STUDIES ON THE BIOSYNTHESIS OF NEOMYCINS

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by

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ABSTRACT
FACULTY OF SCIENCE
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STUDIES ON THE BIOSYNTHESIS OF NEOMYCINS
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This Thesis is concerned with aminoglycoside antibiotics. Novel antibiotics have been produced using idiotrophs of antibiotic-producing organisms and the biosynthesis of the neomycins has been studied using both wild-type and idiotrophs of antibiotics-producing organisms.

By incubating 2-deoxystreptamine-idiotrophs of *Streptomyces* with the 2-deoxystreptamine analogues 2,4-dideoxystreptamine and 2,4,5-trideoxystreptamine active aminoglycoside antibiotics, lacking the free hydroxyl groups on the aminocyclitol moiety, have been synthesized. It was demonstrated that the compounds produced have antibacterial spectra similar to the parent antibiotics but are less potent.

4-*O*-β-ribosyl-2,6-dideoxystreptamine was also converted into antibiotic by 2-deoxystreptamine idiotrophs of *Streptomyces*. The antibiotic synthesized by the 2-deoxystreptamine-idiotroph of a neomycin-producing organism was shown to be identical to deoxyneomycin. It was concluded that 4-*O*-β-ribosyl-2,6-dideoxystreptamine was converted into antibiotic following hydrolysis to produce 2,4-dideoxystreptamine.

When a 2-deoxystreptamine-idiotroph of the paromomycin-producing organism was incubated with neamine, neomycin was produced. Results from experiments involving the conversion of (^{3}H:^{14}C) neamine into neomycin showed that neamine was converted into antibiotic intact and without prior hydrolysis.

From this latter observation a pathway for neomycin biosynthesis, involving the intermediacy of neamine is proposed.

Acknowledgements

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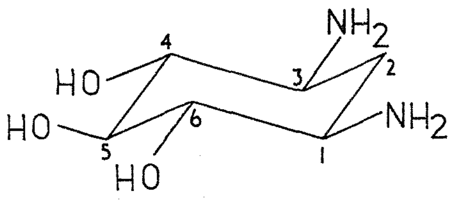
Chapter 1

The metabolism of aminoglycoside-antibiotics by bacteria

1.1 Introduction

The aminoglycoside-antibiotics are a class of compounds each of which is comprised of an aminocyclitol to which is linked sugar derivatives. The majority of the naturally occurring aminoglycosides contain the aminocyclitol 2-deoxystreptamine (Fig. 1.1) to which sugars are joined by glycosidic linkages.

Figure 1.1 The structure of 2-deoxystreptamine



The 2-deoxystreptamine-containing antibiotics can be classified according to which hydroxyl groups are substituted. One group of compounds contain 2-deoxystreptamine with sugar residues linked to the 4-hydroxyl group. Examples of such compounds are neamine and apramycin (Figs. 1.2 & 1.3). In a second group the 2-deoxystreptamine is disubstituted at positions 4 and 5; examples of such compounds are neomycin, paromomycin, ribostamycin and butirosin (Figs. 1.4, 1.5, 1.6 & 1.7). In a third group the 2-deoxystreptamine is again disubstituted, but in this case positions 4 and 6 are involved in the glycosidic linkages. Members of this group include gentamicin, kanamycin and sisomicin (Figs. 1.8, 1.9 & 1.10).

For the sake of clarity the various subunits of the antibiotics have been assigned Roman numerals; thus 2-deoxystreptamine is always referred to as ring II, the 4-substituent is ring I whilst the 5-or 6-substituent is referred to as ring III, and if another sugar is

Figure 1.2 The structure of neamine

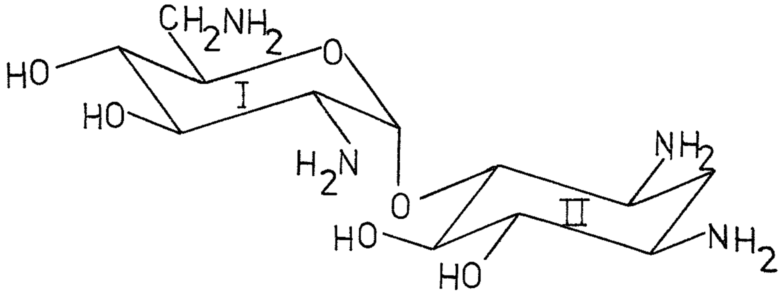


Figure 1.3 The structure of apramycin

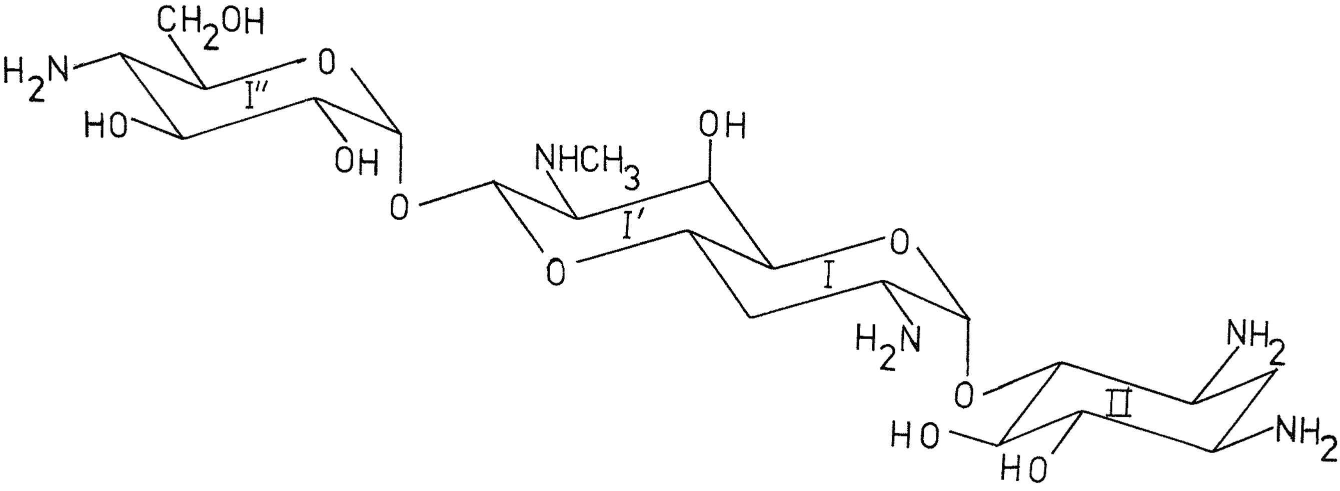
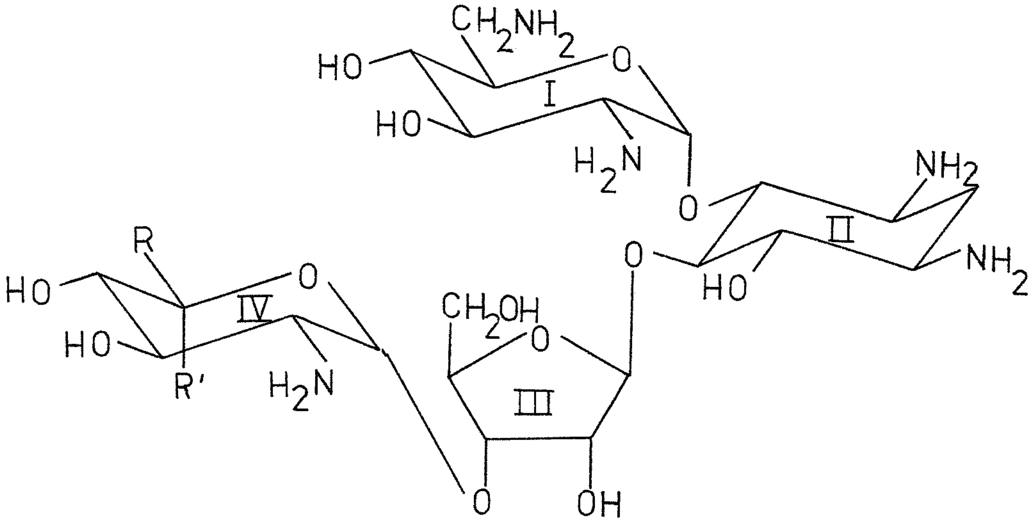
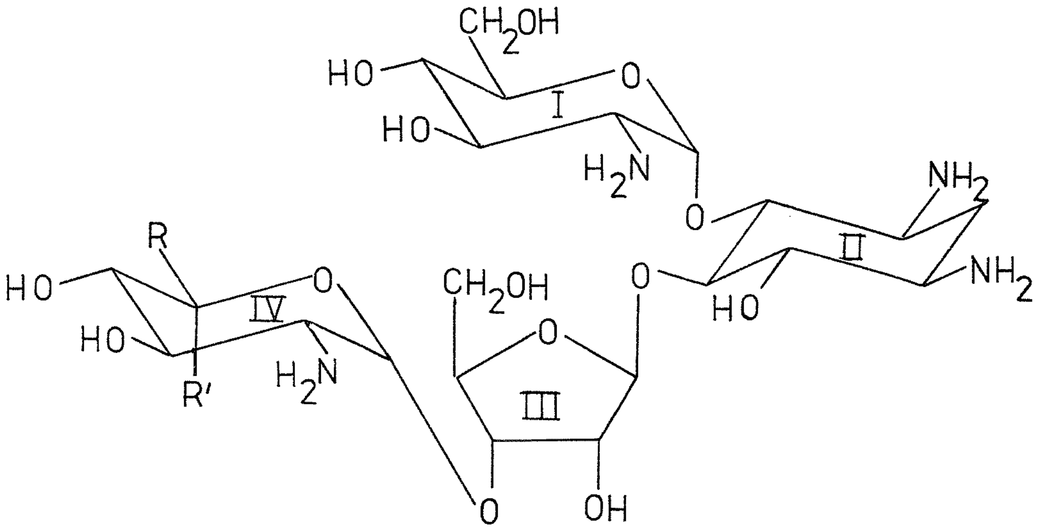


Figure 1.4 The structure of neomycin



	R	R'
neomycin B	H	CH_2NH_2
neomycin C	CH_2NH_2	H

Figure 1.5 The structure of paromomycin



	R	R'
paromomycin I	H	CH_2NH_2
paromomycin II	CH_2NH_2	H

Figure 1.6 The structure of ribostamycin

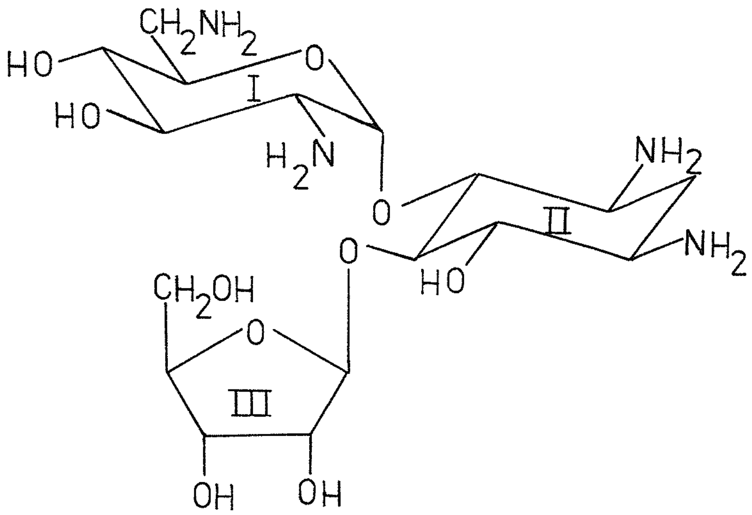


Figure 1.7 The structure of butirosin B

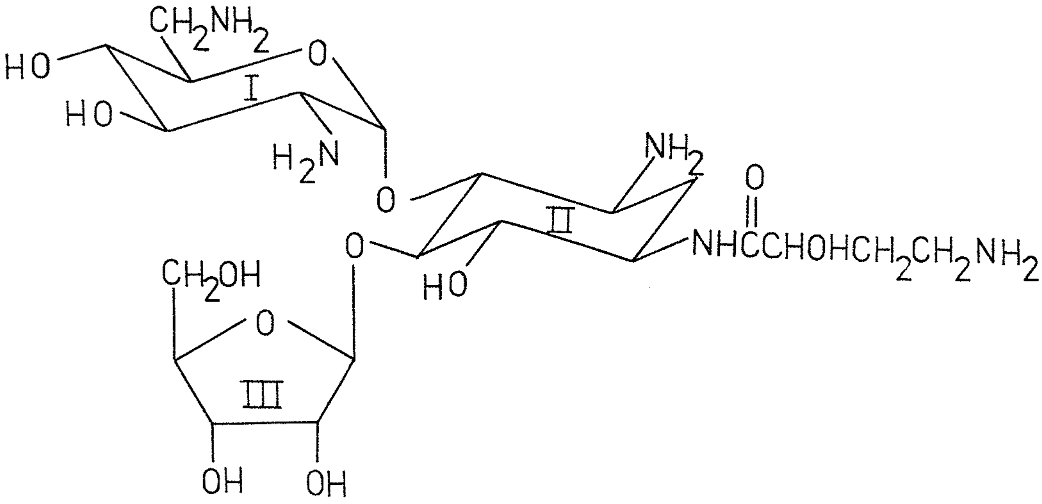


Figure 1.8 The structure of gentamicin C_2

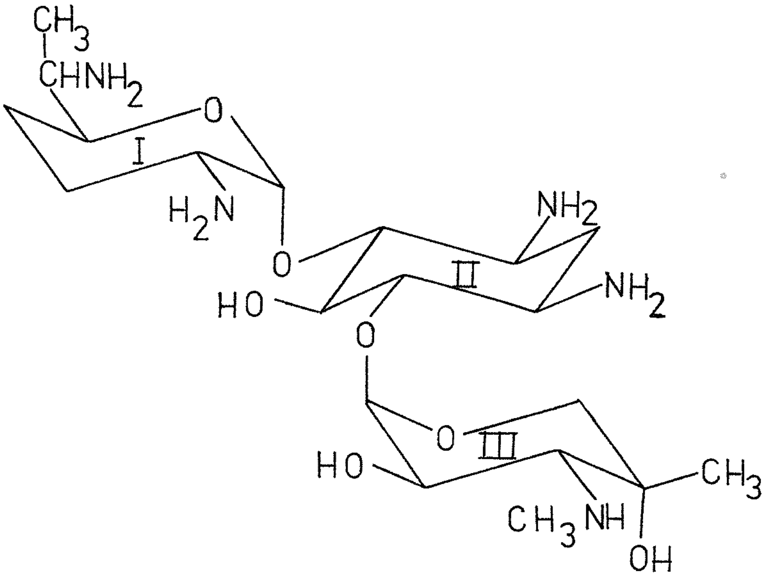


Figure 1.9 The structure of kanamycin B

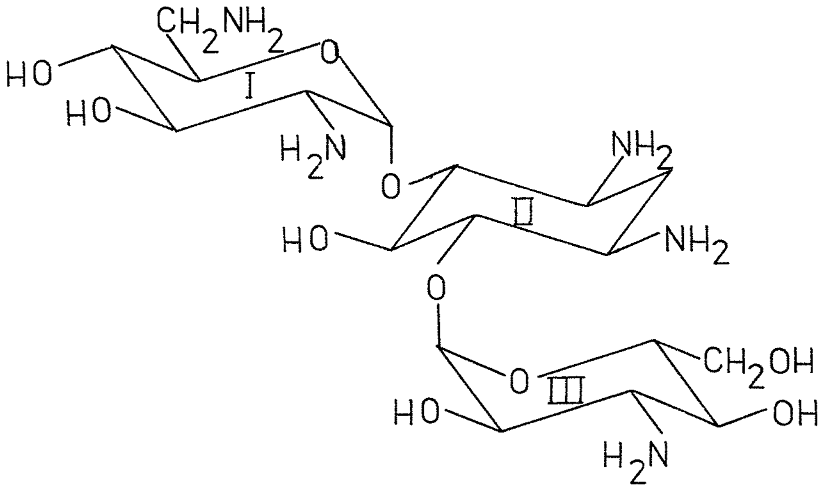
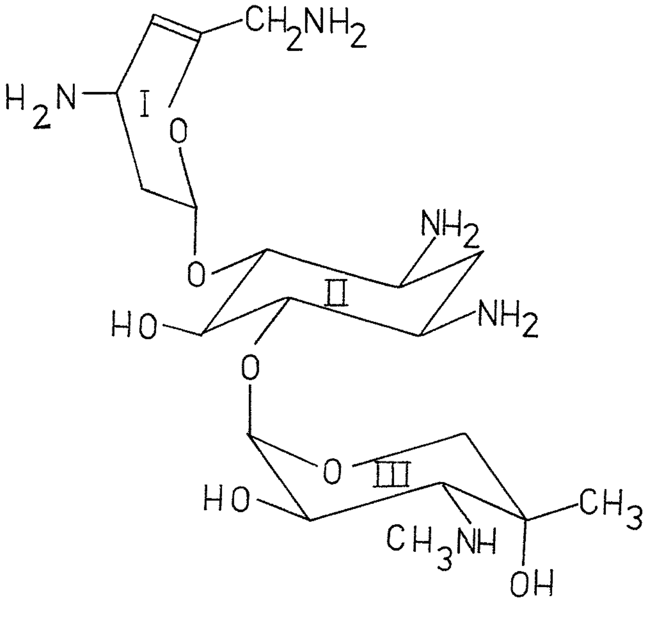


Figure 1.10 The structure of sisomicin



attached to ring III, as in the case of neomycin, this is referred to as ring IV.

Other aminoglycoside-antibiotics are the streptidine-containing streptomycin (Fig. 1.11) and the actinamine-containing spectinomycin (Fig. 1.12).

The vast majority of the aminoglycoside-antibiotics are produced by Actinomycetes. Butirosin is the only known example which is not; this is synthesized by Bacillus circulans.

This chapter is concerned with the biosynthesis of the aminocyclitol antibiotics by bacteria, with emphasis being placed on the biosynthesis of neomycin by Streptomyces fradiae.

1.2 The biosynthesis of neomycin

Neomycin B (Fig. 1.4) is a pseudotetrasaccharide containing two amino sugars, 2,6-diamino-2,6-dideoxy-D-glucose*, (ring I), and 2,6-diamino-2,6-dideoxy-L-idose*, (ring IV); an aminocyclitol 2-deoxystreptamine (ring II); and a five-carbon sugar, D-ribose (ring III). The following studies on the biosynthesis of neomycin (Sebek, 1955; Foght, 1963; Schimbor, 1966; Falkner, 1969) involved incubation of the antibiotic-producing organism S. fradiae with ^{14}C-labelled compounds followed by isolation and degradation of the synthesized neomycin in order to determine where, if at all, the label appeared in the molecule.

1.3 Precursors of the carbon skeleton of neomycin

The first clue as to the origin of the carbon skeleton was provided by the work of Sebek (1955) who reported that 19% of the radioactivity associated with D-[^{14}C]glucose added to one-day old S fradiae cultures was incorporated into neomycin. Although there has been no further report of such high incorporation of D-glucose into neomycin, other workers have shown that D-glucose can provide the complete carbon skeleton.

It was demonstrated by Foght (1963) and by Schimbor (1966) that [1-^{14}C]glucose and [6-^{14}C] glucose were incorporated into neomycin and that each subunit was labelled approximately equally.

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*2,6-diamino-2,6-dideoxy-D-glucose is referred to as neosamine C, and 2,6-diamino-2,6-dideoxy-L-idose is referred to as neosamine B, throughout this thesis.

Figure 1.11 The structure of streptomycin

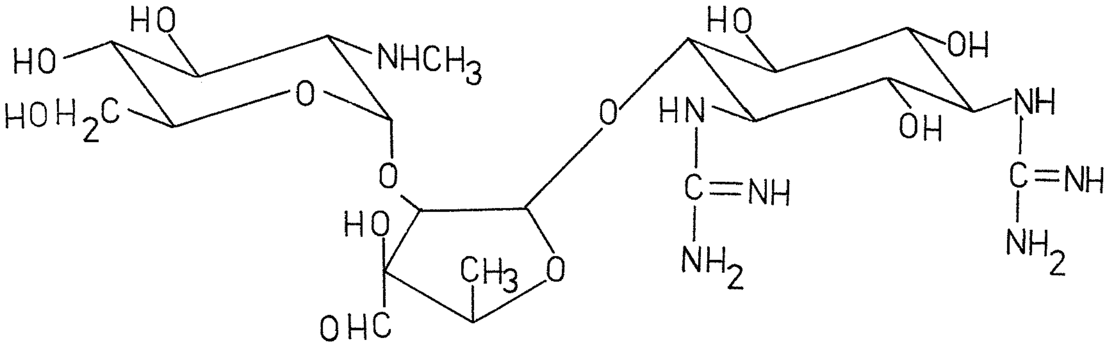
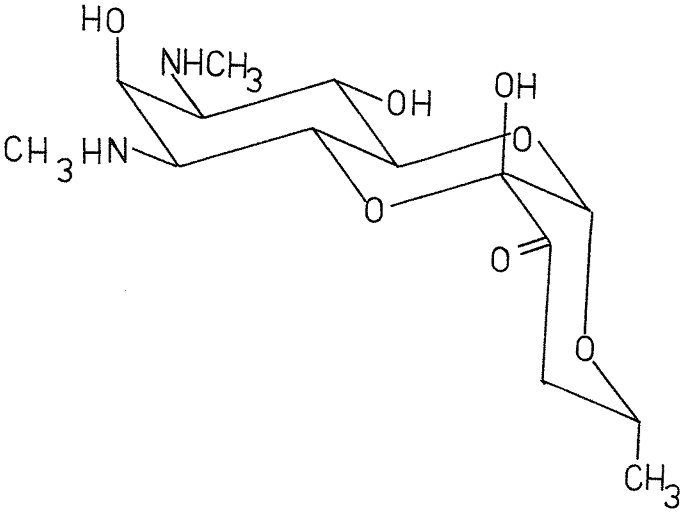


Figure 1.12 The structure of spectinomycin



Foght (1963) also showed that D-[1-^{14}C]glucosamine was incorporated into neomycin to a greater extent than was D-glucose (14.4% compared with 1.4 to 3.6% for glucose). 45% of the total ^{14}C incorporated into neomycin B from D-[1-^{14}C]glucosamine was found in the neosamine B (ring IV) moiety. D-ribose (ring III) contained 1.6% of the incorporated label and the other two subunits (I and II) each contained approximately 25%. D-ribose was also a good precursor for neomycin biosynthesis and was equally incorporated into ribose, which contained 32% of the total ^{14}C in neomycin, and 2-deoxystreptamine, which contained 30% of the total ^{14}C, while neosamine B (ring IV) contained 15% and neosamine C (ring I) contained 25% of the total.

