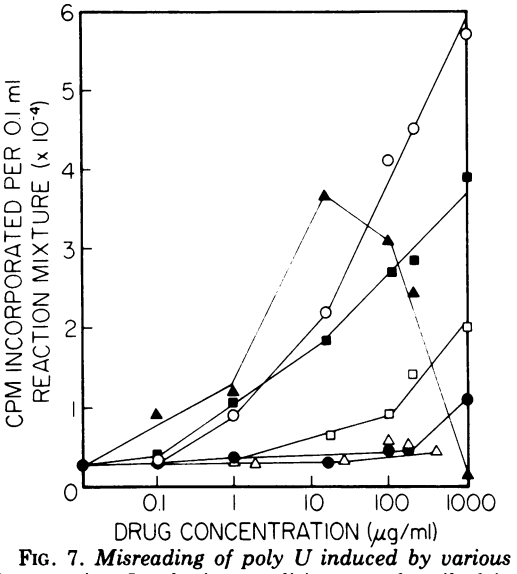



FIG. 7. *Misreading of poly U induced by various kanamycins. Incubation conditions are described in Materials and Methods. Symbols:* ○, *kanamycin A;* ▲, *kanamycin B;* ■, *kanamycin C;* ●, *6'-N-acetyl kanamycin A;* □, *6'-N-acetyl kanamycin B;* △, *4,6-di-O-(6-amino-6-deoxy-α-D-glucopyranosyl)-deoxystreptamine.*

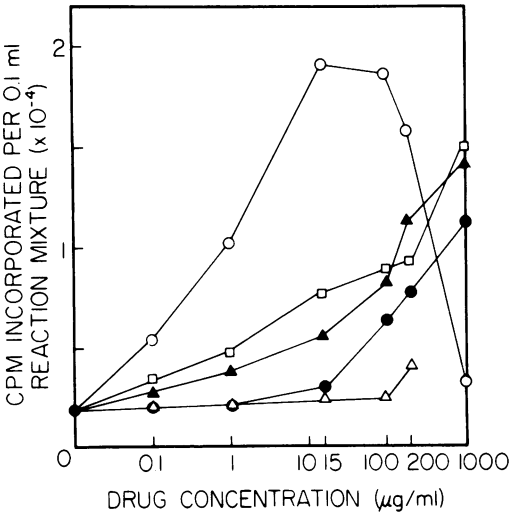



FIG. 8. *Misreading of poly U induced by gentamicin $C_{1a}$ and its enzymatically modified derivatives. Symbols:* ○, *gentamicin $C_{1a}$;* □, *2'-N-acetyl gentamicin $C_{1a}$;* ▲, *6'-N-acetyl gentamicin $C_{1a}$;* ●, *3-N-acetyl gentamicin $C_{1a}$;* △, *gentamicin $C_{1a}$-adenylylate.*

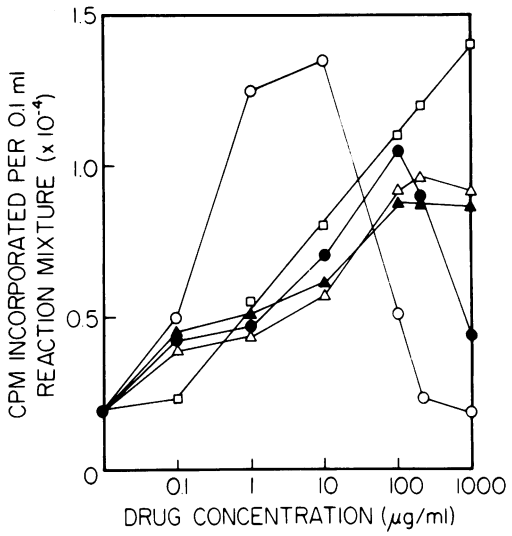



FIG. 9. *Misreading of poly U induced by the neomycin-type antibiotics. Symbols:* ○, *neomycin B;* ●, *neamine;* △, *6'-N-acetylneomycin B;* ▲, *paromomycin;* □, *ribostamycin.*

clear from the data presented in Table 1 that the 1,2 substituted deoxystreptamines are the more potent antibiotics. Since several of the aminoglycosides have close structural similarities, certain conclusions can be drawn about the effects of various amino and hydroxyl moieties on the biological activity of these compounds.