When 2-[1-^{14}C]deoxystreptamine was incorporated into neomycin (52% incorporation), as expected the majority of ^{14}C was in the 2-deoxystreptamine moiety of this molecule.

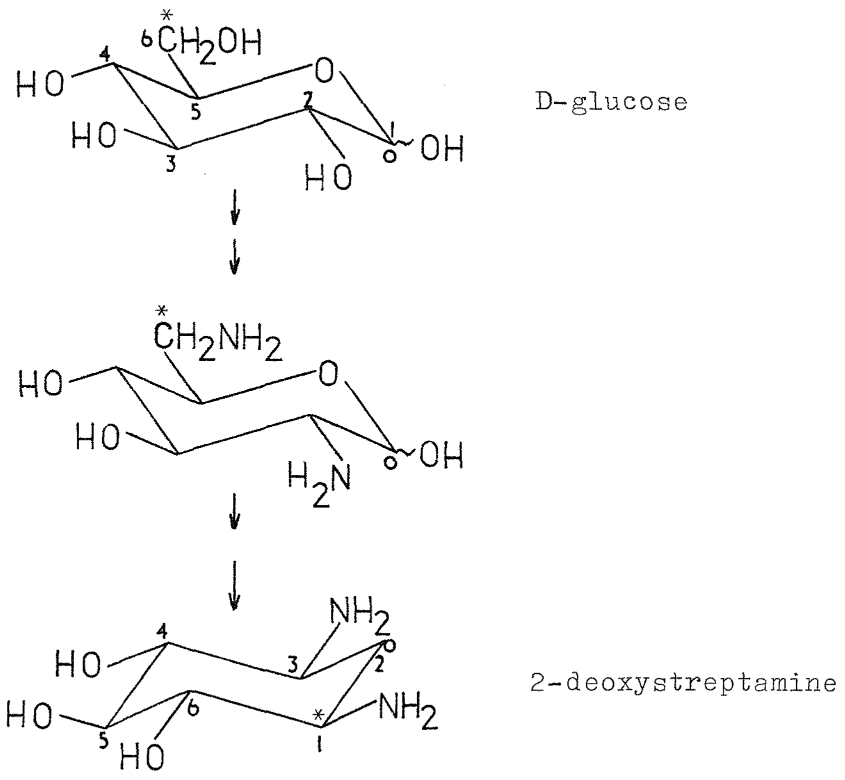
1.4 The biosynthesis of 2-deoxystreptamine

As mentioned above D-glucose is a precursor for the 2-deoxystreptamine moiety of neomycin as synthesized by *S. fradiae* (Foght, 1963; Schimbor, 1966). 2-deoxystreptamine contained 29% of radioactivity from the D-[U-^{14}C]glucose incorporated into neomycin. The demonstration that D-glucosamine (Foght, 1963) and D-ribose (Schimbor, 1966) are incorporated into 2-deoxystreptamine to a greater extent than is D-glucose may be because these former compounds are intermediates in the conversion of D-glucose to 2-deoxystreptamine and as such are more efficiently used. Alternatively the greater incorporation of D-ribose and D-glucosamine into neomycin may be explained by a more efficient uptake of these compounds by *S. fradiae*.

Degradation of neomycin biosynthesized from radioactive precursors showed that D-[1-^{14}C]glucose and D-[6-^{14}C]glucose both labelled 2-deoxystreptamine in the half of the molecule consisting of carbons 1 to 3 (Foght, 1963). This is in agreement with the hypothesis that D-glucose or a derivative of D-glucose cyclises and that a new bond is formed between carbons 1 and 6. Conclusive evidence for this was provided by experiments which demonstrated that D-[1-^{13}C]glucosamine and D-[6-^{13}C]glucose label the adjacent carbon atoms 1 and 2 respectively of 2-deoxystreptamine (Rinehart *et al.*, 1974a).

These observations preclude a pathway in which D-glucose is first converted into D-glucosamine and then cyclised to produce an aminocyclitol (Rinehart, 1964; Fig. 1.13). If this pathway operated D-[1-^{13}C]glucosamine would label position 2 of 2-deoxystreptamine and D-[6-^{13}C]glucose would label position 1 of 2-deoxystreptamine.

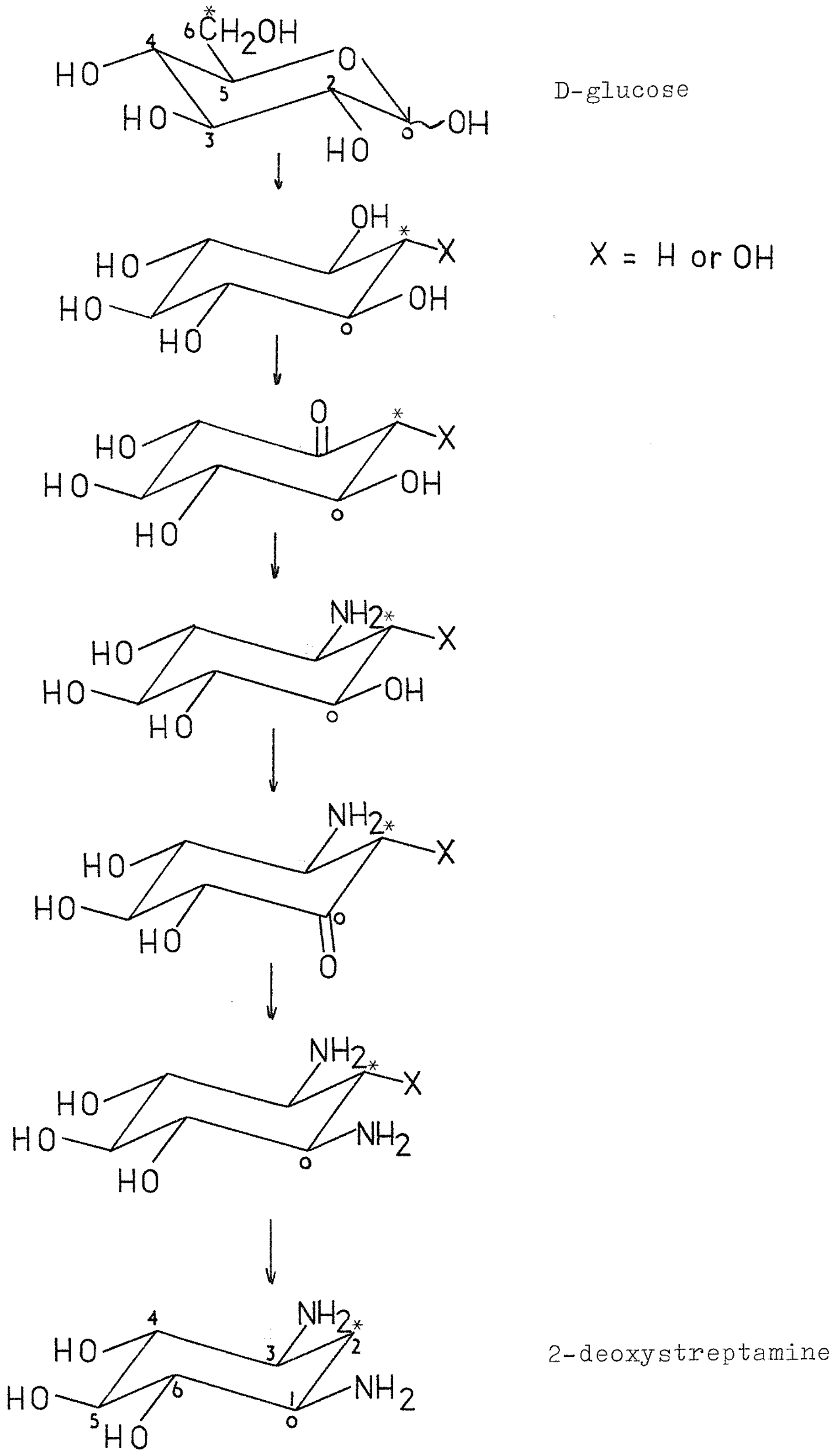
Figure 1.13 2-deoxystreptamine biosynthesis involving functionalized D-glucose and predicted labelling from C-1(o) and C-6(*) of D-glucose



If D-glucose is cyclised prior to amination then D-glucosamine must be deaminated prior to conversion into 2-deoxystreptamine and it has been shown that this does indeed occur (Rinehart *et al.*, 1974b; Rolls *et al.*, 1975). D-[^{15}N]glucosamine added to *S. fradiae* cultures only labelled rings I and IV of the biosynthesized neomycin and was not incorporated into 2-deoxystreptamine. It is therefore possible that radioactive D-glucosamine is converted to D-glucose prior to incorporation into 2-deoxystreptamine.

The exact nature of the cyclitol produced from D-glucose is not known. It has been shown that during the biosynthesis by *S. fradiae* of the streptidine moiety of streptomycin from glucose,

Fig. 1.14 The biosynthesis of 2-deoxystreptamine from D-glucose showing the predicted labelling from radioactive D-glucose

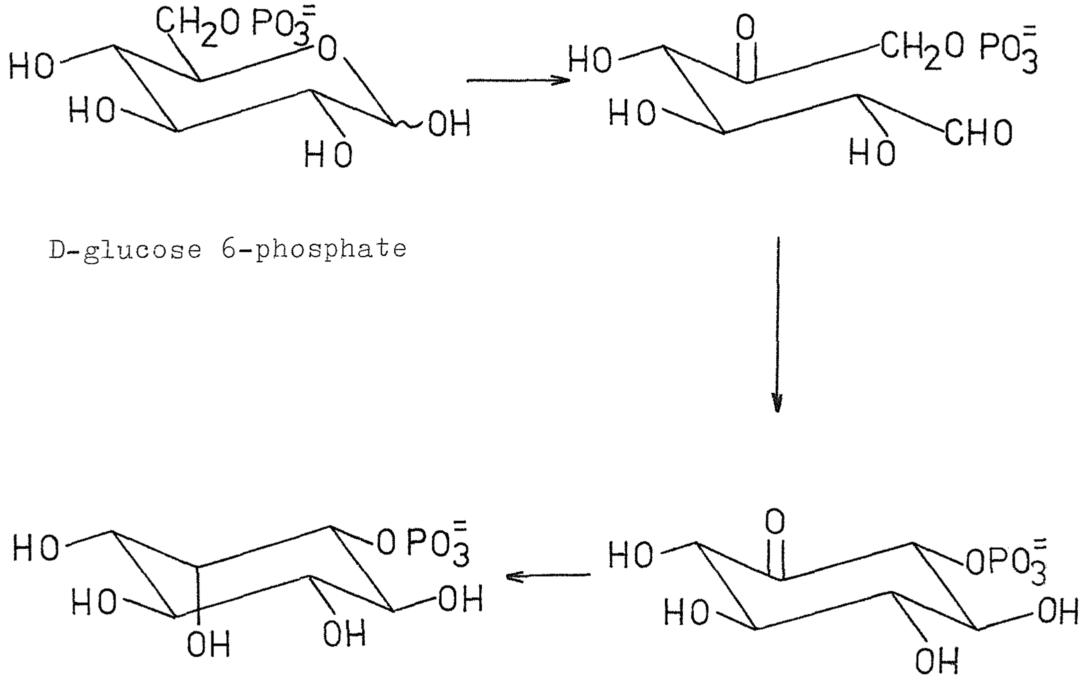


myo-inositol is probably an intermediate (Heding, 1964; Majumdar & Kutzner, 1962) (Fig. 1.17). However, Falkner (1969) failed to demonstrate the incorporation of *myo*- 2-^{14}C inositol into neomycin, thus suggesting that this compound is possibly not an intermediate of 2-deoxystreptamine biosynthesis. Other reasonable explanations for added *myo*-inositol not being incorporated into 2-deoxystreptamine are that it does not enter the organism; alternatively the metabolism of *myo*-inositol may involve enzyme-bound intermediates.

A biosynthetic scheme which explains the data has been proposed by Rinehart & Stroshane (1976) (Fig. 1.14). This reaction sequence involves the cyclisation of glucose or a derivative to an inositol- or inosose-type of intermediate, which is then aminated.

Alternatively 1-phosphate-*myo*-inosose-2 (Fig. 1.15), which has been suggested to be an intermediate in the conversion of D-glucose 6-phosphate into *myo*-inositol 1-phosphate (Loewus & Kelly, 1962), might be aminated (Fig. 1.16), this producing an intermediate similar to the monoaminated compound in Fig. 1.14 but possessing a 2-*O*-phosphate group.

Figure 1.15 Mechanism of inositol cyclase



myo-inositol 1-phosphate

Figure 1.17 Key intermediates involved in the conversion of D-glucose into streptidine

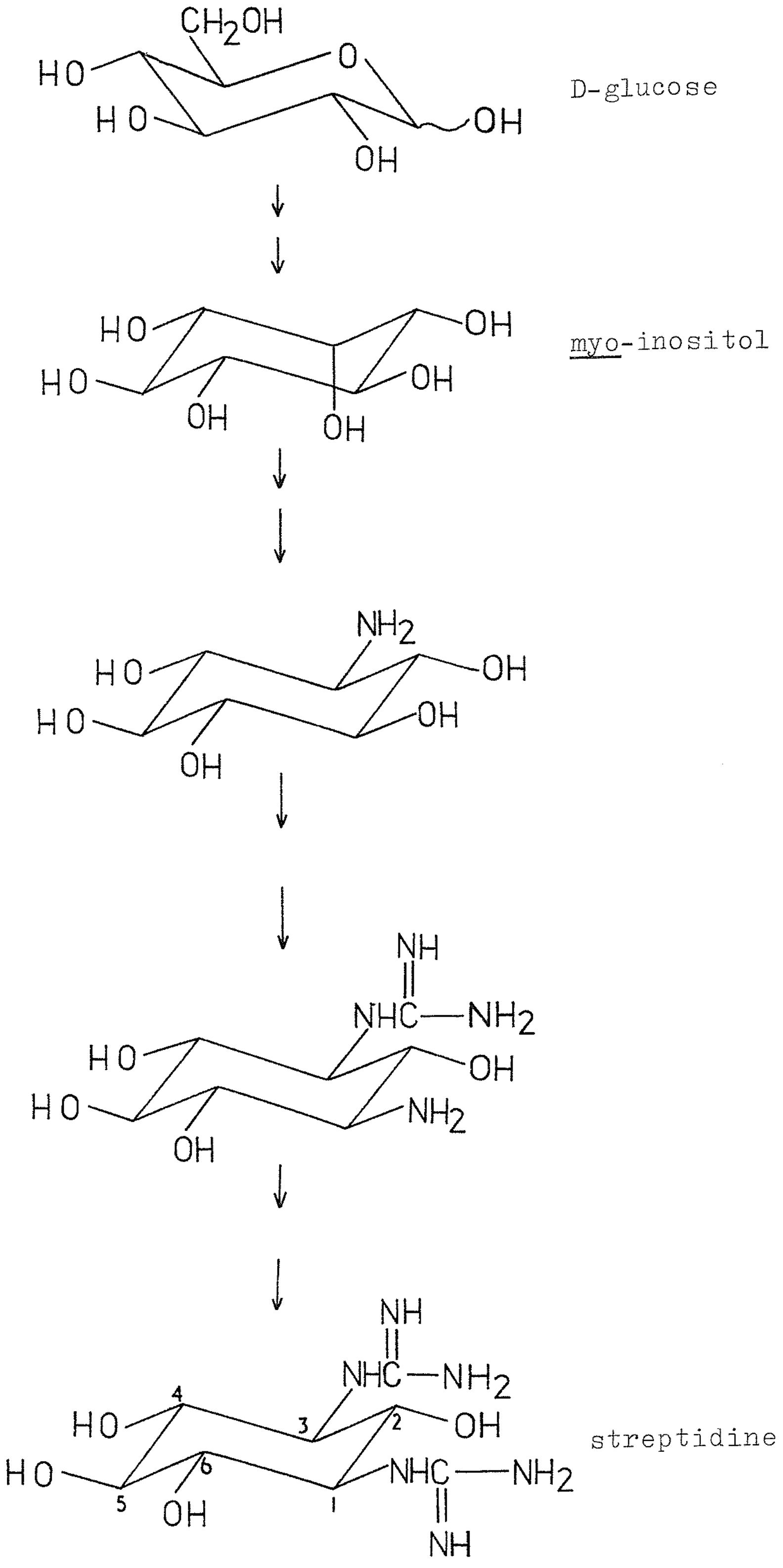
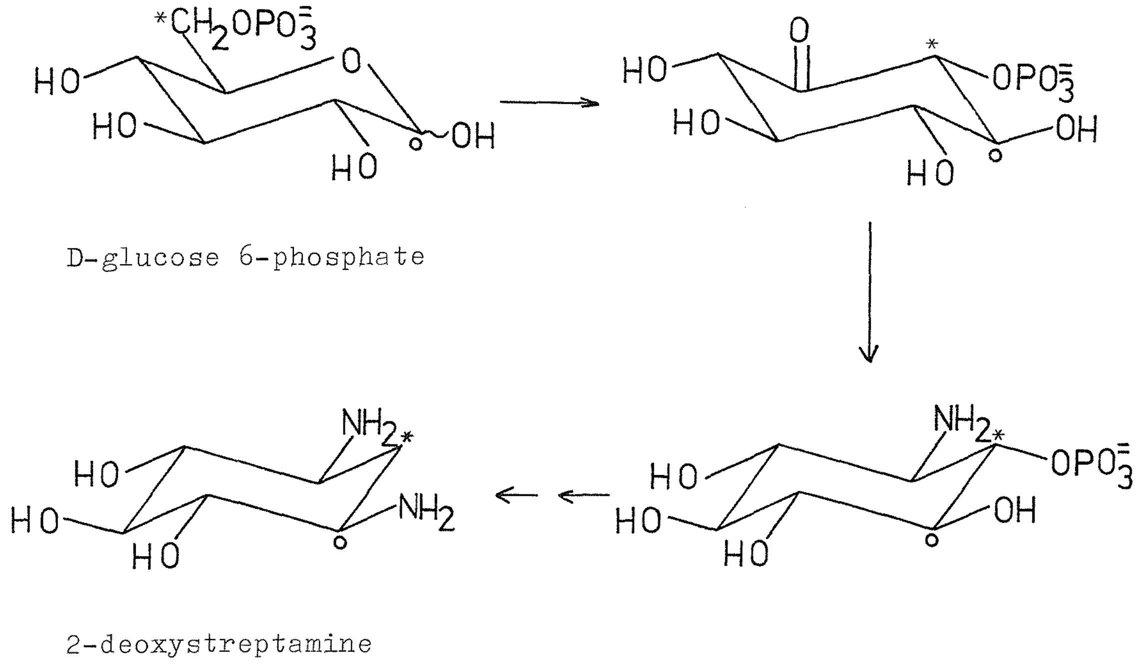


Figure 1.16 The possible role of an inosose derivative in 2-deoxystreptamine biosynthesis



In both of the pathways described in Figs. 1.14 and 1.16 amination at carbon-3 occurs prior to amination at carbon-1, which is favoured by analogy with streptidine biosynthesis (Fig. 1.17). An alternative pathway involves amination of carbon-1 prior to amination of carbon-3 (Fig. 1.18).

Figure 1.18 Alternative route for amination of 2-deoxystreptimine

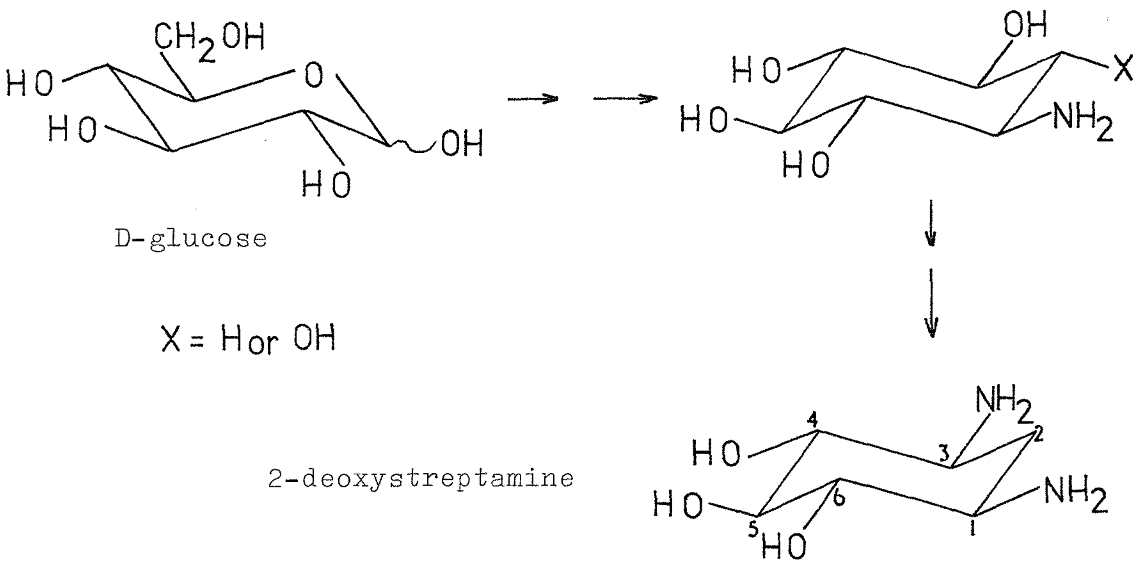
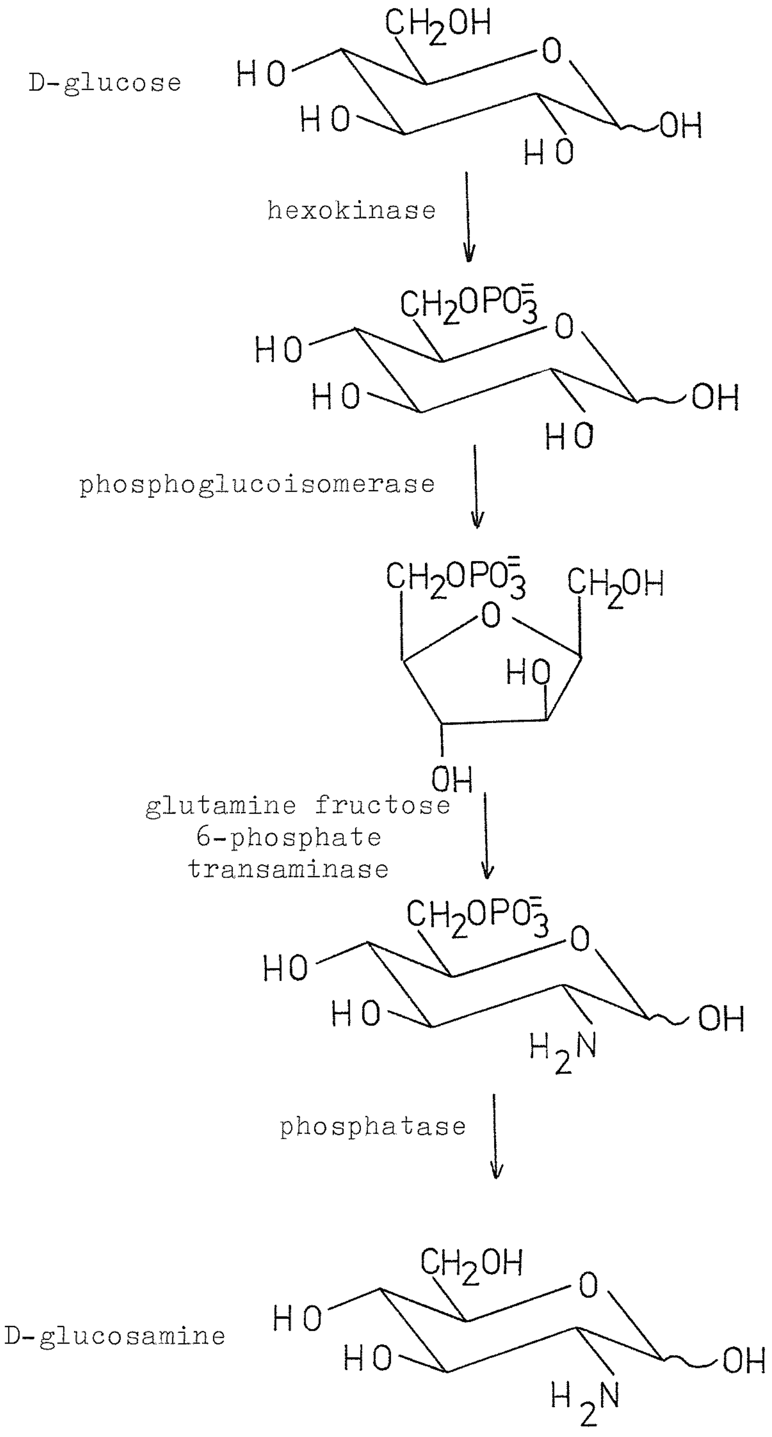


Figure 1.19 The conversion of D-glucose into D-glucosamine



In summary there is evidence that D-glucose is a precursor of 2-deoxystreptamine and is cyclised prior to functionalization. It is not known what the product of cyclisation is and there is no evidence to suggest which of the two amino-groups is added first.

1.5 The biosynthesis of the diamino-hexose moieties of neomycin

D-glucose has been shown to be a precursor of both neosamine C (ring I) and neosamine B (ring IV of neomycin B) (Foght, 1963; Schimbor, 1966). Carbons 1 and 6 of D-glucose were shown to label carbons 1 and 6 respectively of neosamine C and neosamine B demonstrating that the glucose skeleton is incorporated intact (Falkner, 1969).

The incorporation of D-glucosamine into these subunits was greater than the incorporation of D-glucose (Foght, 1963). D-glucosamine is more efficiently incorporated because either it is more readily taken up by the organism or because it is further along the biosynthetic route leading to the neosamines. The distribution of ^{13}C in the subunits biosynthesized from ^{13}C-precursors by S. fradiae confirmed earlier observations that D-glucose and D-glucosamine are incorporated intact into neosamines and that D-glucosamine labels neosamines to a greater extent than does D-glucose (Rinehart *et al.*, 1974a).

An investigation into the incorporation of D-[^{15}N]glucosamine into neomycin by S. fradiae demonstrated that ^{15}N was found only in the neosamine moieties of the antibiotic and suggests that D-glucosamine is incorporated without prior deamination.

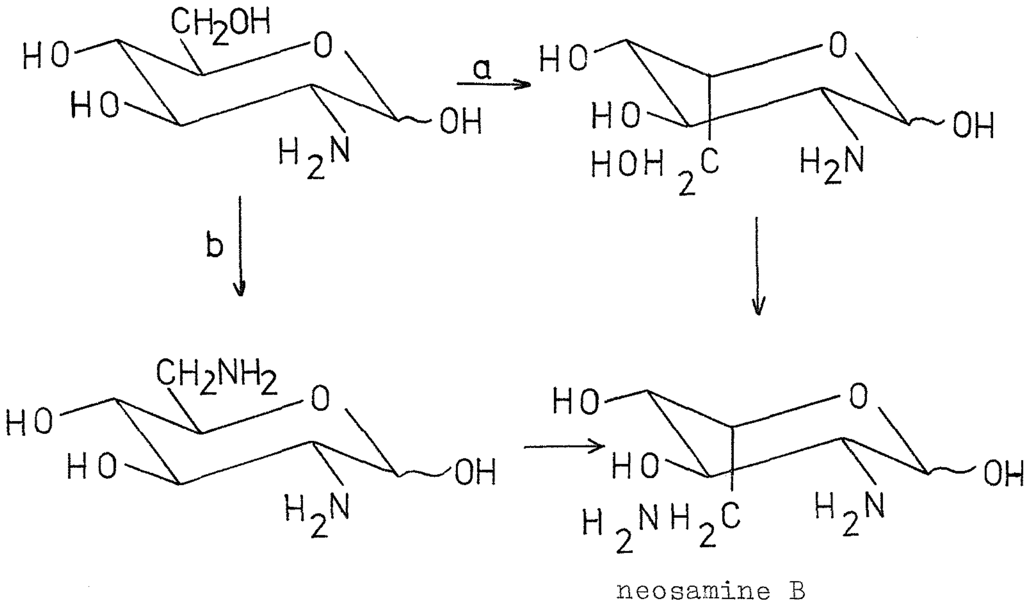
Although nothing else is known about the biosynthesis of the neosamines from D-glucose it is tempting to speculate on the reactions involved.

The conversion of D-glucose into D-glucosamine occurs by a well known pathway (Fig. 1.19). It is possible that S. fradiae synthesizes D-glucosamine from D-glucose by a similar route. The conversion of D-glucosamine into neosamine C involves the replacement of the 6-hydroxyl group with an amino-group. Neosamine B biosynthesis from D-glucosamine is more complicated and requires, in addition to amination at position 6, epimerization to produce the L-idose-configuration.

There are two possible pathways for the conversion of

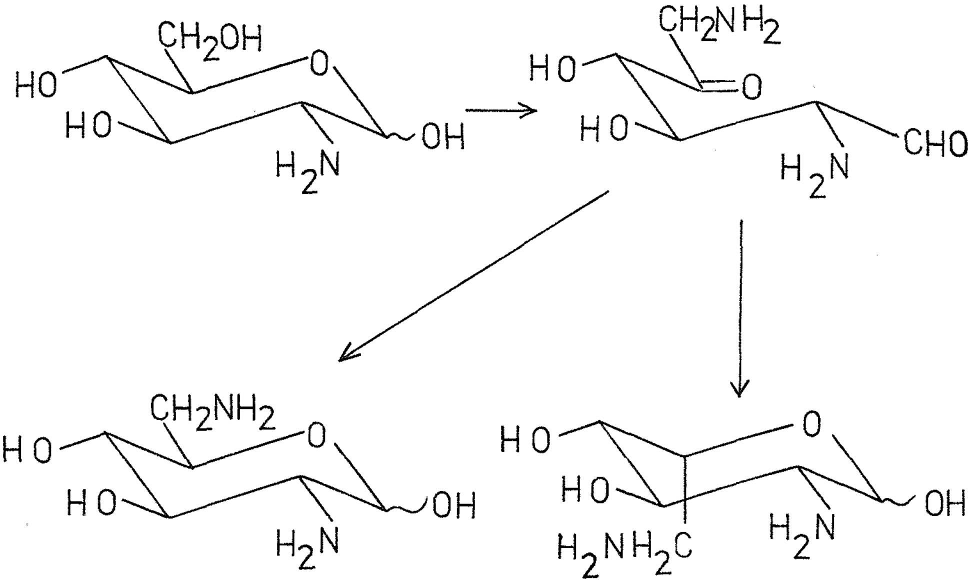
D-glucosamine into neosamine B. In the first (Fig. 1.20a) the carbon skeleton is isomerized to produce an L-idosamine-derivative and this is then aminated to produce neosamine B. In an alternative pathway (Fig. 1.20b) a 2,6-diamino-intermediate is produced prior to isomerization to the L-idose configuration.

Figure 1.20 The conversion of D-glucosamine into neosamine B



Rinehart (1964) favours this second mechanism and also suggests that neosamines B and C are produced by similar pathways differing only in the last reaction (Fig. 1.21). Thus D-glucosamine is aminated at position 6 and converted into a 5-keto-derivative. The reduction of the 5-keto group will produce either neosamine B or C.

Figure 1.21 The biosynthesis of the neosamines from D-glucosamine



neosamine C

neosamine B

1.6 Biosynthesis of the D-ribose-moiety of neomycin

Early observations that carbon-1 of D-ribose may be derived from carbon-1 of D-glucose, and that to a lesser extent carbon-5 of D-ribose may be derived from carbon-6 of D-glucose (Foght, 1963), were later confirmed using ^{13}C precursors (Rinehart *et al.*, 1974a).

These results are explained by the operation of two known pathways. The biosynthesis of D-ribose from D-glucose by the hexose monophosphate pathway (Fig. 1.22) explains the labelling of carbon-5 from D-[6-^{14}C]glucose and the operation of the glucuronate pathway (Fig. 1.23) explains the labelling of carbon-1 of ribose from D-[1-^{14}C]glucose.

Figure 1.22 The labelling of D-ribose synthesized from D-glucose via the hexose monophosphate pathway

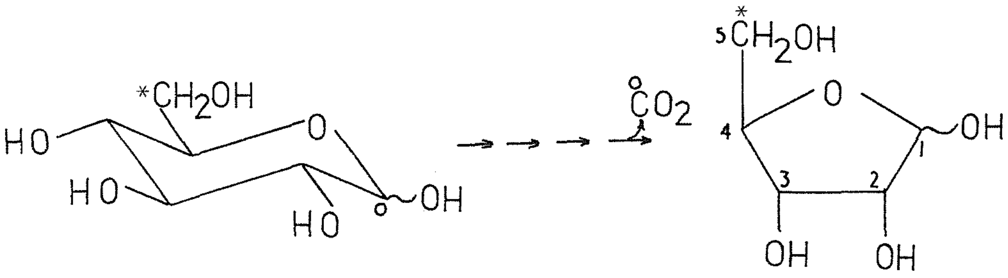
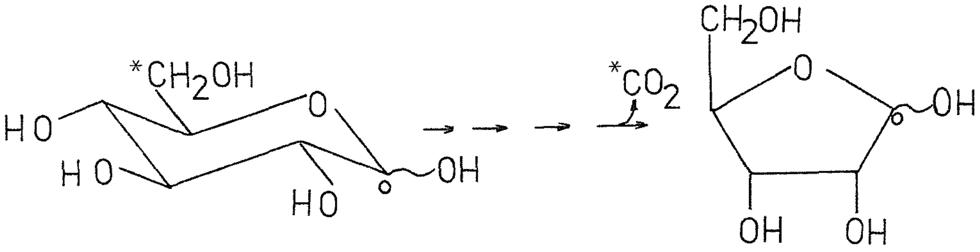


Figure 1.23 The labelling of D-ribose synthesized from D-glucose via the glucuronate pathway



1.7 The subunit-assembly of the neomycins

It is probable that during neomycin-biosynthesis individual subunits are elaborated and these are then assembled to produce the final antibiotic or an immediate precursor. Little is known about the order in which these subunits are assembled.

Schimbor (1966) demonstrated that 35% of ^{14}C from added [^{14}C]neosamine C (ring I) was incorporated by S. fradiae into neomycin, between 75 and 80% of this being associated with the neosamine C moiety, the remainder being found in neosamine B (ring IV) and 2-deoxystreptamine (ring II). It has been suggested (Falkner, 1969) that the neomycin isolated in these experiments was contaminated with neosamine C precursor. In contrast to the results above Falkner (1969) found that less than 8% of added [6-^{14}C] neosamine C was incorporated into neomycin and, whilst the majority of the ^{14}C was associated with the neosamine C moiety, degradation of this subunit showed that the label was accumulated at carbon-1 instead of carbon-6. This is a most unexpected result and warrants re-investigation.

Foght (1963) has demonstrated an incorporation of [^{14}C] neosamine B into neomycin although only 12% of the incorporated radioactivity was associated with this moiety. The reliability of this observation was questioned by Schimbor (1966) who claimed that the precursors used were impure.

When added 2-[1-^{14}C]deoxystreptamine was incorporated by S. fradiae into neomycin (52% incorporation), 85% of the label was in the 2-deoxystreptamine-moiety (Foght, 1963). Further evidence that free preformed 2-deoxystreptamine is a precursor for neomycin biosynthesis is the isolation of 2-deoxystreptamine-idiotrophs of S. fradiae (Shier et al., 1969; see Section 1.11).

Experiments with D-[1-^{14}C]ribose have shown that of the ^{14}C incorporated into the D-ribose-moiety of neomycin by S. fradiae virtually all remained at position C-1, thus suggesting that D-ribose is probably incorporated as a preformed subunit (Schimbor, 1966).

Thus although some results conflict, there is evidence that all of the subunits are biosynthesized independently prior to incorporation into neomycin. In the case of the neosamines, however, further investigation is required.

Figure 1.24 The structure of neobiosamine B

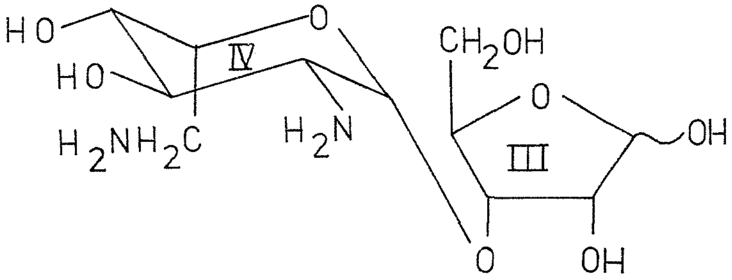
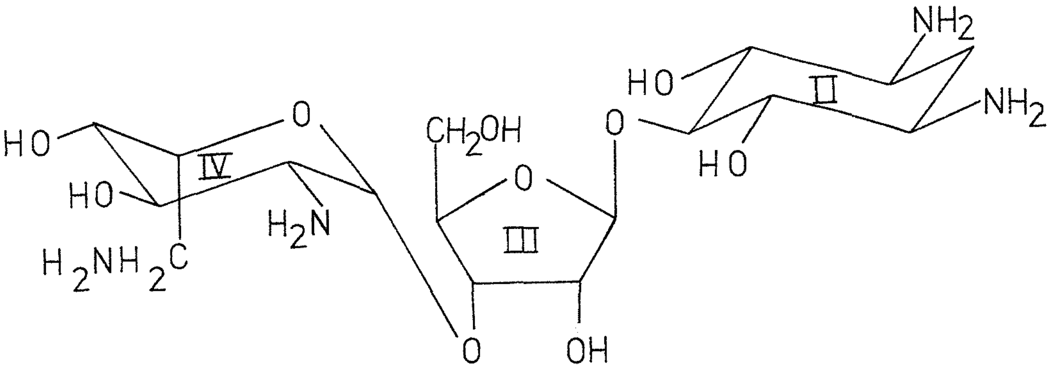


Figure 1.25 The structure of the compound consisting of rings II-III-IV of neomycin B



A result which contrasts with this conclusion is the isolation of paromomycin (which differs from neomycin only with respect to ring I being D-glucosamine) from neomycin samples (Hessler et al., 1970). It is possible that this is a precursor for neomycin-biosynthesis. This would suggest that the biosynthesis of neosamine C may be completed subsequent to the assembly of a basic structure. The completion of subunit-biosynthesis subsequent to the assembly of a basic structure has been proposed to occur during the production of gentamicins and sisomicins (see Section 1. 9). However, as paromomycin was never found to constitute more than 1% of the total neomycin sample it seems unlikely that it is a precursor of the latter antibiotic.

Of the possible intermediates of neomycin biosynthesis from preformed subunits neamine (Fig. 1.2) was the first to be isolated (Peck et al., 1949). Furthermore it was shown that this was not a product of neomycin degradation occurring during the isolation procedure (Perlman & O'Brien, 1953). These workers demonstrated that when S. fradiae was grown on medium to which meat extract or the inorganic fraction of meat extract was added neamine was the principle aminoglycoside produced, neomycin B and C being synthesized in very small amounts. When the meat extract was omitted from the medium neomycin B and C were the principle aminoglycosides produced.

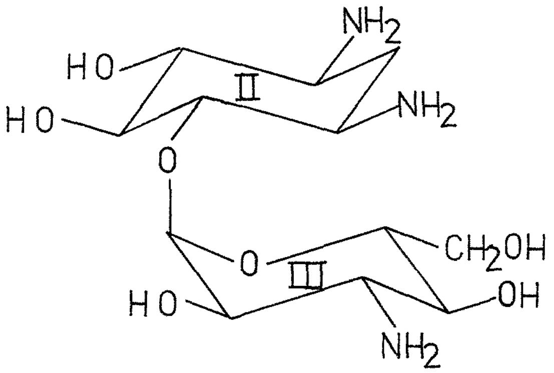
There is no evidence that the other half of the neomycin molecule, neobiosamine (rings III-IV; Fig. 1.24), is produced as a free intermediate by S. fradiae cultures.

A compound corresponding to rings II-III-IV (Fig. 1.25) of neomycin has been isolated from a commercial sample of neomycin (Claes et al., 1974). These authors suggest that the linkage of neobiosamine (ring III-IV) to 2-deoxystreptamine may occur. Alternatively the compound consisting of rings II-III-IV of neomycin could be produced by the linkage of ring IV to rings II-III. It is unfortunate that no comparison between the amount of aminoglycoside consisting of rings II-III-IV and the amount of neamine in the sample was made, although it seems that the former compound is present only in very small quantities.

Ribostamycin, which contains rings I-II-III of neomycin (Fig. 1.6), has been shown to exist in nature, being synthesized by Streptomyces ribosidificus (Shomura et al., 1970; Akita et al.,1970).

2-deoxystreptamine-idiotrophs, organisms unable to produce antibiotic unless 2-deoxystreptamine is added to the growth medium (see Section 1.11), have been used to examine the subunit-assembly of various antibiotics. Shier _et_ _al_. (1974) incubated 2-deoxystreptamine-idiotrophs of _S. fradiae_, a neomycin-producing organism, _S. rimosus_ forma _paromomycinus_, a paromomycin-producing organism, and _S. kanamyceticus_, a kanamycin-producing organism, with the potential intermediates neamine (rings I-II of neomycin and kanamycin B), paromamine (rings I-II of paromomycin and kanamycin C) and 6-kanosamidino-2-deoxystreptamine (rings II-III of the kanamycins; Fig. 1.26). None of the organisms converted any of these compounds into antibiotics. The _S. fradiae_ mutant also failed to convert ribostamycin into any other aminoglycoside.

Figure 1.26 The structure of 6-kanasamidino-2-deoxystreptamine



Various reasons were proposed why the compounds are not converted into antibiotics (Shier _et_ _al_., 1974). The precursors may fail to enter the cell. This is not very probable as neamine is an antibiotic against the producing organisms at a concentration of 100μg/ml. It is possible that neamine is a precursor for the antibiotics but that the antibacterial activity of neamine masks any modification into other antibacterial compounds. Another possibility for the failure of the compounds to support antibiotic production is that the pathway for subunit assembly does not include the compound tested. However in the experiments using the 2-deoxystreptamine-idiotroph of S. kanamyceticus, neither of the two possible intermediates (rings I-II and rings II-III) are converted into antibiotic, thus suggesting that this possibility is unlikely in this case.

Other reasons suggested for the compounds not being converted into antibiotics are that derivatized intermediates may be involved in the biosynthesis or that the intermediates may be enzyme bound. In support of the former proposal is the observation that aminoglycoside-phosphates are produced by antibiotic-synthesizing cultures (see Section 1.8) and hence these phosphate derivatives may be the true intermediates.

In conclusion it has been speculated that neamine is an intermediate in the pathway for neomycin biosynthesis (Rinehart, 1964; Falkner, 1969), although there is no direct evidence for this. Both of these authors also suggest that rings III and IV are joined to produce neobiosamine which is then added to neamine to produce neomycin. Evidence for the existence of neobiosamine as an intermediate is only indirect (Foght, 1963; Schimbor, 1966; Falkner, 1969).

The sequence of subunit-assembly involved in neomycin biosynthesis is discussed at greater length later in this thesis (Section 6.10).

1.8 The role of alkaline phosphatase in aminoglycoside biosynthesis

It is possible that phosphorylated-intermediates are involved in the biosynthesis of aminoglycosides. Aminoglycoside-phosphates are produced by certain antibiotic-biosynthesizing organisms and hydrolysis of these compounds by alkaline phosphatase, also produced by the antibiotic-biosynthesizing organism, releases the antibiotic.