**Effects of amino groups in the hexose linked to the 4-position of deoxystreptamine on the biological activity of the aminoglycosides.** Kanamycins A, B, and C differ only in the number and location of the amino groups in the hexoses in the 4-position (Fig. 2). Kanamycin A has a 6'-amino-6'-deoxy-D-glucose ring, kanamycin C has a 2'-amino-2'-deoxy-D-glucose, and kanamycin B has a 2',6'-diamino-2'6'-dideoxy-D-glucose ring. The extent of inhibition of R17 RNA-directed polypeptide synthesis by these antibiotics is shown in Fig. 5. As can be seen, kanamycin B is a more potent antibiotic than either kanamycins A or C. The presence of a diamino hexose, therefore, results in a compound that is a better inhibitor of protein synthesis than one containing only one amino group. This same conclusion is obtained by comparing neomycin and paromomycin. The data in Table 1 indicate that neomycin is a more potent antibiotic than paromomycin. The only difference between these compounds is that the former contains a 2',6'-diamino-2'6'-dideoxy-D-glucose ring, and the latter a 2'-amino-2-deoxy-D-glucose moiety, at the 4-position of deoxystreptamine (Fig. 1). Kanamycin A is a better inhibitor of protein synthesis than kanamycin C (Fig. 5). Therefore, when only one amino group is present, an antibiotic that contains a 6-amino substituent is more active than one containing a 2-amino substituent.

One of the ways that the aminoglycosides can be modified is by acetylation of various amino groups by enzymes that are present in bacterial strains resistant to these antibiotics (1). It has been observed that the acetylation of an aminoglycoside does not always eliminate biological activity (1). The only aminoglycoside that is inactivated by enzymatic *N*-acetylation of the aminohexose ring in the 6-position is kanamycin A; 6'-*N*-acetylkanamycin A is not an antibiotic (Fig. 5). However, 6'-*N*-acetylkanamycin B still retains considerable biological activity. Since 6'-*N*-acetylkanamycin B very closely resembles kanamycin C in the extent of inhibition of protein synthesis, it seems that acetylation simply neutralizes the contribution of amino groups. As would be expected, 6'-*N*-acetylneomycin B is very similar to paromomycin in biological activity. It can thus be concluded that at least one amino group is required in the aminohexose at the 4-position for the compound to retain biological activity.

This same pattern of relative activity is observed with the gentamicins and their acetylated derivatives (Fig. 6). Gentamicin $C_{1a}$, which contains two amino groups in the purpurosamine ring (Fig. 3), is the most potent antibiotic, followed by 2'-*N*-acetylgentamicin $C_{1a}$ (which has a free 6'-amino group) and by 6'-*N*-acetylgentamicin $C_{1a}$ (which has a free 2'-amino group). To summarize: antibiotic activity can be related to the number and location of amino groups in the hexose moiety glycosidically linked to the 4-position of deoxystreptamine as follows (in decreasing order of potency):

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

The patterns of misreading activity are also strongly dependent on the number of amino groups. Those antibiotics with a 2,6-diamino sugar (tobramycin, kanamycin B, gentamicins $C_{1a}$, $C_1$, or $C_2$, neomycins B or C) show a unique pattern of misreading. There is a progressive increase in the level of misreading over a broad concentration range (0.1 to 15 μg/ml, about $10^{-7}$ to $10^{-5}$ M), followed by a marked inhibition of misreading at higher concentrations (Fig. 7–9). The compounds with one amino group (kanamycins A and C, paromomycin, 6'-*N*-acetylneomycin B, 6'-*N*-acetylkanamycin B, 6'-*N*-acetylgentamicin $C_{1a}$, and 2'-*N*-acetylgentamicin $C_{1a}$) do not show this inhibition of

misreading between 15 and 1,000 μg/ml. Compounds with no amino groups (6′-*N*-acetylkanamycin A) do not cause any misreading at concentrations up to 200 μg/ml (Fig. 7).