Nomi et al. (1968) have isolated a compound, the L-component, from streptomycin-producing cultures which, on exposure to another component (H) having phosphatase characteristics, was converted into streptomycin. Furthermore Miller & Walker (1969) examined kinase activity in Streptomyces bikiniensis and found an enzyme which phosphorylated streptidine and streptomycin using adenosine triphosphate as phosphate-donor. Similarly Walker & Skorvaga (1973) have shown *Streptomyces griseus* has a kinase capable of *O*-phosphorylating position 3 of the *N*-methyl-L-glucosamine-moiety of streptomycin. These latter workers also demonstrated streptomycin 6-phosphate phosphatase which removes the phosphate from streptomycin 6-phosphate (*O*-phosphorylated at position 6 of streptidine) and from streptomycin 3"-phosphate (*O*-phosphorylated at position 3 of *N*-methyl-L-glucosamine).

A possible advantage of biosynthesizing streptomycin from streptidine phosphate is that the streptomycin phosphate thus synthesized is inactive and could be excreted as such. A high phosphate concentration in the medium would be expected to inhibit dephosphorylation and it has been observed that in these conditions streptomycin biosynthesis was inhibited (Dulaney, 1948).

It has been shown that when *S. fradiae* was grown in the presence of certain amino-acids, and in particular serine or glycine, phosphorylated neomycins B and C accumulated (Majumdar & Majumdar, 1970). When *S. fradiae* mycelia were incubated in the presence of 500μg/ml of these phosphorylated compounds the final neomycin concentration was 325μg/ml, whilst in the absence of the phosphorylated compounds the final neomycin concentration was only 102μg/ml.

It is unlikely that the enzyme found in *S. fradiae* which phosphorylates the 3-hydroxyl group of neomycin (Miller & Walker, 1969) is responsible for the synthesis of the neomycin phosphate observed in the work of Majumdar & Majumdar (1970), which probably has the phosphate linked to the nitrogen of one of the amino groups of neomycin.

There is more evidence that neomycin phosphate is a genuine precursor of neomycin than there is for streptomycin phosphate being a precursor of streptomycin. When *S. fradiae* was grown on three different media it was found that neomycin-formation varied directly with alkaline phosphatase activity (Majumdar & Majumdar, 1971). It was further observed that one of the alkaline phosphatase activities found in mycelial extracts was specific for neomycin phosphate.

The correlation between alkaline phosphatase and neomycin biosynthesis was examined in more detail by Bandyopadhyay & Majumdar (1974). Using washed mycelia of *S. fradiae* they showed that alkaline phosphatase activity and neomycin biosynthesis are both maximal with calcium and magnesium present. D-glucose was shown to inhibit alkaline phosphatase synthesis and neomycin production, and arabinose was shown to stimulate both. Asparagine was shown to be a very good inducer of alkaline phosphatase and stimulated neomycin biosynthesis. Finally chloramphenicol inhibits the production of alkaline phosphatase and neomycin.

In summary there is evidence for neomycin phosphate being an intermediate in neomycin biosynthesis, the last step of the production of this latter compound being catalysed by alkaline phosphatase.

1.9 The biosynthesis of gentamicins and sisomicins

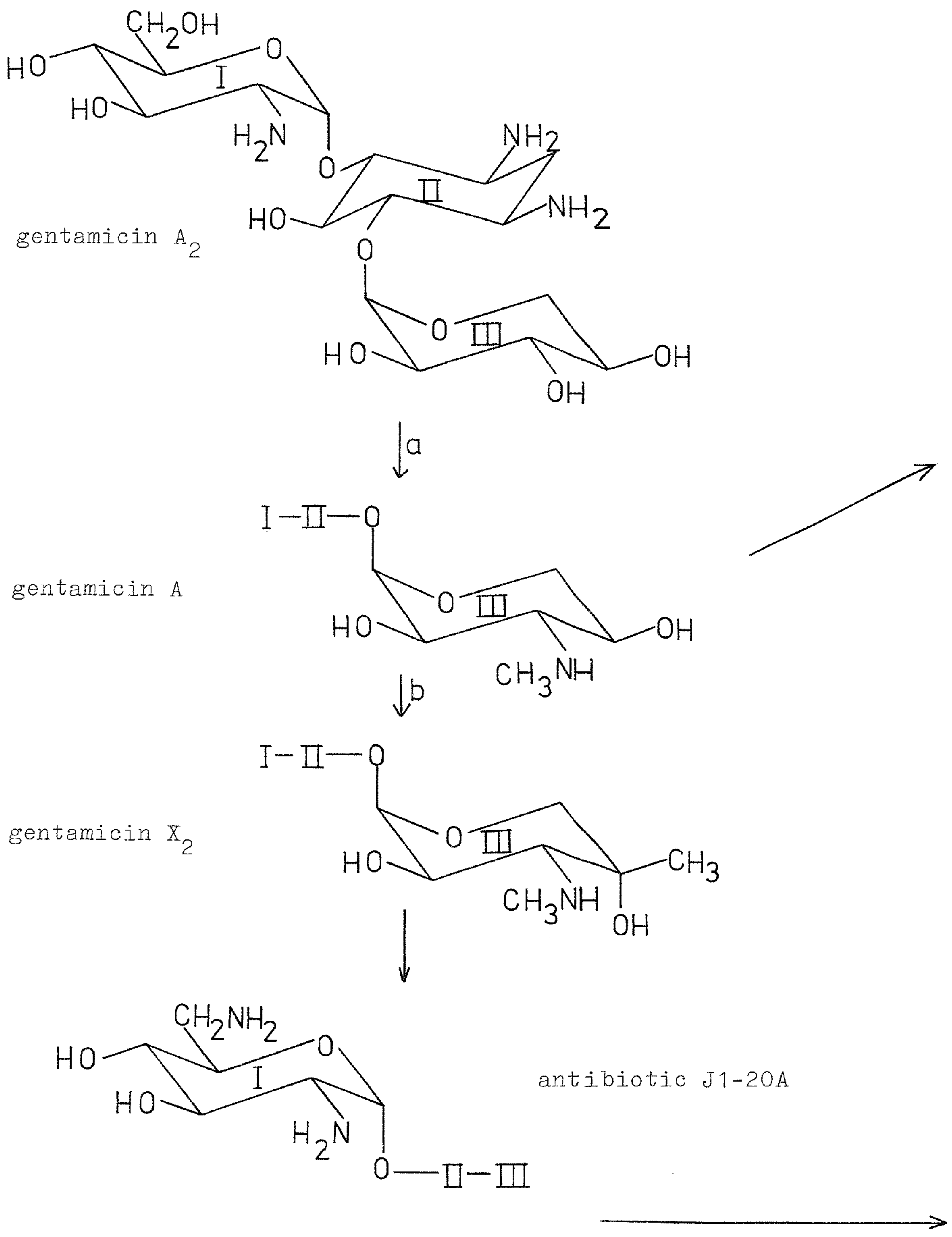
It has been proposed that the individual components of neomycin are biosynthesized and then joined to form the final molecule (Rinehart, 1964; Section 1.7), or the intermediate neomycin phosphate (Majumdar & Majumdar, 1970; Section 1.8) which is dephosphorylated to produce neomycin. In contrast an alternative route has been proposed for the biosynthesis of other aminoglycoside antibiotics in which precursors of the subunits are joined to produce a basic structure. This is then modified to produce the final molecule.

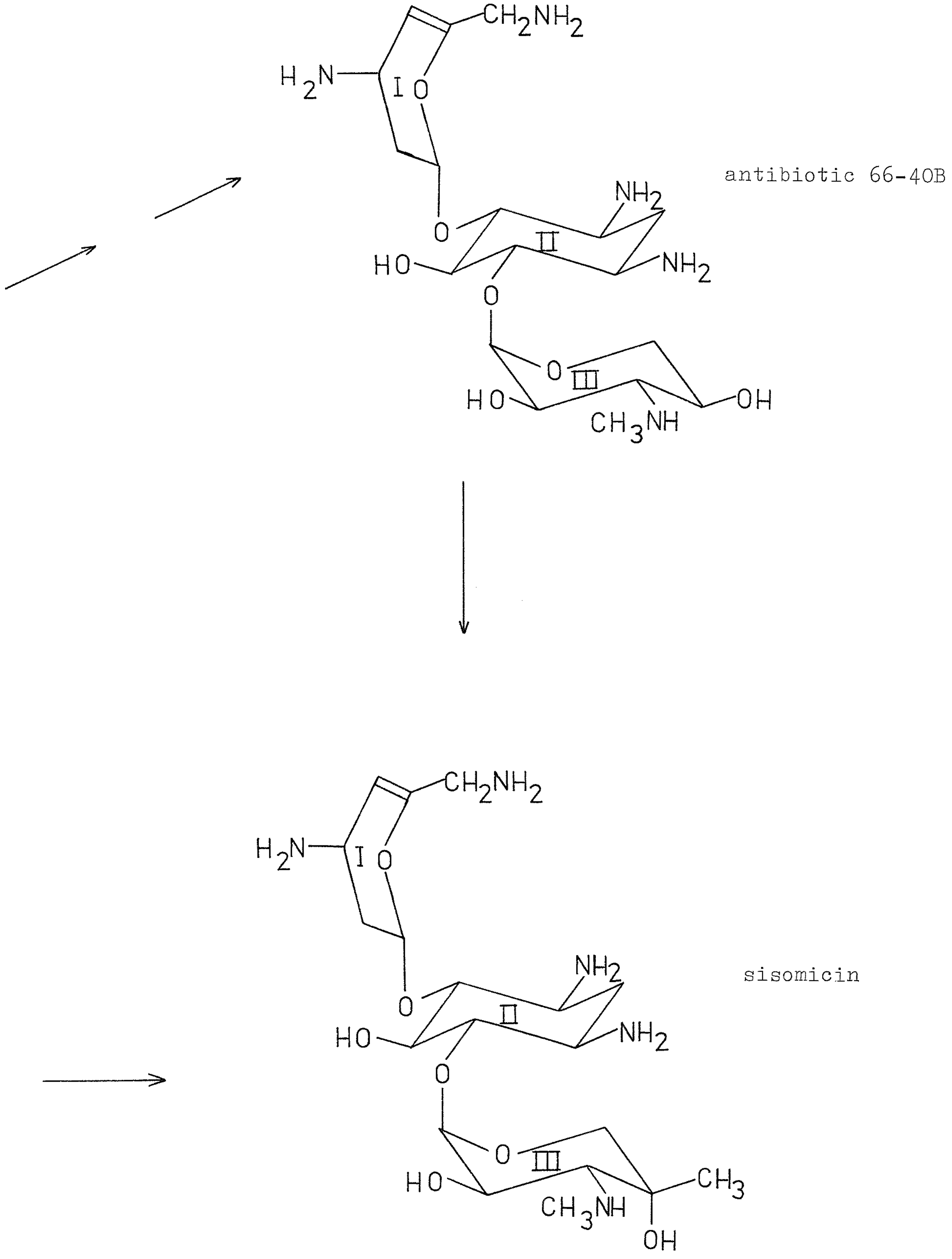
For example it has been suggested that the N-methyl group of the N-methyl-L-glucosamine-moiety of streptomycin is added after N-dimethylstreptomycin has been synthesized (Heding, 1968; Heding & Bajpai, 1973). A similar type of pathway has been proposed for the biosynthesis of the gentamicins and sisomicins.

Small quantities of gentamicins A, A_2 and X_2, and antibiotics J1-20A and 66-40B (see Fig. 1.27 for structures) have been isolated from cultures of the sisomicin-producing organism Micromonospora inyoensis. It has been suggested that these compounds are intermediates of sisomicin biosynthesis and, rather than this latter antibiotic being produced by the assembly of pre-formed subunits, a basic structure is synthesized (probably gentamicin A_2; Fig. 1.27) and this is then functionalized (Testa & Tilley, 1975).

Evidence for this was obtained using a 2-deoxystreptamine-idiotroph (see Section 1.11) of M. inyoensis. This organism was cultured in the presence of possible precursors of sisomicin and modification of these precursors monitored. Sisomicin was produced

Figure 1.27 Sisomicin biosynthesis




CH2NH2
H2N
I
O
O
NH2
II
HO
NH2
O
O
III
HO
OH
CH3NH
antibiotic 66-40B
CH2NH2
H2N
I
O
O
NH2
II
HO
NH2
O
O
III
HO
CH3
CH3NH
OH
sisomicin

from gentamicins A_2, A and X_2, and antibiotics J1-20A and 66-40B (see Fig. 1.27 for structures). Only the product from gentamicin A was shown to be sisomicin by any technique other than chromatography. In this case the product was identified using mass spectrometry. From these results the pathway outlined in Fig. 1.27 was proposed for sisomicin biosynthesis (Testa & Tilley, 1975).

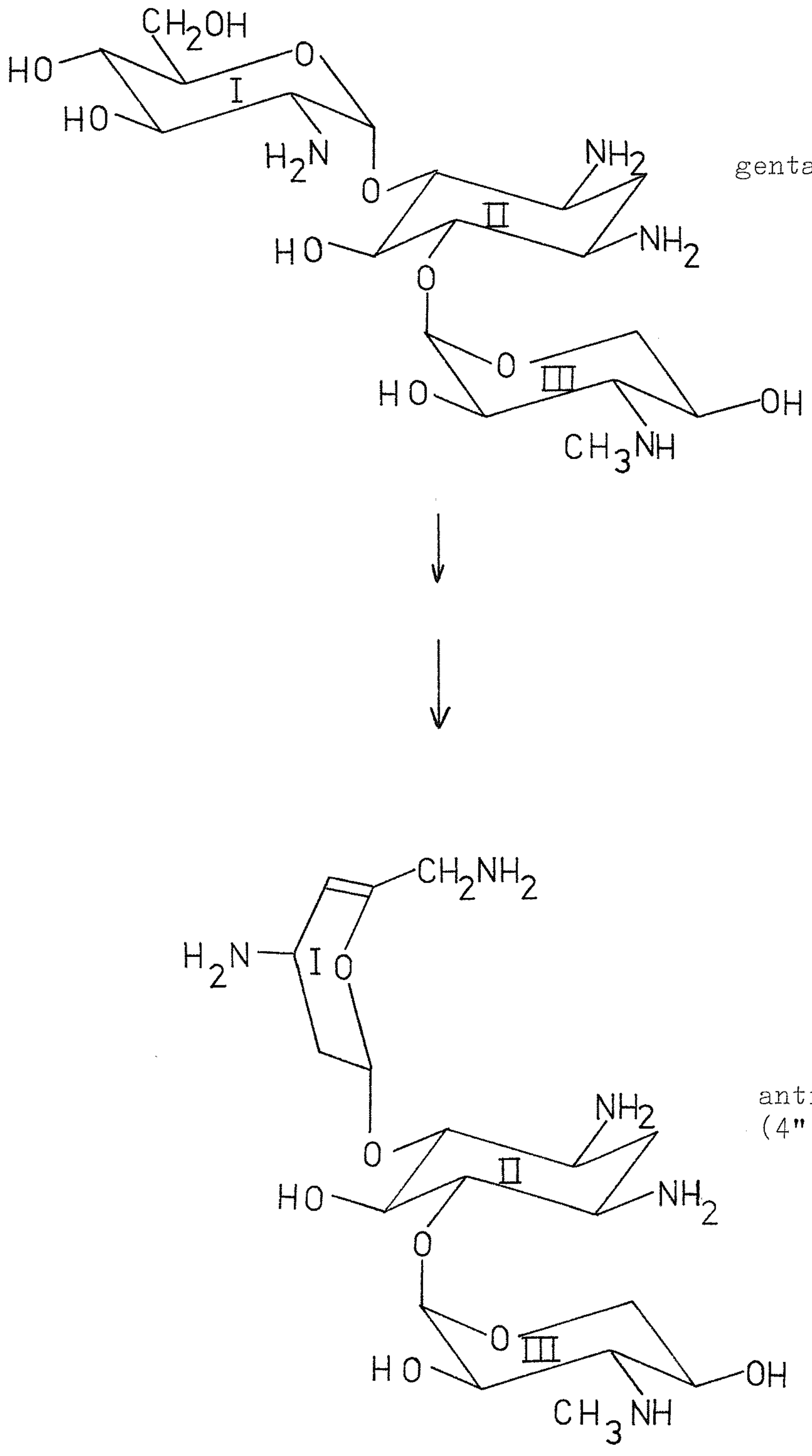
However antibiotic production from the various compounds can be explained by the hydrolysis of the precursor followed by the incorporation of the liberated 2-deoxystreptamine into sisomicin. The results from only one experiment performed by Testa & Tilley suggest this may not be so; that in which gentamicin A_1 was converted into antibiotic 66-40D (Fig. 1.28). If gentamicin A_1 had been hydrolysed prior to conversion into antibiotic the compound produced should be sisomicin. That gentamicin A_1 was converted into antibiotic 66-40D suggests hydrolysis had not occurred.

The first two steps (a and b) in the proposed sequence for sisomicin biosynthesis from gentamicin A_2 (Fig. 1.27) are methylation reactions. Lee _et al_. (1976b) have demonstrated that the label from L-[_methyl_-^{14}C] methionine was incorporated into sisomicin by _M. inyoensis_ and suggest that the amino acid provides the methyl groups of this antibiotic. Moreover shortly after the addition of L-[_methyl_-^{14}C] methionine to _M. inyoensis_ cultures label appeared in gentamicin A and antibiotic 66-40B (Fig. 1.27). Following further incubation ^{14}C appeared in sisomicin also. From these results it was concluded that gentamicin A and antibiotic 66-40B are precursors for sisomicin biosynthesis.

The ^{14}C gentamicin A isolated in the experiment above was incubated with a 2-deoxystreptamine-idiotroph of _M. inyoensis_. The aminoglycosides were purified and it was found that 98.7% of the recovered ^{14}C was associated with gentamicin A and 1.3% was associated with a sisomicin-like compound, again suggesting that gentamicin A is a precursor of the latter antibiotic. This result could also be obtained if gentamicin A were degraded and sisomicin synthesized from the degradation products.

It has been demonstrated that the gentamicins may be biosynthesized in a way similar to sisomicin. Testa & Tilley (1976) isolated a mutant of _Micromonospora purpurea_ which was unable to synthesize gentamicin but could produce paromamine. The mutant was

Figure 1.28 The biosynthesis of antibiotic 66-40D from gentamicin A_1



gentamicin A_1

antibiotic 66-40D
(4" demethyl sisomicin)

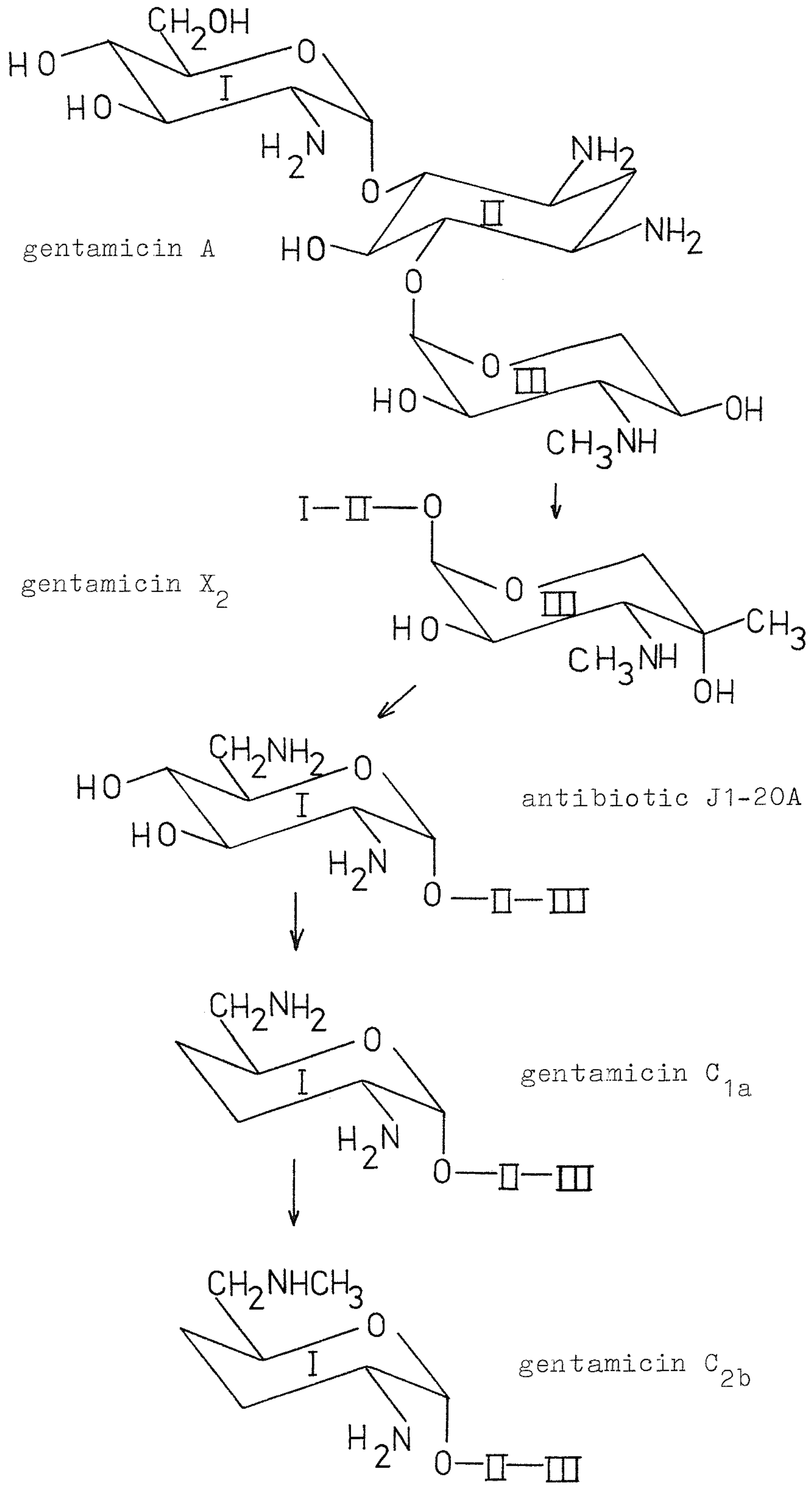
incubated in the presence of various naturally-produced gentamicins. After incubation the compounds were examined for conversion into other gentamicins. Gentamicin C_1 was produced from gentamicin C_2 (see Fig. 1.29 for structures), gentamicin C_{2b} was produced from gentamicin C_{1a}, gentamicin C_{1a} and C_{2b} were produced from antibiotic J1-20A, and gentamicins C_2 and C_1 were produced from both antibiotic G-418 and J1-20B. Gentamicins C_{1a}, C_2 and C_1 were produced from gentamicin A and X_2. from these results the authors propose a branched pathway for the biosynthesis of gentamicins (Fig. 1.29).