Streptomycin shows only a very gradual increase in misreading of poly U as the antibiotic concentration is increased from $10^{-6}$ to $10^{-3}$ M (5). Bacterial mutants with altered ribosomes that are resistant to either streptomycin or paromomycin have been obtained, but no ribosomal resistant mutants have been isolated for gentamicins $C_{1a}$, $C_1$, $C_2$, kanamycin B, or neomycin. Perhaps these latter antibiotics have more than one binding site on the ribosome; the second site may be the one responsible for the inhibition of misreading at high concentrations (5). Only those antibiotics with a 2,6-aminohexose show biphasic inhibition and misreading curves. Two mutations might therefore be needed to obtain a strain containing ribosomes resistant to these antibiotics. It may be possible to select for a strain that is resistant to neomycin by starting with one that is resistant to paromomycin. Similarly, kanamycin B- or gentamicin $C_{1a}$-resistant strains might be obtained by selecting first for ribosomal resistance to kanamycins A or C or to gentamicin A (or 2′-*N*-acetylgentamicin $C_{1a}$), respectively. It is not possible to state what role the amino groups play in the interaction of the antibiotic with the ribosome; they could be involved in hydrogen bonding or ionic interactions.

**Effect of hydroxylation of the hexose glycosidically linked to the 4-position of 2-deoxystreptamine.** Kanamycin B and tobramycin are identical in structure except that the latter lacks a 3′-hydroxyl moiety (compare Fig. 2 and 4). Both are equally effective in inhibiting protein synthesis (Table 1). Therefore, the presence or absence of a hydroxyl group at the 3′-position of this hexose does not have a great effect on the activity of these antibiotics. Gentamicin $C_{1a}$ contains purpurosamine (a 3′,4′-dideoxy sugar) (Fig. 3) and is as good an antibiotic as kanamycin B. These three antibiotics differ only slightly in misreading activity (Fig. 7 and 8), they all produce greater misreading with increasing concentrations up to approximately 10 μg/ml, and a further increase in concentration is then strongly inhibitory. It is apparent that the degree of hydroxylation of the sugar in the 4-position of deoxystreptamine has no effect on the activity of these antibiotics.

That the presence or absence of hydroxyl groups does not have a marked effect on the biological activity of these aminoglycosides can also be seen by comparing the activities of neamine, tobramine (3′-deoxy), and gentamine $C_{1a}$ (3′,4′-dideoxy) (see Table 1). These three compounds, which consist of only one diaminohexose linked to deoxystreptamine, have essentially identical biological activity in vitro.

The aminoglycosides lacking the 3′-hydroxyl group are not substrates for neomycin-kanamycin phosphotransferase, an enzyme found in bacteria resistant to neomycin and kanamycin, since this enzyme phosphorylates that hydroxyl group in the aminoglycosides. Since the 3′-deoxy compounds are potent antibiotics, they are currently being used to treat infections involving bacteria that are resistant to kanamycin and neomycin. Phosphorylation of the 3′-hydroxyl leads to complete inactivation since the phosphorylated antibiotic can no longer bind to the ribosome (13). The studies described here suggest that inactivation occurs because the hydroxyl group is replaced by a bulky charged group and not as a result of blocking a functionally important hydroxyl group.

**Effect of other substituents on the deoxystreptamine ring on the biological activity of the aminoglycosides.** Kanamycin B, tobramycin, and gentamicin $C_{1a}$ also differ in the nature of the substituent at the 6-position of deoxystreptamine. The former two compounds have a kanosamine substituent (Fig. 2 and 4), and the latter has garosamine. Since there is very little difference in the in vitro biological activity of tobramycin, kanamycin B, and gentamicin $C_{1a}$, and between gentamicin A and kanamycin C, we conclude that although kanosamine and garosamine have substantial roles in determining antibiotic potency (i.e., compare tobramycin with tobramine and gentamicin $C_{1a}$ with gentamine $C_{1a}$), they are very similar in this respect.

These substituents are important, however, in the recognition of these compounds by the various aminoglycoside inactivating enzymes. For example, the enzyme which acetylates the 3-amino group in the deoxystreptamine ring of gentamicin $C_{1a}$ (Fig. 3) does not recognize this same group in kanamycin B or tobramycin. This specificity is presumably determined by differences in the substituents on the deoxystreptamine ring (3).

The neomycins are structurally different from the kanamycins and gentamicins in that substituents are on adjacent hydroxyls (4, 5) of deoxystreptamine (Fig. 1). Various degradation products of the neomycins allow an assessment of the requirements for biological activity in this series. Table 1 shows that the order of decreasing potency in inhibiting protein synthesis is

neomycin B, neomycin C, butirosin, ribostamycin, neamine, and methyl neobiosaminide B. Therefore, the presence of all four rings yields maximal activity, followed by three rings, and then two. Neamine is a good antibiotic, and methyl neobiosaminide B is not; the latter compound inhibits phage RNA-directed protein synthesis by only 10% at 50 μg/ml. The lack of activity of methyl neobiosaminide B is possibly due to the fact that the $C_6$ substituent is inverted (dihydrosisomicin is inactive) rather than to the replacement of deoxystreptamine by ribose. In this regard it would be interesting to examine the antibacterial activity of methyl neobiosaminide C.

**Role of 2-deoxystreptamine in determining the biological activity of the aminoglycosides.** It has been proposed that the deoxystreptamine ring is the primary determinant of the biological activity of the aminoglycoside antibiotics (7, 11). However, it has been shown that deoxystreptamine can be replaced by streptamine (4), and it is apparent from our results that the sugar substituents on the deoxystreptamine ring play the most important role. We have confirmed this supposition by studies with synthetic compounds provided by R. Lemieux.

These compounds contain a 2-deoxystreptamine ring substituted with various sugars and amino sugars. 4,6-Di-*O*-(α-D-glucopyranosyl)-deoxystreptamine, which consists of two D-glucose moieties glycosidically linked to 2-deoxystreptamine, is completely inactive in inhibiting protein synthesis (Table 1). 4,6-Di-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-deoxystreptamine is only weakly active. Therefore, the presence of a deoxystreptamine moiety is not enough to guarantee antibacterial activity; the deoxystreptamine has to be linked to the appropriate amino sugars. Thus the location of the amino group in the 3-amino-D-glucose moiety (kanosamine) of kanamycin A is important for biological activity. When that sugar is changed to 6-amino-6-deoxy-D-glucose, the compound loses almost all biological activity.

2-Deoxystreptamine has two amino groups. When one of them is enzymatically acetylated by an enzyme found in a gentamicin-resistant strain of *Pseudomonas aeruginosa* (3) to yield 3-*N*-acetylgentamicin $C_{1a}$, the compound is no longer an antibiotic (Fig. 6 and 8). In butirosin, the 1-amino group of deoxystreptamine is substituted with 4-amino-2-hydroxybutyric acid, and this does not substantially reduce biological activity in our in vitro assays. This suggests that the 1-amino group of 2-deoxystreptamine may be unimportant for biological activity; however, studies of other compounds with substituents at this position will be necessary to establish this. Similar substitution of kanamycin results in an antibiotic (BBK-8) which differs little in activity from kanamycin. The importance of this substitution is that it increases anti-*Pseudomonas* activity and makes the compound inert to various forms of inactivation (9).

**General conclusions.** We have shown that the number and location of amino groups in the sugars attached to deoxystreptamine profoundly affect the biological activity of the aminoglycoside antibiotics. The presence of at least one amino group in these sugars is needed for biological activity. The addition of extra substituents to the deoxystreptamine-aminosugar combination (even a ribose moiety as in ribostamycin) can further increase the biological activity of these compounds.

As new aminoglycosides and additional enzymatically modified derivatives become available, it may be possible to derive additional conclusions as to the biological activity of these antibiotics. The structural requirements for substrate activity for the nine aminoglycoside inactivating enzymes that have so far been discovered have been reviewed (R. Benveniste and J. Davies, Annu. Rev. Biochem., in press). Fortunately, the requirements for activity as an enzyme substrate are somewhat different from those for antibiotic activity, or the design of new aminoglycosides that could inhibit resistant bacterial strains would not be possible.

The ribosome is a very complicated macromolecule, and genetic studies with ribosomes from antibiotic-resistant strains of bacteria have proved useful in characterizing certain of the protein components (6). Among the aminoglycoside-aminocyclitol antibiotics there are distinct binding sites for the streptomycin-type compounds, spectinomycin, and kasugamycin, which have been established by studies of resistant mutants. In the case of the antibiotic groups typified by kanamycin and neomycin, it is not known if these two groups share the same binding site or have separate sites. The binding of neomycin to ribosomes affects the binding of streptomycin (13); kanamycin has no effect. The effects of both neomycin and kanamycin B on protein synthesis appear to be the result of multiple binding. The binding of aminoglycoside-aminocyclitols to the ribosome would seem to be the role predominantly of amino groups in these antibiotics.

We have completely ignored one important aspect of the aminoglycoside antibiotics—their toxicity. There is a need for a simple and direct assay which would relate structural require-

ments for antibacterial activity to those responsible for the various toxic effects of aminoglycosides (e.g., nephro- and ototoxicity). If this could be done, the rational design of new and useful aminoglycoside antibiotics would be greatly facilitated.

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