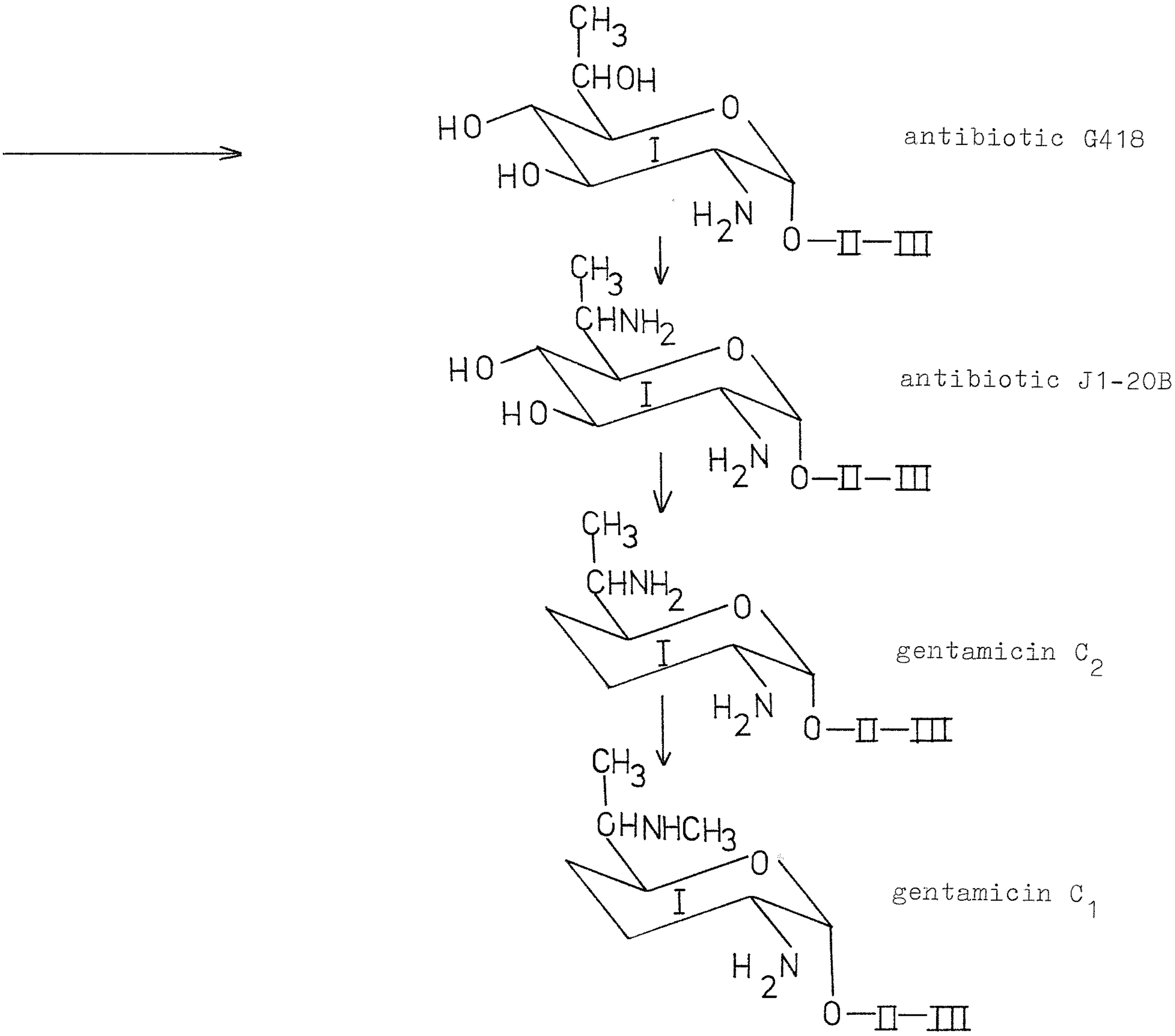
In the proposed pathway (Fig. 1.29) for the conversion of gentamicin A to gentamicin C_1 three methylation-reactions occur and during the conversion of gentamicin A to gentamicin C_{2b} two methylation-reactions occur. Lee <u>et al</u>. (1973, 1974) showed that ^{14}C from L-[<u>methyl</u>-^{14}C]methionine was incorporated into gentamicins C_{1a}, C_2 and C_1 and it was demonstrated (Lee <u>et al</u>., 1975) that the radioactive precursor was labelling at least some of the methyl groups of these antibiotics.

Small quantities of gentamicins A, X_2 and antibiotic G-418 (see Fig. 1.29 for structures) are produced by <u>M. purpurea</u>. The addition of L-[methyl-^{14}C]methionine to one-day old cultures of <u>M. purpurea</u> resulted in a small amount of label appearing in gentamicins A, X_2 and antibiotic G-418 after 15 minutes incubation (Lee <u>et al</u>., 1976a). At this time gentamicins C_{1a}, C_2 and C_1 remained unlabelled. After incubating the culture for 24 hours ^{14}C appears in gentamicins C_{1a}, C_2 and C_1 as well as gentamicin A, X_2 and antibiotic G-418. These results support the proposal that gentamicins A and X_2 are precursors of gentamicins C_{1a}, C_2 and C_1, and that antibiotic G-418 is a precursor of gentamicins C_1 and C_2.

However when ^{14}C gentamicins C_{1a}, C_2 and C_1, synthesized from L-[<u>methyl</u>-^{14}C] methionine, were incubated with <u>M. purpurea</u> they were found to label gentamicin X_2 and antibiotic G-418 to a considerable degree. This is an unexpected result and emphasises the need for a more detailed examination of the relationship between these antibiotics. Gentamicin X_2 and antibiotic G-418 could be produced from gentamicins C_{1a}, C_2 and C_1 either directly or via a hydrolysis mechanism.

Fig. 1.29 Gentamicin biosynthesis




CH3
CHOH
HO
HO
O
I
H2N
O—II—III
antibiotic G418
CH3
CHNH2
HO
HO
O
I
H2N
O—II—III
antibiotic J1-20B
CH3
CHNH2
O
I
H2N
O—II—III
gentamicin C2
CH3
CHNHCH3
O
I
H2N
O—II—III
gentamicin C1

1.10 Modification of aminoglycoside antibiotics by antibiotic-resistant organisms

One of the problems encountered using aminoglycoside (and other) antibiotics is the increasing population of antibiotic-resistant organisms. The resistant mechanisms of these organisms have been examined in the hope that information obtained can be used to predict structures to which these organisms will be sensitive.

In bacteria isolated from nature it has been found that antibiotic-resistance is commonly due to the presence of enzymes which modify, and thereby inactivate, the antibiotics. There are three mechanisms by which the aminoglycosides are known to be inactivated (Benveniste & Davies, 1973; Price et al., 1974). These are phosphorylation or adenylylation of the hydroxyl groups and acetylation of amino groups. Ten aminoglycoside inactivating enzymes have been discovered; eight of these inactivate 2-deoxystreptamine containing antibiotics and the remaining two inactivate streptomycin and spectinomycin (Table 1.1; Figs. 1.30, 1.31 & 1.32).

Knowledge of the mechanisms and substrate specificities of these inactivating enzymes has made possible the prediction of structures which will not be modified. For example Umezawa et al. (1967) discovered that certain kanamycin-resistant R factor carrying organisms produced one of the aminoglycoside phosphotransferase (3') enzymes (Table 1.1), which inactivates kanamycin by phosphorylation at position 3 of ring I. As expected 3'-deoxykanamycin, the kanamycin analogue lacking the 3 hydroxyl group phosphorylated by aminoglycoside phosphotransferase (3'), is an active antibiotic against organisms with this enzyme (Umezawa et al., 1971).

Paromomycin is also inactivated by phosphorylation at position 3 of ring I, this modification again being catalysed by aminoglycoside phosphotransferase (3'). However lividomycin, which is a paromomycin analogue lacking the 3 hydroxyl group, is also inactivated by aminoglycoside phosphotransferase (3'), although in this case phosphorylation occurs at position 5 of ring III (Fig. 1.32).

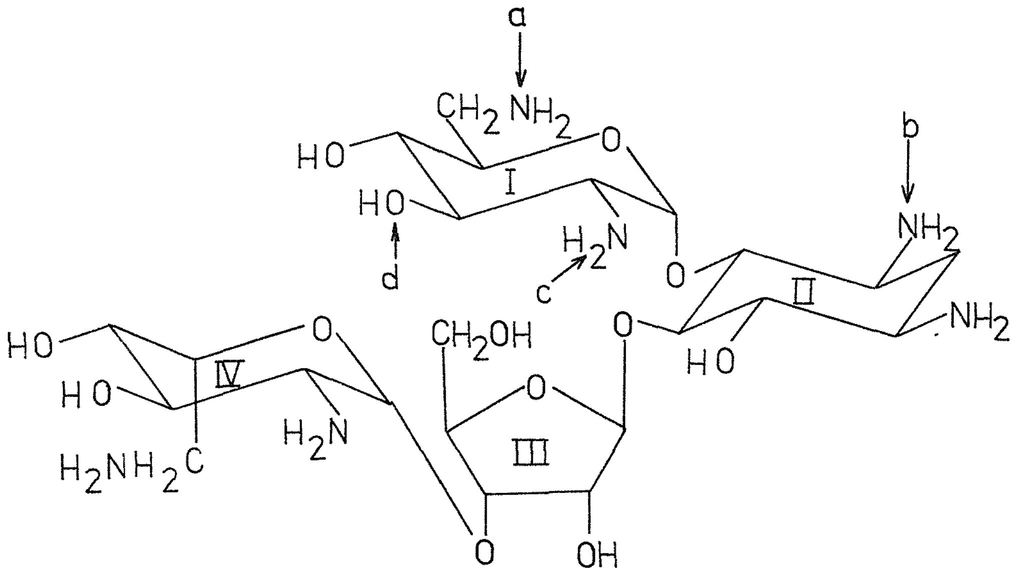
Alteration of part of the antibiotic away from the site of modification can have an effect on whether the antibiotic is a substrate for an inactivating enzyme. Amikacin (Fig. 1.33), which is a kanamycin analogue with an L(-)-γ-amino-α-hydroxybutyryl moiety

Table 1.1 Aminoglycoside inactivating enzymes
(Benveniste & Davies, 1973; Price *et al.*, 1974)

Enzyme	Antibiotics inactivated
Aminoglycoside phosphotransferase (3')-I	neomycins, paromomycins, ribostamycins, lividomycins, kanamycins
Aminoglycoside phosphotransferase (3')-II	neomycins, paromomycins, ribostamicins, butirosins, kanamycins
Aminoglycoside phosphotransferase (5")	lividomycin
Aminoglycoside nucleotidyltransferase (2")	gentamicins, kanamycins
Aminoglycoside acetyltransferase (6')	neomycin, ribostamycin butirosins, kanamycins A & B, gentamicins C_{1a} & C_2
Aminoglycoside acetyltransferase (3)-I	gentamicins, kanamycin B*
Aminoglycoside acetyltransferase (2')	gentamicins, neomycins*, paromomycins*, kanamycins B* & C*, ribostamycin*, butirosins*, lividomycins*
Aminoglycoside acetyltransferase (3)-II	neomycins, paromomycins, ribostamycin, kanamycin, gentamicins, lividomycins
Streptomycin phosphotransferase	streptomycin
Streptomycin adenylyltransferase	streptomycin, spectinomycin

*These compounds are poor substrates

Figure 1.30 Sites of modification by neomycin B-inactivating enzymes



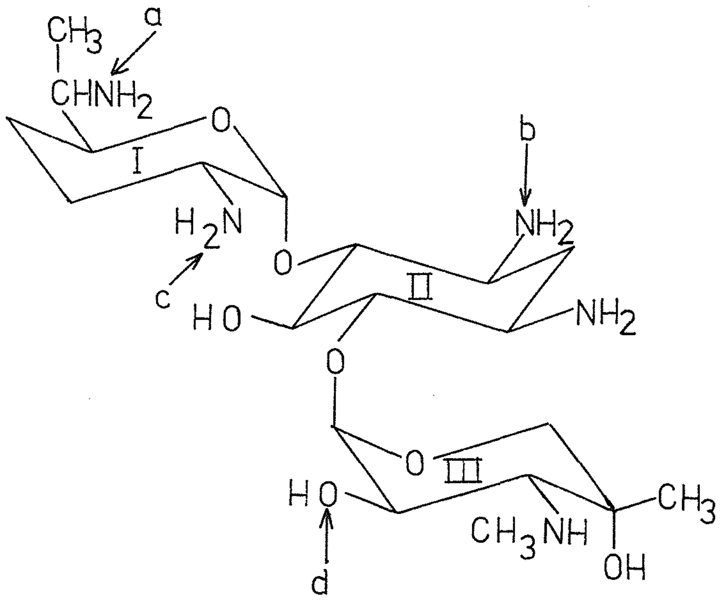
a aminoglycoside acetyltransferase (6')

b aminoglycoside acetyltransferase (3)-II

c aminoglycoside acetyltransferase (2')

d aminoglycoside phosphotransferase (3')-I and II

Figure 1.31 Sites of modification by gentamicin C_2-inactivating enzymes



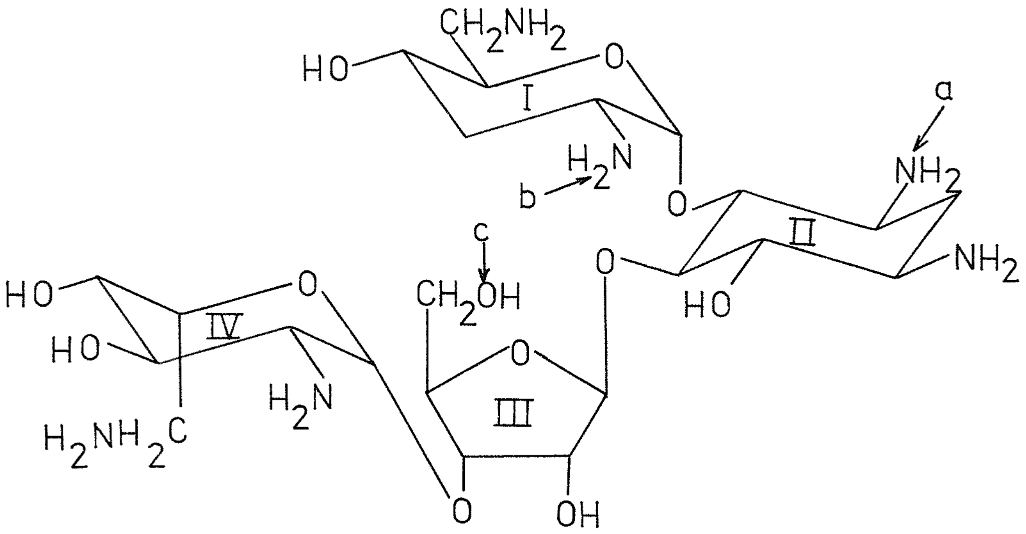
a aminoglycoside acetyltransferase (6')

b aminoglycoside acetyltransferase (3)-I and II

c aminoglycoside acetyltransferase (2')

d aminoglycoside nucleotidyl transferase (2")

Figure 1.32 Sites of modification by lividomycin B-inactivating enzymes

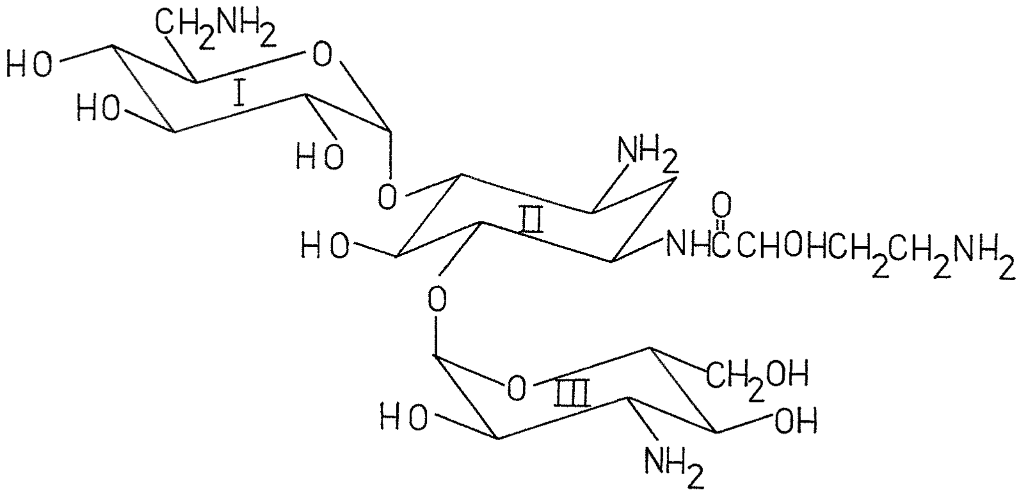


a aminoglycoside acetyltransferase (3)-II

b aminoglycoside acetyltransferase (2')

c aminoglycoside phosphotransferase (3')-I and aminoglycoside phosphotransferase (5")

Figure 1.33 The structure of amikacin



on the 1-amino group of 2-deoxystreptamine, is inactivated by only one of the four kanamycin-inactivating enzymes (Benveniste & Davies, 1973). Amikacin is a substrate for aminoglycoside acetyltransferase (6') (Table 1.1), an enzyme which acetylates the 6-amino group of ring I.

It has also been shown that an alteration in the aminocyclitol ring of sisomicin can produce antibiotics which are more active than the parent compound against organisms containing aminoglycoside-inactivating enzymes (Testa *et al.*, 1974).

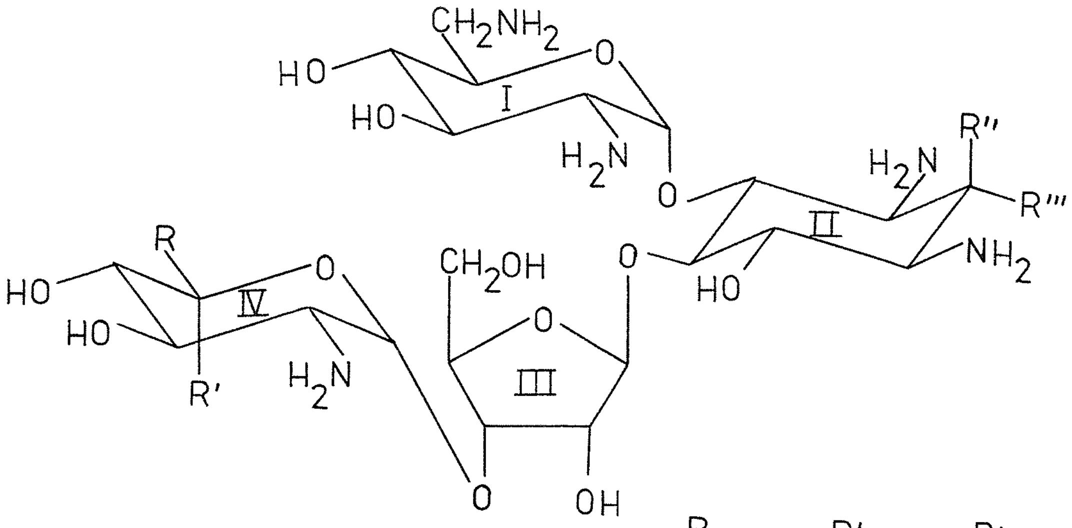
1.11 Aminocyclitol-idiotrophs of antibiotic producing organisms

Organisms have been isolated which can only produce antibiotic if an essential compound is present. Thus Shier *et al.* (1969) reported the isolation of mutants of *S. fradiae* which would only produce neomycin when added 2-deoxystreptamine was present. This type of mutant was later given the name *idiotroph* (Nagaoka & Demain, 1975), a defination of which is a mutant requiring a special nutrient to produce a product peculiar to that organism. Shier *et al.* (1969) found that by incubating the 2-deoxystreptamine-idiotroph in the presence of the 2-deoxystreptamine-analogues streptamine and *epi*-streptamine, neomycin-analogues were produced (hybrimycins A and B; Fig. 1.34) in which 2-deoxystreptamine was replaced by the added analogue.

Since these experiments were described there have been reports of idiotrophs of various antibiotic-producing organisms being used to synthesize analogues of paromomycin (Shier *et al.*, 1974; Fig. 1.35) kanamycin (Kojima & Satoh, 1973; Figure 1.36), ribostamycin (Kojima & Satoh, 1973; Fig. 1.37), sisomicin (Testa *et al.*, 1974; Figs. 1.38 & 1.39), butirosin (Claridge *et al.*, 1974; Taylor & Schmitz, 1976; Figs. 1.40, 1.41 & 1.42) and streptomycin (Nagaoka & Demain, 1974, 1975; Fig. 1.43).

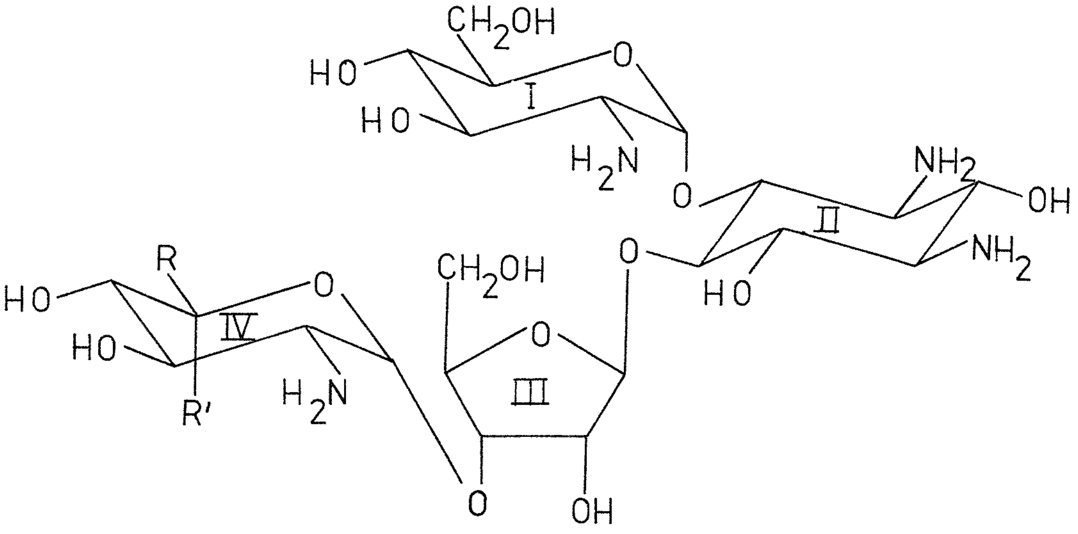
It has been observed that 2-deoxystreptamine-idiotrophs of the organisms producing the 4,5-disubstituted 2-deoxystreptamine antibiotics (neomycin, paromomycin, ribostamycin and butirosin) incorporate 2-deoxystreptamine-analogues into antibiotics similar to the parent compound, excepting that the natural aminocyclitol (ring II) is replaced by the synthetic analogue. In contrast, when the 2-deoxystreptamine-idiotrophs of the organisms which produce 4,6-disubstituted 2-deoxystreptamine antibiotics (sisomicin and

Figure 1.34 The structure of hybrimycins A and B



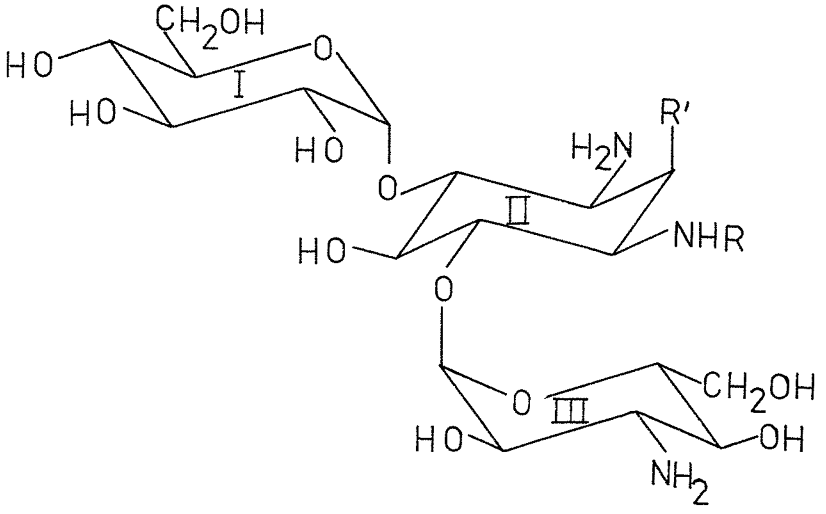
	R	R'	R''	R'''
hybrimycin A_1	H	CH_2NH_2	H	OH
hybrimycin A_2	CH_2NH_2	H	H	OH
hybrimycin B_1	H	CH_2NH_2	OH	H
hybrimycin B_2	CH_2NH_2	H	OH	H

Figure 1.35 The structure of hybrimycins C



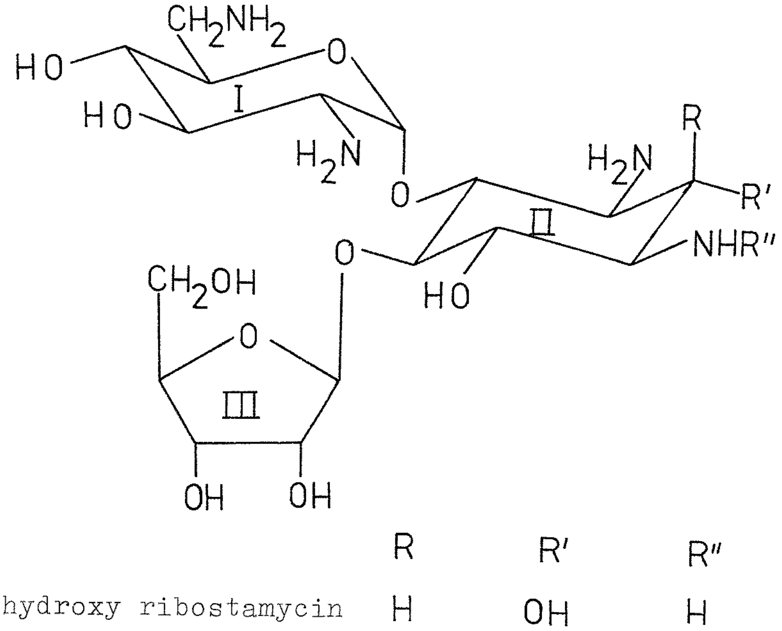
	R	R'
hybrimycin C_1	H	CH_2NH_2
hybrimycin C_2	CH_2NH_2	H

Figure 1.36 The structure of kanamycin-analogues



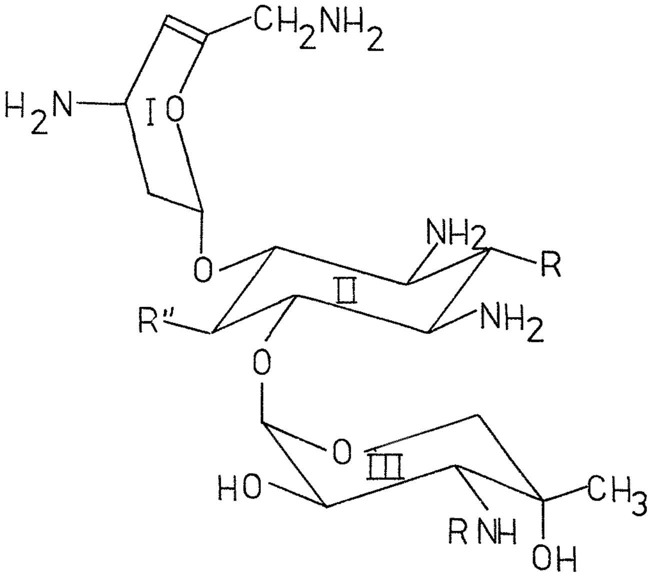
	R	R'
6'-deamino-6'-hydroxy-1-N-methyl kanamycin A	CH_3	H
6'-deamino-6'-hydroxy-2-hydroxy kanamycin A	H	OH

Figure 1.37 The structure of ribostamycin-analogues



	R	R'	R''
2-hydroxy ribostamycin	H	OH	H
2-*epi*-hydroxy ribostamycin	OH	H	H
1-N-methyl ribostamycin	H	H	CH_3

Figure 1.38 The structure of sisomicin-analogues



	R	R'	R''
mutamicin 1	OH	CH_3	OH
mutamicin 1a	OH	$COCH_3$	OH
mutamicin 1b	OH	H	OH
mutamicin 2	H	CH_3	H

Figure 1.39 The structure of mutamicin 2a

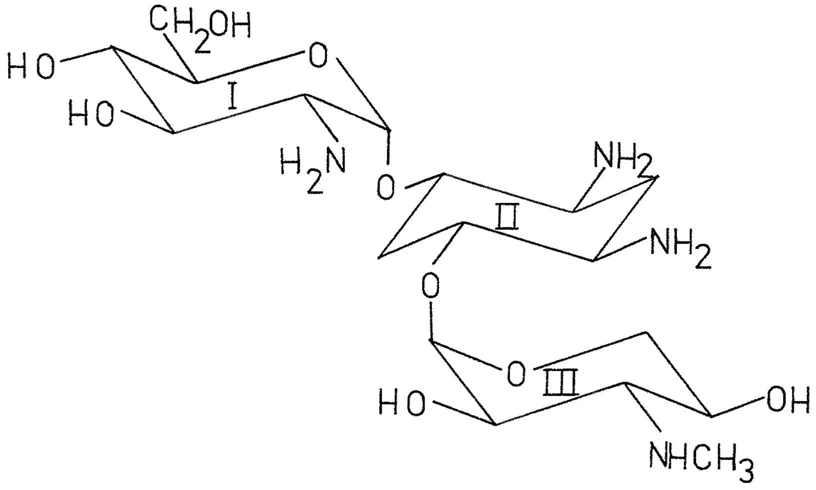


Figure 1.40 The structure of 5-deoxybutirosamine

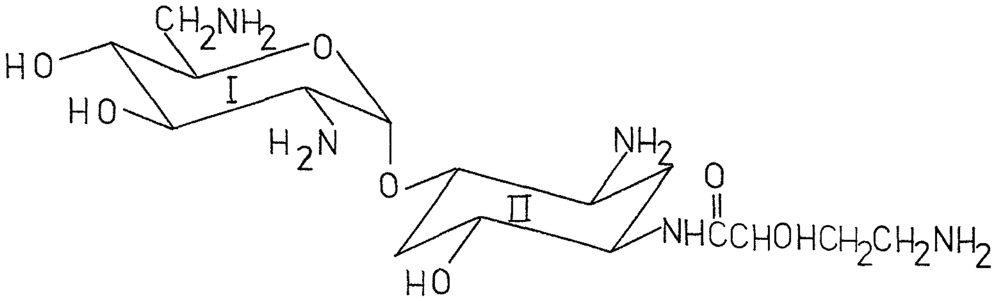


Figure 1.41 The structure of 2-hydroxybutirosin B

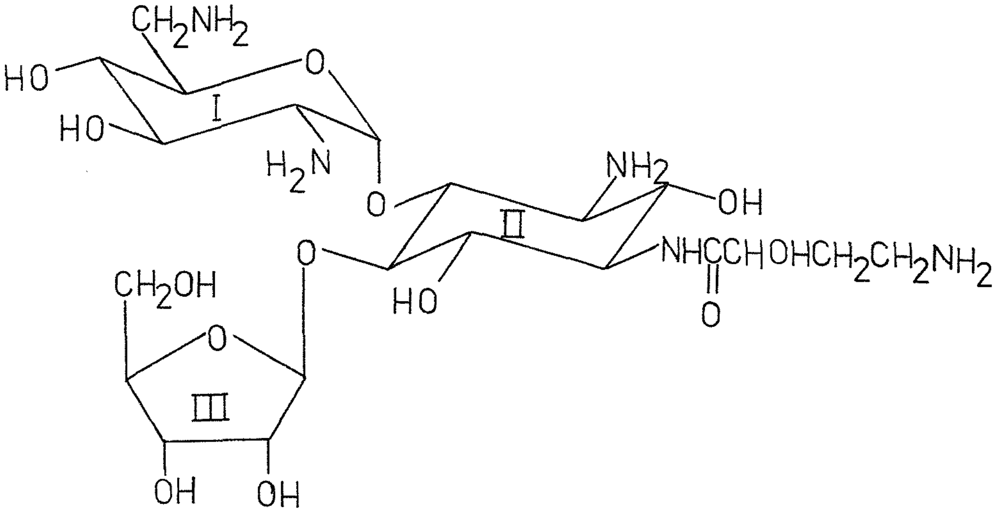


Figure 1.42 The structure of the streptidine-containing analogue of butirosin B

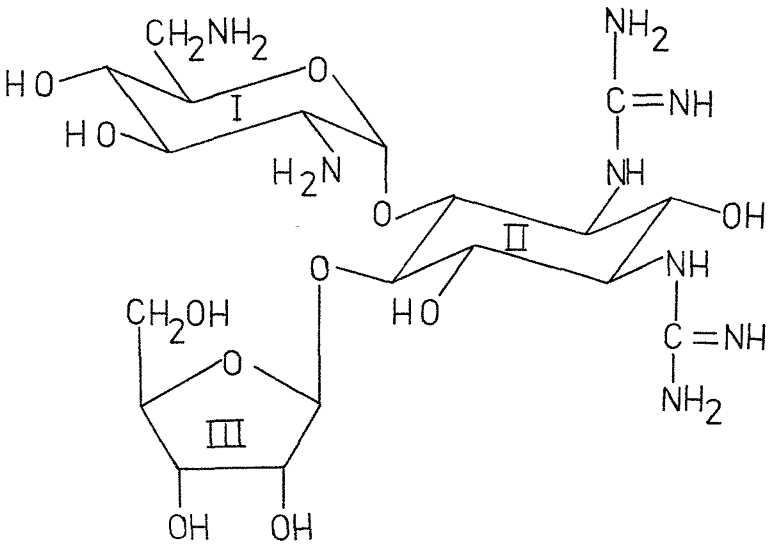
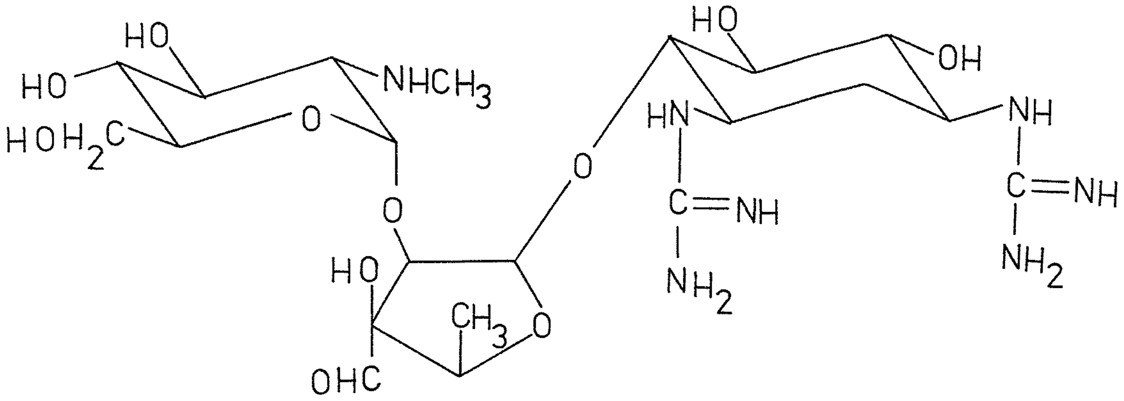


Figure 1.43 The structure of 2-deoxystreptomycin



kanamycin) are incubated with 2-deoxystreptamine-analogues, as well as producing analogues of the parent antibiotics in which the aminocyclitol is altered, the organisms synthesized compounds in which the functionalization of ring I and ring III is incomplete (Kojima & Satoh, 1973; Testa et al., 1974). There is evidence to suggest that in the case of the 4,6-disubstituted 2-deoxystreptamine-antibiotics, sisomicin and gentamicin, functionalization of rings I and III may occur after the synthesis of a basic structure (see Section 1.9). On the basis of this last observation the results obtained using the 2-deoxystreptamine-idiotrophs supplemented with 2-deoxystreptamine-analogues could be explained by the analogue being incorporated into a basic structure which is not a substrate for the functionalization-enzymes.

1.12 Aims of this work

The work described in this thesis is concerned with the elucidation of the order of subunit-assembly during the biosynthesis of neomycins and with the synthesis of novel antibiotics; some of this work has been published elsewhere (Olesker et al., 1975; Pearce et al., 1976; Cleophax et al., 1976).

Chapter 2

Materials and Methods

2.1 Chemicals

Standard laboratory chemicals used were of reagent grade and were obtained from BDH Chemicals, Poole, Dorset, U.K. D-[6-$^3H_2$] glucose and D-[U-^{14}C] glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Neomycin sulphate was from Boehringer Corporation (London), London W.5, U.K. Neomycin B sulphate and neomycin C sulphate were prepared from neomycin sulphate using paper chromatography developed with 2-methylpropan-2-ol/butan-2-one/methanol/6.5M-NH_3 solution (3:16:1:6, by vol.). Neamine was prepared from neomycin sulphate by the method of Dutcher & Donin (1952) (see Section 2.14). Paromomycin sulphate was a gift from Parke-Davis, Hounslow, Middlesex, U.K. Paromomycin I sulphate and paromomycin II sulphate were prepared from paromomycin sulphate using the technique employed to produce the B and C components of the neomycin sulphate mixture.

The aminocyclitols and their derivatives were gifts from Dr. S. D. Gero, Institut de Chimie des Substances Naturelles, C.N.R.S., 91190 Gif-sur-Yvette, France.

Diagnostic sensitivity test agar base (DST agar), nutrient agar and nutrient broth were from Oxoid, London S.E.1, U.K.

Celite hyflo super gel was from Kochlight Laboratories Ltd., Colnbrook, Bucks, U.K.

2.2 Organisms; source and properties

All of the antibiotic-producing organisms were obtained from the American Type Culture Collection (A.T.C.C.), c/o Order Department, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.

Streptomyces fradiae wild type (A.T.C.C. 10745) synthesizes neomycin. The 2-deoxystreptamine-idiotroph of *Streptomyces fradiae* (A.T.C.C. 21401) synthesizes neomycin only in the presence of added 2-deoxystreptamine. The 2-deoxystreptamine-idiotroph of *Streptomyces rimosus* forma *paromomycinus* (A.T.C.C. 21484) synthesizes paromomycin only in the presence of added 2-deoxystreptamine. The 2-deoxystreptamine-idiotroph of *Streptomyces kanamyceticus* (A.T.C.C. 21486) synthesizes kanamycin only in the presence of added 2-deoxystreptamine.

The following organisms were used for antibiotic assay. The number in parenthesis is that given the organism for the experiments reported in this thesis only.

Escherichia coli Bristol (PTI) was the standard organism used both to detect and assay antibiotic and was a gift from Professor P. Watt, Department of Medical Microbiology, of this university. *Escherichia coli* W3110 was a gift from M.D. Watson of this department. *Escherichia coli* (gentamicin-resistant due to presence of aminoglycoside nucleotidyl transferase 2") (PT2), *Proteus mirabilin* (PT3), the neomycin-resistant *Staphylococcus aureus* strains 12336 (PT4) and 5507 (PT5), the neomycin-sensitive *Staphylococcus aureus* (PT6) and *Pseudomonas aeruginosa* (PT7), were all gifts from Professor P. Watt. *Shigella sonnei* C631978 and *Salmonella typhimurium* LT2 were gifts from Dr. W. T. Drabble of this department. *Escherichia coli* strains K-12 ML1629, K-12 ML1410 and JR66/W677 and *Pseudomonas aeruginosa* H9 were all gifts from Dr. S. Kondo, Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo, Japan. *E. coli* JR66/W677 and *P.aeruginosa* H9 are neomycin-resistant due to the presence of aminoglycoside phosphotransferase (3') type II (Matsuhashi *et al*., 1975), and *E. coli* K-12 ML1629 and K-12 ML1410 are neomycin-resistant due to the presence of aminoglycoside phosphotransferase (3') type I (Umezawa *et al*., 1973).

2.3 Growth of *Streptomyces*

Streptomyces spores were produced on medium containing starch, 1%; K_2HPO_4, 0.1%; $MgSO_4.7H_2O$, 0.1%; NaCl, 0.1%; $(NH_4)_2SO_4$, 0.2%; $CaCO_3$, 0.2%; agar, 1% and 0.1% of a 0.1% solution in water of the following; $FeSO_4.7H_2O$, $MnCl_2.4H_2O$ and $ZnSO_4.7H_2O$. 10ml aliquots of the above medium were poured into sterile plastic petri dishes. These were dried, inoculated and incubated at 30°C for three days and then stored at room temperature. Spore were transfered to fresh plates every three weeks.

Streptomyces fradiae (A.T.C.C. 10745 and 21401) was grown in the following liquid media.

a) *Sebek complex medium* (Sebek, 1955) contained soybean meal, 2.5%; yeast extract, 0.5%; NaCl, 0.5% $CaCO_3$, 0.2% and glucose, 1%. The pH was adjusted to 7.5 using NaOH solution.

b) Sebek defined medium (Sebek, 1955) contained glucose, 1%; casein hydrolysate, 1%; $(NH_4)_2HPO_4$, 1%; $MgSO_4.7H_2O$, 0.05%; $FeSO_4.7H_2O$, 0.005%; $CuSO_4.5H_2O$, 0.005% and $CaCO_3$, 1%. The pH was adjusted to 7.2-7.3 with NaOH solution.

c) Majumdar and Majumdar defined medium (1971) contained maltose, 1.5%; $NaNO_3$, 0.51%; K_2HPO_4, 0.1%; $MgSO_4.7H_2O$, 0.05%; $CaCl_2.2H_2O$, 0.004%; $FeSO_4.7H_2O$, 0.0005%; $ZnSO_4.7H_2O$, 0.00005%. The pH was adjusted to 7.5 using NaOH.

The 2-deoxystreptamine-idiotroph of the paromomycin-producing organism, Streptomyces rimosus forma paromomycinus, was grown in a medium of the following composition (Schier et al., 1972). Soybean meal, 1%; casein hydrolysate, 0.25%; $CaCO_3$ 0.5%; glucose, 1%; NaCl, 0.5% and NH_4Cl, 0.167%. The pH was adjusted to 7.5 using NaOH solution.

The liquid media described above were sterilized at 121°C for 15 minutes in a steam autoclave prior to use.

The organisms were grown at 30°C in either a 500ml Erlenmeyer flask containing 50ml of medium, a 2litre Erlenmeyer flask containing 200ml of medium or a 125ml conical flask containing 10ml medium. The flasks were incubated on a rotary shaker; in the case of the 500ml and 125ml flasks the shaker produced a 2.5cm diameter rotation 150 times/minute, and the shaker used for cultures in the 2 litre flasks produced a 4cm diameter rotation 120 times/minute.

2.4 Biosynthesis of antibiotics

For the production of deoxyneomycin, the antibiotic synthesized in the presence of 4-O-β-ribosyl-2,6-dideoxystreptamine and dideoxyneamine, the 2-deoxystreptamine-idiotroph of S. fradiae was cultured in 50ml Sebek complex medium (Section 2.3) for 2 to 3 days and 10ml of this was used to inoculate 200ml of Sebek complex medium to which had been added the amino-cyclitols in sterile aqueous solutions. The cultures were incubated for five days at which time the antibiotic production was maximal.

For the production of deoxyparomomycin and the conversion of neamine into antibiotic by the 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus, the organism was grown for three days in the medium described above. To either 50ml in the case of the neamine conversion into neomycin experiments, or 200ml in the case of deoxyparomomycin production, of the same medium, supplemented with the relevant aminocyclitol derivative, was added 2.5ml or 10ml respectively of the 3-day old culture. The flasks were then incubated for five days at which time the antibiotic production was maximal.

2.5 Screening of compounds for their ability to support the production of antibiotic

The following method is based on that reported by Schier et al. (1973). Aqueous solutions of the compounds to be tested were sterilized in a steam autoclave at 103KPa for 15 minutes. These were added to a solution (2.8%) of sterilized Oxoid nutrient agar at approximately 50°C and 10ml aliquots poured into sterile plastic petri dishes. The plates were dried at 37°C and then inoculated with a single streak of Streptomyces spores taken from a culture grown on a starch/salts agar plate (Section 2.3). The plates were incubated at 30°C for three days. Antibiotic production was determined by overlaying the plates with 8ml of a solution (4%) of Oxoid diagnostic sensitivity testing agar at 45°C containing 2.5% by volume of an overnight culture of Escherichia coli Bristol in nutrient broth. The plates were incubated at 37°C overnight and then examined for the inhibition of growth of E. coli around the Streptomyces streak.

2.6 Antibiotic extraction

The cultures containing antibiotics were centrifuged at 10,000 rpm for 30 minutes. The supernatant was poured twice through a column containing Amberlite IRC50 resin. The column was prepared in the following way. Amberlite IRC50 (H^+) was suspended in water and poured into a glass column of 2.5cm diameter. The volume of resin used was about 1ml for every 2.5mg of antibiotic to be extracted; this quantity of antibiotic was usually present in 5 to 12.5ml of culture supernatant. 2ml of 4M -NH_3 solution per ml of resin was passed through the column in order to generate the ammonium phase. The resin was then washed with distilled water until the pH of the eluant was 7.0. The column was then used for antibiotic extraction.

The resin through which the supernatant had been passed was washed four times, each time with a quantity of water equal to the resin volume, and the antibiotic eluted using four times the resin volume of 2M-NH_3 solution. The eluant containing antibiotic from the resin was dried using a rotary evaporator.

2.7 Purification of antibiotics using paper chromatography

The usual system for purification of antibiotics involved descending paper chromatography using Whatman 3MM paper and the solvent system MeOH/NH_3 (sp. gr. 0.88) (4:1, V/V). If the solvent was to be allowed to run off the end of the chromatogram the paper was serrated ensuring an even solvent front. Chromatography was carried out in glass tanks 25cm x 30cm x 55cm which were equilibrated with solvent for approximately 1 hour before starting. Chromatograms were dried in a drying cabinet at 40°C.

Antibiotics were eluted from the chromatogram using the apparatus described in Diagram 2.1. The elution was completed after approximately 2.5ml had been collected; initially this was checked by assaying for the presence of antibiotic activity in the paper.

2.8 Purification of antibiotics using gel filtration chromatography

The column was prepared for purifying deoxyneomycin from a crude extract containing the antibiotic and 2,4-dideoxystreptamine as major components.

100gm Sephadex G10 was boiled in 200ml 0.1M-NH_3 solution. The slurry was poured in one go into a column 2.5cm diameter with a glass scinter base covered with a 0.5cm layer of celite hyflo super gel. The column was left overnight to settle; the final column was 45cm high. The homogeneity of the column was checked by applying 2mg dextran blue to the top of the column and eluting this with 0.1M-NH_3 solution. The dextran blue passed through the column in a narrow blue band. o-nitro phenol and dextran blue dissolved in 1ml 0.1M-NH_3 solution, were applied to the top of the column and developed using 0.1M-NH_3 solution. 5ml fractions were collected. The dextran blue eluted in fractions 13, 14 and 15 and the o-nitro phenol in fractions 30 to 38. When 50mg of neomycin and 20mg of 2-deoxystreptamine dissolved in 1ml were applied to the top of the

Diagram 2.1 Elution apparatus

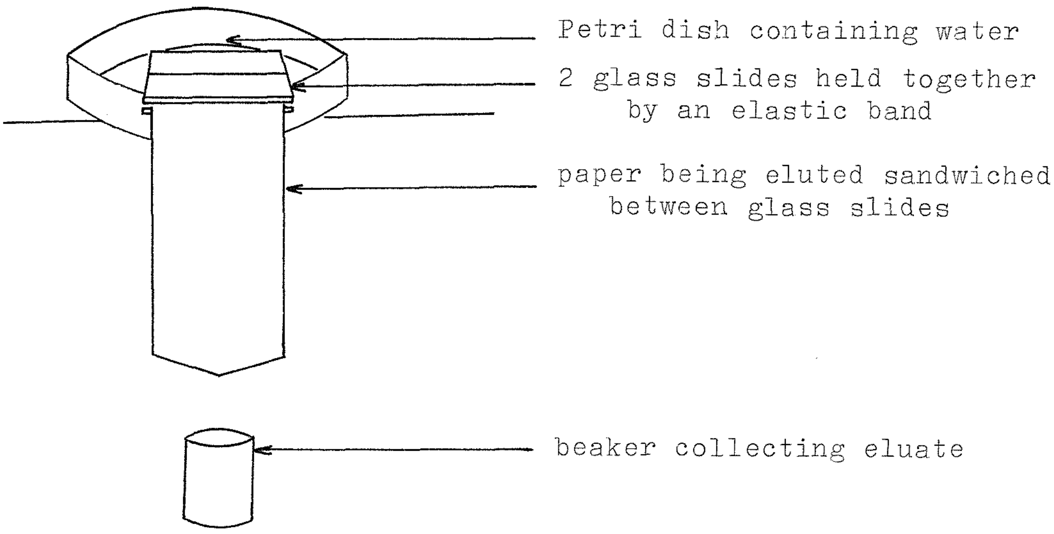
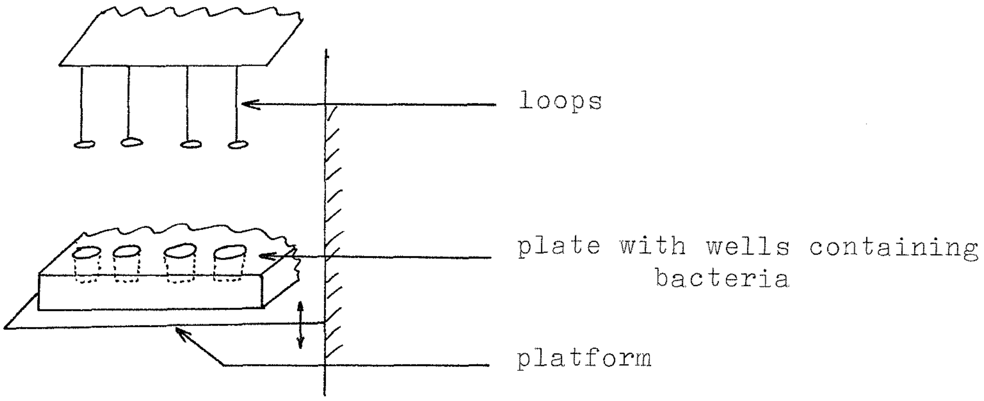


Diagram 2.2 Simplified diagram of the apparatus used during M.I.C. determinations



When the platform is raised the loops dip into the wells containing bacteria. The plate with the wells is lowered and replaced with an agar plate containing antibiotic. The platform is raised again until the loops touch the agar surface. In this way the agar plate is inoculated.

column and developed using 0.1M-NH_3 solution, and 5ml fractions collected, the majority of neomycin appeared in fraction 14-19 and the 2-deoxystreptamine appeared in fraction 21 onwards.

2.9 Antibiotic detection using a staining technique

The neomycins were detected on paper chromatograms using the method described by Pan & Dutcher (1956). This method detects amino and amide groups.

The paper chromatograms were air dried and sprayed with a 0.5% solution of sodium hypochlorite in water. When the chromatogram had dried it was sprayed with absolute ethanol. After this had evaporated the chromatogram was sprayed with a solution containing equal volumes of a 1% aqueous soluble starch solution and a 1% aqueous potassium iodide solution.

On spraying with the final solution the neomycins immediately appear as deep blue spots against a colourless background.

2.10 Antibiotic detection using a bioautogram

This method was used to detect active antibiotics on paper chromatograms.

A strip 1cm wide was cut from the chromatogram along the path the migrating antibiotic had taken. This was cut into 4cm pieces and placed on the surface of Oxoid diagnostic sensitivity testing agar three mm in depth and containing 2.5% by volume of an overnight culture of E. coli Bristol. The plates were incubated overnight at 37°C and the zones of growth inhibition measured.

2.11 Determination of antibiotic potency

Minimum inhibitory concentrations for antibiotics were determined using an agar dilution technique. A solution (4%) of diagnostic sensitivity testing agar was sterilized in a steam autoclave at 103KPa for 15 minutes and cooled to approximately 50°C. Aqueous solutions of antibiotic were added to 10ml aliquots of the molten agar which were then poured into sterile plastic petri dishes. When the agar had solidified the plates were dried at 37°C for 2 hours. The plates were inoculated with organisms using a multi-inoculator (Diagram 2.2). Organisms suspended in glycerol (40%) were placed in the wells of the multi-inoculator. The loops were sterilized using 70% aqueous ethanol followed by flaming.

The inoculated plates were incubated overnight at 37°C and then examined for growth. The minimum concentration of antibiotic which inhibited the growth of an organism is referred to as the minimum inhibitory concentration (M.I.C.) for that organism.

The bacteria used in the M.I.C. determination (Section 2.2) were grown in Oxoid nutrient broth in a bijou bottle at 37°C and an equal quantity of 80% aqueous glycerol (sterilized for 15 minutes at 103KPa) was added. The suspensions were stored in a deep freeze at -20°C and required no further preparation before use.

2.12. Antibiotic assays

These were carried out using a paper disc technique. 25μl of a solution of antibiotic to be tested was applied to a Whatman no.17 chromatography paper disc six mm in diameter which had been placed on the surface of an Oxoid diagnostic sensitivity testing agar plate seeded with 2.5% by volume of an overnight broth culture of E. coli Bristol. The plates were incubated at 37°C overnight and the diameter of the zones of growth inhibition around the paper disc measured. A plot of the diameter of the circular zone of growth inhibition against the logarithm of the concentration of antibiotic gave a straight line.

Standard solutions of neomycin were prepared in 0.1M-phosphate buffer, pH7.9. The assay was used to measure antibiotic concentration between 32μg and 1mg neomycin sulphate/ml (1mg neomycin sulphate is equivalent to 665μg neomycin base). Cultures containing more than this were diluted using 0.1M-phosphate buffer.

In the neamine incorporation experiments (Chapter 5) the antibiotic produced was less active than 32μg neomycin/ml and was assayed using a well technique. Oxoid diagnostic sensitivity testing agar plates were prepared as in the disc assay above except the agar was six mm deep. Using a 10 mm diameter cork borer, holes were cut into the agar. 0.1ml of the solution to be assayed were added to the wells. The plates were incubated overnight and the zones of inhibition measured as in the paper disc-type assay. In these experiments the standard solutions contained both neomycin and neamine. The standard corresponding to zero conversion of neamine into neomycin contained 50μg neamine/ml and no neomycin. The standard corresponding to 100% conversion of neamine into neomycin contained neomycin sulphate (100µg/ml) but no neamine. Although neamine is an antibiotic 50µg/ml was not detected using either the well or disc technique.

2.13 Ribose assay

The purity of the neomycins was determined by assaying their ribose content using the procedure of Dutcher et. al. (1953). The method is based on the observation that when neomycin is heated with strong mineral acid, furfural is produced from the ribose (ring III). The furfural is measured by the amount of absorption at 280mµ.

The assay was carried out as follows. Either 0.5 or 0.25ml (containing 180µg to 1.5mg) of a solution in water of neomycin sulphate was made up to a total volume of 5ml with 40% aqueous sulphuric acid in a boiling tube and heated in a boiling water bath for 1.5 hours. After cooling the extinction of the solution was measured in silica cells using a Unicam SP1800 ultra violet spectrophotometer at 280mµ.

A plot of the quantity of neomycin used against the extinction at 280mµ produced gives a straight line.

2.14 Methanolysis of antibiotics

This was carried out using the procedure described by Dutcher & Donin (1952).

1g of neomycin sulphate was added to 100ml of dry methanol and sufficient methanolic hydrogen chloride (see Section 2.15) to produce a final HCl concentration of approximately 0.35M. The suspension was refluxed over an oil bath at 100°C for 2.5 hours, filtered hot through Whatman no. 1 filter paper and the filtrate cooled in an ice bath. 40ml of anhydrous diethyl ether was added, this producing a white precipitate of neamine (or deoxyneamine from deoxyneomycin). The precipitate was collected on a glass-scinter filter covered with a 5mm layer of Celite hyflo super gel. The precipitate was washed with cold methanol, dissolved in water and filtered, and finally evaporated to dryness.

Typically this gave a yield of approximately 200mg of neamine hydrochloride.

This methanolysis procedure, scaled down as necessary, was used to produce deoxyneamine from deoxyneomycin.

The procedure for the methanolysis of radioactive neomycin, deoxyparomomycin and paromomycin was similar to that described above except isolation of the products was carried out using paper chromatography. A typical reaction mixture contained 16mg deoxyparomomycin and 5ml of 0.35M-HCl in methanol. This was refluxed for 2.5 hours at 100°C and the reaction mixture dried using a rotary evaporator. The residue was dissolved in water and dried again. This was repeated to remove all traces of HCl. The residue was finally dissolved in water and chromatogrammed on Whatman 3MM paper using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). The products, detected using the staining technique (Section 2.9), were eluted from the paper using water. Typical Rf values for the products were neamine, 0.25; deoxyparomamine, 0.45; paromamine, 0.35; and methyl neobiosaminide, 0.6.

2.15 Preparation of methanolic hydrogen chloride

Dry methanol (100ml) was put into a 250ml round bottom flask and cooled in an ice bath. HCl (gas) was bubbled through for 1.5 hours. HCl (gas) was obtained from either a cylinder or from an HCl generator. The concentration of HCl in the methanol was determined by titration with 1M-NaOH. The solution was stored in a round bottom flask with a sealed stopper at 0°C.

2.16 Preparation of radioactive neamines

a) The synthesis of [ring I-^3H; ring II-^3H] neamine

S. fradiae wild type (A.T.C.C.10745) was grown in Sebek defined medium (Section 2.3) for three days and 2.5ml of the culture was added to 50ml of Sebek complex medium (Section 2.3) in a 500ml Erlenmeyer flask. After three days incubation 50μCi of D-[6-$^3H_2$] glucose (25μmoles) was added to the culture and after a further two days, when antibiotic production was maximal, neomycin was isolated (Section 2.6) and purified (Section 2.7). Usually 25 to 30mg of neomycin was produced. Incorporation of ^3H into neomycin (generally labelled in rings I to IV) was 3 to 4%.

[ring I-^3H; ring II-^3H] neamine was prepared from the generally labelled [ring I-^3H; ring II-^3H; ring III-^3H; ring IV-^3H]

neomycin by a method involving methanolysis in methanolic HCl followed by purification of the products using paper chromatography (Section 2.14). The yield of neamine was usually 7.5 to 10mg. By the method of preparation the neamine was ^{3}H-labelled in both rings I and II and is designated [ring I-^{3}H; ring II-^{3}H] neamine.

b) The synthesis of [ring I-^{14}C] neamine

The 2-deoxystreptamine-idiotroph of S. fradiae (A.T.C.C.21401) was grown in Sebek defined medium (Section 2.3) for three days. Then 10ml of this culture was transferred to a 2-litre Erlenmeyer flask containing 200ml of Sebek complex medium (Section 2.3). After three days incubation a solution containing 50mg of 2-deoxystreptamine, which had been sterilized for 15 minutes at 103KPa in a pressure cooker, and 50μCi of D-[U-^{14}C] glucose (25 moles) was added to the culture. This was incubated for a further two days and the neomycin extracted (Section 2.6) and purified (Section 2.7). This yielded 20 to 40mg neomycin. Neamine was produced as in Section 2.16a. By the method of preparation this species of neamine is labelled with ^{14}C only in ring I and is designated [ring I-^{14}C] neamine.

c) The preparation of [ring I-^{14}C,^{3}H; ring II-^{3}H] neamine

Solutions containing [ring I-^{3}H; ring II-^{3}H] neamine and [ring I-^{14}C] neamine were mixed to give [ring I-^{14}C,^{3}H; ring II-^{3}H] neamine.

2.17 Radioactivity measurement

^{3}H and ^{14}C were assayed using a Philips liquid scintilation analyser. Solutions in water of the samples containing radioactivity were added to 10ml of scintilant. The scintilation fluid contained zylene, 2 litres; synperonic NXP, 1 litre; 2,5-diphenyloxazole, 12g; and 1,4-bis (4-methyl-5-phenyloxazol-2-yl) benzene, 0.9g. If necessary the scintilant was clarified using water.

2.18 Electrophoresis

Electrophoresis at pH3.5 was carried out using a Gilson high voltage electrophorator, model D. The buffer contained pyridine: acetic acid : water (1:10:280, by vol.). Electrophoresis at pH10.0 was carried out using a Locarte high voltage electrophorator. The buffer contained 50mM triethylamine in water with the pH adjusted to 10.0 using acetic acid.

2.19 N-acetylation

The method of N-acetylation was based on the procedure of Carter et al. (1961).

Neamine free base was prepared by passing 100mg neamine hydrochloride in 10ml water through 4ml IRA400 resin in the hydroxyl phase. The resin was washed with water and the eluates pooled and evaporated to dryness. The product was suspended in 12ml methanol and cooled in an ice bath. 4ml acetic anhydride was added and the suspension cleared. This was left at 0°C overnight and then poured into 40ml of anhydrous diethyl ether. This produced a white precipitate which was collected and dried.

Deoxyneamine and deoxyparomamine were N-acetylated using a similar technique, scaled down as necessary.

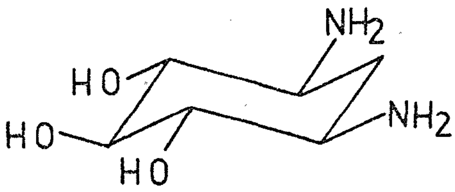
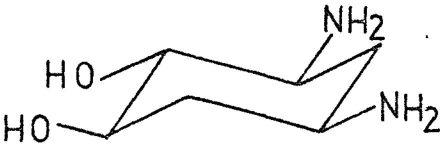
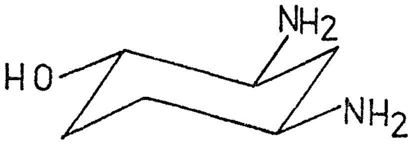
2.20 Hydrolysis of N-acetyl compounds

The following method is based on the technique reported by Carter et al. (1961).

1mg of N-acetyl compound was refluxed with 0.5ml of 2.5M-HCl at 100°C for 10 hours. The HCl was removed using suction and the residue dissolved in a minimum of water. The products were then subjected to paper chromatography.

Figure 3.1 The conversion of aminocyclitols into antibiotic by deoxystreptamine-idiotrophs of antibiotic-synthesizing organisms

The following compounds were tested for their ability to support antibiotic production (Section 2.5). + denotes antibiotic production and - denotes no antibiotic production.

Compound (250µg/ml)	Antibiotic production		
	S. fradiae	S. rimosus	S. kanamyceticus
2-deoxystreptamine 	+	+	+
2,4-dideoxystreptamine 	+	+	-
2,4,5-dideoxystreptamine 	+	-	-

Chapter 3
The biosynthesis of novel antibiotics

3.1 Introduction

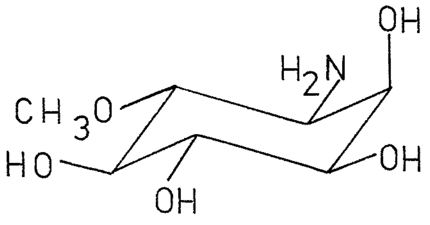
Aminocyclitol-idiotrophs of aminoglycoside-producing organisms have been previously used to produce antibiotics in which the natural aminocyclitol is replaced by a synthetic analogue. Thus 2-deoxystreptamine has been replaced by streptamine, *epi*-streptamine, streptidine and 2,5-dideoxystreptamine in a number of antibiotics (Schier *et al*., 1969; Kojima & Satoh, 1973; Testa & Tilley, 1975; Claridge *et al*., 1974; Taylor & Schmitz, 1976). Also the streptidine moiety of streptomycin has been replaced by 2-deoxystreptidine using similar techniques (Nagaoka & Demain, 1975).

This section is an account of the biosynthesis of novel antibiotics of the neomycin and neamine type using a similar approach.

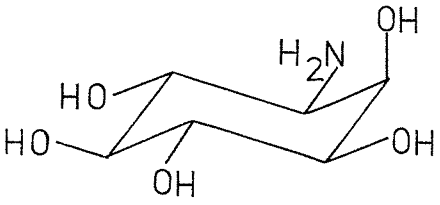
3.2 The conversion of aminocyclitols into antibiotics by 2-deoxystreptamine-idiotrophs of antibiotic-synthesizing streptomyces

Using the technique described in Section 2.5 the compounds shown in Fig. 3.1 and Fig. 3.2 were tested for their ability to support the production of antibiotic active against *E. coli*. Three of the compounds were able to support antibiotic synthesis (Fig. 3.1). Thus when the 2-deoxystreptamine-idiotrophs of neomycin- and paromycin-producing organisms were grown in the presence of 2,4-dideoxystreptamine, antibiotic was produced. The 2-deoxystreptamine-idiotroph of the neomycin-producing organism was also capable of synthesizing antibiotic in the presence of 2,4,5-trideoxystreptamine, whereas the paromomycin-producing organism failed to do so. The kanamycin-producing 2-deoxystreptamine-idiotroph only produced antibiotic from 2-deoxystreptamine. The other two idiotrophs used also produced antibiotic from 2-deoxystreptamine. None of the compounds had any effect on the antibiotic activity produced from 2-deoxystreptamine except *myo*-inosose-2 (see Appendix for an account of the effect of *myo*-inosose-2 on the neomycins).

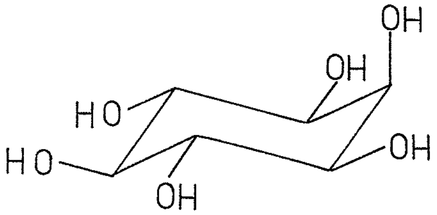
Figure 3.2 Structures of aminocyclitols not able to support antibiotic biosynthesis



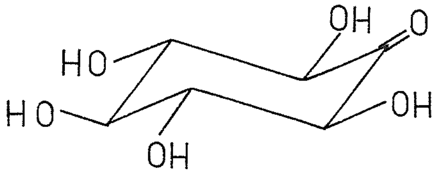
2-O-methyl-1L-1-amino-1-deoxy-*myo*-inositol



1L-1-amino-1-deoxy-*myo*-inositol



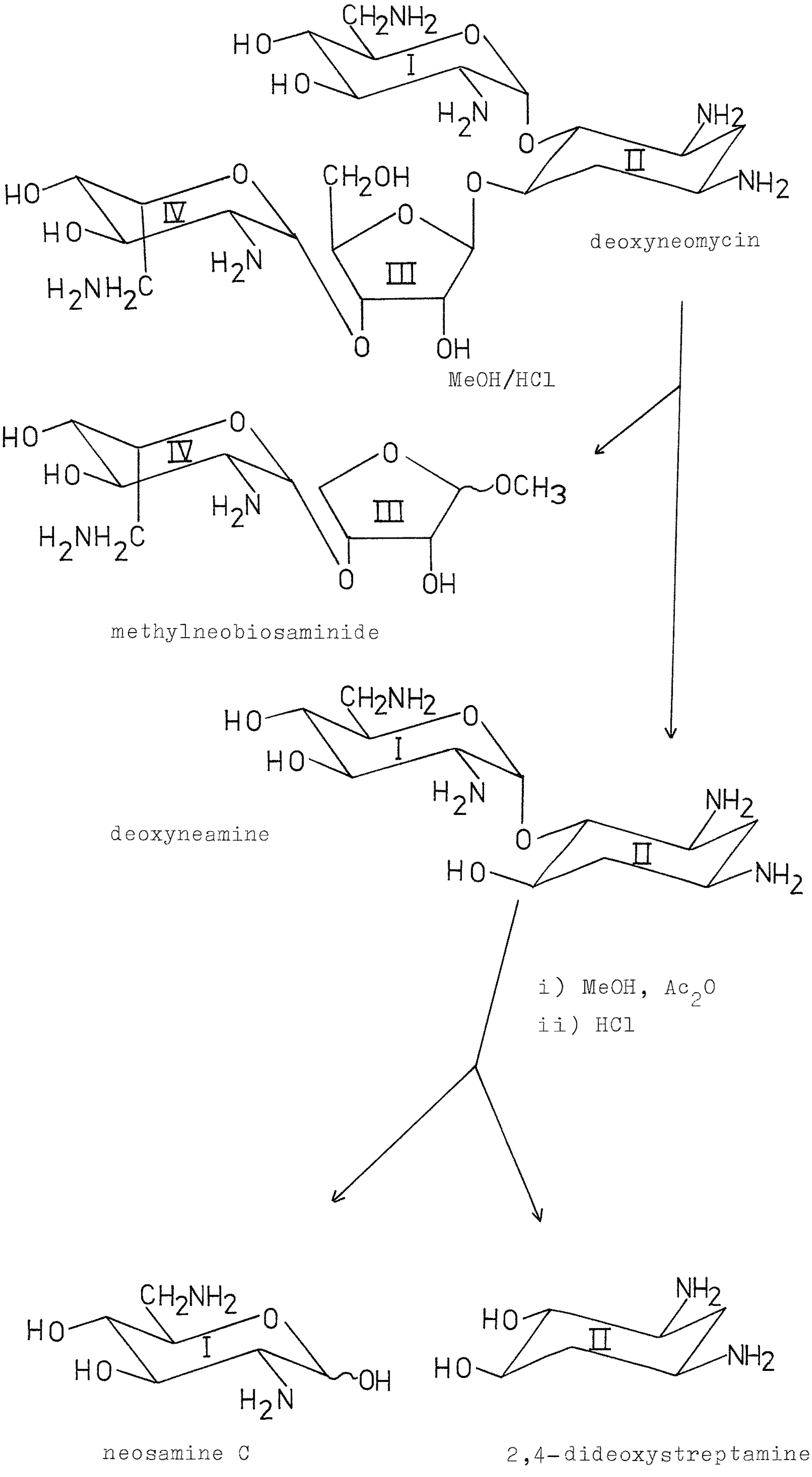
myo-inositol



myo-inosose-2*

*Interpretation of results with *myo*-inosose-2 is difficult because this compound inactivates neomycin and would probably inactivate any antibiotic produced (see Appendix).

Scheme 3.1 Reactions involved in deoxyneomycin degradation (For the sake of clarity only the B isomer is shown)



3.3 The biosynthesis of deoxyneomycin

The experiments described below demonstrate that 2,4-dideoxystreptamine is incorporated into a neomycin-type antibiotic such that it replaces 2-deoxystreptamine. The antibiotic produced is thus deoxyneomycin (structure shown in Scheme 3.1).

a) Biosynthesis and isolation of deoxyneomycin

A sample of the antibiotic produced by the 2-deoxystreptamine-idiotroph of S.fradiae (A.T.C.C.21401) from 2,4-dideoxystreptamine (250μg/ml) was prepared, extracted from the culture and purified as described in Sections 2.4, 2.6 and 2.7. The chromatogrammed antibiotic, which was detected by staining (Section 2.9) and using a bioautogram (Section 2.10) was eluted from the paper (Section 2.7) with water and was dried using a rotary evaporator, this producing a yellowish residue. At no stage in the procedure was the temperature allowed to rise above 50°C.

As an alternative to purification using paper chromatography, gel filtration chromatography was examined (Section 2.8). Crude deoxyneomycin (50mg) eluted from the IRC-50 resin was applied to the top of the column in 1ml and eluted with 0.1M -NH_3 solution. 2,4-dideoxystreptamine was eluted in fraction 35-41 and the antibiotic was eluted in fractions 23-33 (fraction volume 3ml). The fractions containing antibiotic were pooled and concentrated using a rotary evaporator.

The antibiotic could be separated into two active components by paper chromatography and the solvent t-butanol/butan-2-one/methanol/6.5M -NH_3 solution (Table 3.1a). The components were designated deoxyneomycin B and deoxyneomycin C depending on their properties in comparison to neomycin B and C. Thus deoxyneomycin B migrated a greater distance from the origin in two different chromotography conditions than did deoxyneomycin C. Deoxyneomycin B was a more active antibiotic than deoxyneomycin C.

Although the Rf values for the B and C components are different using MeOH/NH_3 solution (sp. gr. 0.88) as solvent (Table 3.1b) in practice chromatography with this solvent is not a satifactory way to prepare individual components because of imperfect separation.

Table 3.1 Chromatographic properties of deoxyneomycin

a) Deoxyneomycin was chromatogrammed on paper using the solvent t-butanol/butan-2-one/methanol/6.5M -NH_3 solution (3:16:1:6). The chromatogram was developed for 72hours. Antibiotics were detected using the staining technique (Section 2.9) and using a bioautogram (Section 2.10).

Compound	Distance migrated (cm)
deoxyneomycin B	16
deoxyneomycin C	9

b) The components separated as above were eluted using water and rechromatogrammed on paper using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). Antibiotics were detected using the staining technique (Section 2.9).

Compound	Rf value
neomycin B	0.25
neomycin C	0.165
deoxyneomycin B	0.29
deoxyneomycin C	0.21

b) Estimation of purity of deoxyneomycin

The purity of the deoxyneomycin complex purified on paper was determined using the ribose assay (Section 2.13) and by using a radiochemical assay.

[^{3}H]-deoxyneomycin was prepared from 2,4-[1-^{3}H] dideoxystreptamine using the technique described in Section 2.4, with the modification that 2,4-[1-^{3}H] dideoxystreptamine (0.11μCi/mg, 1mg/ml) was added as the aminocyclitol supplement. From the specific radioactivity of both the 2,4-dideoxystreptamine and the synthesized deoxyneomycin, it was possible to calculate the purity of the deoxyneomycin. The results in Table 3.2 show that the radiochemical and the ribose assay give similar values for the purity of deoxyneomycin. The chemical assay estimates the antibiotic to be 72% deoxyneomycin and the radiochemical assay estimates the antibiotic to be 66% deoxyneomycin.

In order to determine the structure of the antibiotic the following experiments were performed.

c) Methanolysis of deoxyneomycin

Methanolysis of deoxyneomycin (100mg) (Section 2.14) gave two products (A and B). The yield of deoxyneamine (product A) was 12.5mg and the yield of product B was 16mg. Chromatography showed that methanolysis of both neomycin and deoxyneomycin gave two products (Table 3.3). Product B from deoxyneomycin methanolysis had the same Rf value as product B from neomycin methanolysis, indicating that it is possibly a mixture of methylneobiosaminides (Scheme 3.1). Product A from deoxyneomycin methanolysis had a different Rf value to that of product A from neomycin methanolysis (neamine). These results are expected on the basis that the difference between the two antibiotics is in that half of the molecule containing ring II.

d) Electrophoresis of product B from the methanolysis of neomycin and deoxyneomycin

Product B from the methanolysis of deoxyneomycin behaved in a similar way, under various conditions of electrophoresis, to product B from neomycin which is known to be a mixture of methylneobiosaminides (Table 3.4). From this information and that

Table 3.2 Estimation of purity of deoxyneomycin

[^{3}H]-deoxyneomycin was prepared and purified using the techniques described in Section 2.4, 2.6 and 2.7. The purified deoxyneomycin was analysed using the ribose assay (Section 2.13) and the specific radioactivity was determined by measuring the amount of ^{3}H present in a sample of purified deoxyneomycin (0.1ml of a solution containing 17.5mg deoxyneomycin/ml).

a) Radiochemical assay

Compound	Specific radioactivity (dpm/μMol)
2,4 dideoxystreptamine added to the medium	37,950
deoxyneomycin isolated from the medium	24,925

Therefore the antibiotic contains 66% deoxyneomycin.

b) Ribose assay

0.25ml of a solution containing 4.38mg of deoxyneomycin produced the same quantity of furfural as 3.25mg neomycin. Therefore the antibiotic contains 74% deoxyneomycin.

Table 3.3 Chromatographic properties of the methanolysis products from the neomycins

Approximately 250µg of each methanolysis product was chromatogramed for 4.5 hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). The compounds were detected by staining (Section 2.9).

Starting material	Rf value
neomycin	0.20
deoxyneomycin	0.25

Methanolysis product		Rf value
From neomycin:	product A (neamine)	0.25
	product B (methyl neobiosaminide)	0.6
From deoxyneomycin:	product A	0.35
	product B	0.6

Table 3.4 Electrophoresis of product B from the methanolysis of neomycin and deoxyneomycin

The product B of the methanolysis described in Section 3.3c was purified by paper chromatography using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). The compound with an Rf of 0.6 was eluted and subjected to paper electrophoresis in two different buffer systems (Section 2.18). Compounds were detected by staining (Section 2.9).

a) Electrophoresis was carried out using 4,000 volts for 20 minutes in buffer pH3.5.

Compound	Distance migrated (cm)
Product B from neomycin	17.5 towards cathode
Product B from deoxyneomycin	17

b) Electrophoresis was carried out using 4,000 volts for 20 minutes in buffer pH10.

Compound	Distance migrated (cm)
Product B from neomycin	5 towards cathode
Product B from deoxyneomycin	5

The experiment described in b) above showed a non-migrating spot from both compounds.

obtained from chromatography it is probable that products B from neomycin and deoxyneomycin are identical and are methylneobiosaminides, these being produced from neobiosamine (see Scheme 3.1).

e) Mass spectrometry of product A from the methanolysis of neomycin and deoxyneomycin

The mass spectra of the tetramethylsilyl derivatives of products A from the methanolysis of neomycin (neamine) and deoxyneomycin were similar except that two peaks in the spectrum of neamine appeared 88 mass units lower, at m/e 255 and 372. This decrease in mass would correspond to the loss of an $-OSi(CH_3)_3$ group in the tetramethylsilylated derivatives which corresponds to the replacement of a hydroxyl group with a proton in the original compound.

This confirms that the difference between neomycin and deoxyneomycin lies in the rings I-II fragment, and that this fragment of deoxyneomycin is probably a deoxyneamine.

f) The hydrolysis of deoxyneamine

Neamine and deoxyneamine were both exposed to N-acetylating conditions (Section 2.19). The glycosidic bond joining neosamine C (ring I) to deoxystreptamine (ring II) is relatively stable to acid hydrolysis. This is because the 2-amino group of neosamine C can be protonated and thus provides an electrostatic shielding of the glycosidic bond which inhibits protonation of the glycosidic oxygen. N-acetylation removes the basic amino-group adjacent to the glycosidic link by producing a neutral amide. Hydrolysis of the glycosidic link is then possible (Rinehart, 1964).

The N-acetylated compounds were hydrolysed in 6N-HCl for 10 hours (Section 2.20) and then subjected to paper chromatography together with standards. The hydrolysis of neamine produced a compound corresponding to 2-deoxystreptamine and the hydrolysis of deoxyneamine produced a compound corresponding to 2,4-dideoxystreptamine (Table 3.5). A compound with an Rf value of 0.3 was produced from the hydrolysis of both compounds.

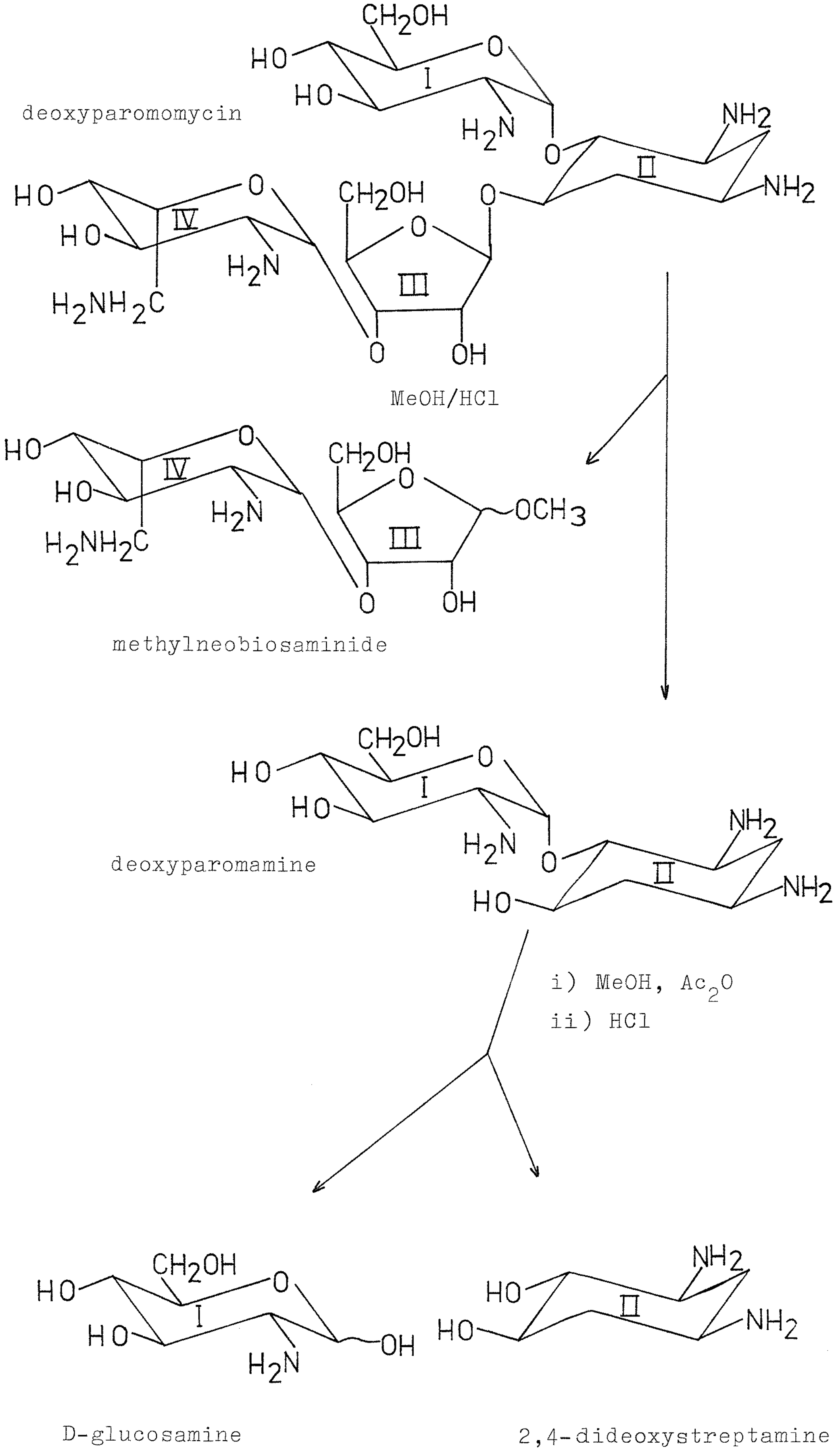
In these conditions the N-acetyl group will be removed from the compounds. The minor spot (Rf value 0.4) produced from the deoxyneamine hydrolysis mixture was probably deacetylated starting material.

Table 3.5 Chromatography of the hydrolysis products from neamine and deoxyneamine

Compounds, hydrolysed in HCl as described in the text, were chromatogrammed on paper for 4.5 hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). Compounds were detected by staining (Section 2.9).

Compound		Rf value
deoxystreptamine		0.55
hydrolysed neamine	i)	0.55
	ii)	0.3
dideoxystreptamine		0.6
hydrolysed deoxyneamine	i)	0.6
	ii)	0.3
	iii) (minor spot)	0.4

Scheme 3.2 Reactions involved in deoxyparomomycin degradation
(For the sake of clarity only the I isomer is shown)



Thus the difference between neamine and deoxyneamine is that the former contains 2-deoxystreptamine and the latter 2,4-dideoxystreptamine.

g) Antibacterial potency of deoxyneomycin

Using the technique described in Section 2.11, the antibacterial potency of the deoxyneomycins and deoxyneamine against various organisms was determined. The results in Table 3.6 show that the deoxyneomycin complex has a similar antibacterial spectrum to, but is slightly less active than neomycin, although deoxyneomycin C is more potent than neomycin C. Deoxyneamine is also an antibiotic but is weak compared with neamine (Table 3.13).

3.4 The biosynthesis of deoxyparomomycin

The experiments described below demonstrate that 2,4-dideoxystreptamine is incorporated into a paromomycin-type antibiotic such that it replaces 2-deoxystreptamine. The antibiotic produced is thus deoxyparomomycin (structure shown in Scheme 3.2).

a) Biosynthesis and isolation of deoxyparomomycin

A sample of the antibiotic synthesized by the 2-deoxystreptamine-idiotroph of S.rimosus forma paromomycinus in the presence of 2,4-dideoxystreptamine (250μg/ml) was prepared, extracted and purified using the techniques described in Section 2.4, 2.6 and 2.7. The antibiotic was separated by further paper chromatography into two components (Table 3.7a).

The components were designated deoxyparomomycin I and deoxyparomomycin II depending on their properties in comparison to paromycin I and II. Thus deoxyparomomycin I migrated a greater distance from the origin in two different chromatography conditions than did deoxyparomomycin II. Deoxyparomomycin I was a more active antibiotic than deoxyparomomycin II.

Although the Rf values for the I and II components were different using MeOH/NH_3 solution (sp. gr. 0.88) as solvent (Table 3.7b) in practice this technique is not a satisfactory way to prepare individual components because of imperfect separation.

The following experiments were carried out in order to determine the structure of the antibiotic.

Table 3.6 Antibacterial potency of deoxyneomycins

The minimum inhibitory concentrations (M.I.C.) of the deoxyneomycin components and neomycin components (prepared from mixtures of the relevant antibiotic using paper chromatography as described in Table 3.1a) against the organisms listed below were determined using the technique described in Section 2.11. The deoxyneomycin mixture was 50% pure (see Section 2.13 for method) and the results for this antibiotic mixture have been corrected accordingly.

Organism	M.I.C. (μg/ml)					
	neomycin			deoxyneomycin		
	mixture	B	C	mixture	B	C
E. coli PT1	0.62	0.62	80	1.25	2.5	5
E. coli PT2	>80	>80	>80	>80	>80	>80
E. coli W3110	2.5	2.5	40	1.25	1.25	80
P. mirabilis PT3	1.25	1.25	10	2.5	5	5
S. aureus PT4	> 80	>80	>80	>80	>80	>80
S. aureus PT5	> 80	>80	>80	>80	>80	>80
S. aureus PT6	0.62	0.62	80	2.5	2.5	20
P. aeruginosa PT7	80	80	>80	80	80	>80
S. sonnei C631-978	80	40	>80	>80	>80	>80
S. typhimurium LT2	2.5	2.5	40	5	5	20
E. coli K-12 ML1629	>80	>80	>80	>80	>80	>80
E. coli K-12 ML1410	>80	>80	>80	>80	>80	>80
E. coli JR66/W699	>80	>80	>80	>80	>80	>80
P. aeruginosa H9	>80	>80	>80	>80	>80	>80

Table 3.7 Chromatographic properties of deoxyparomomycin

a) Deoxyparomomycin was chromatogrammed on paper for 72 hours using the solvent t-butanol/butan-2-one/MeOH/6.5M -NH_3 solution (3:16:1:6, by vol.). Compounds were detected using the staining method (Section 2.9) and using a bioautogram (Section 2.10).

Compound	Distance migrated (cm)
deoxyparomomycin I	20
deoxyparomomycin II	11

b) The components separated as above were eluted and rechromatogrammed for 4.5 hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). Antibiotics were detected using the staining method (Section 2.9).

Compound	Rf value
paromomycin I	0.3
paromomycin II	0.22
deoxyparomomycin I	0.34
deoxyparomomycin II	0.275

b) Methanolysis of deoxyparomomycin

Deoxyparomomycin (16mg) was refluxed in methanolic HCl (Section 2.14). The product was subject to paper chromatography. From the results (Table 3.8) it can be seen that one common fragment is produced by the methanolysis of paromomycin and deoxyparomomycin (product B) and this corresponds to methylneobiosaminide. The other fragment is different; paromamine migrates with an Rf value of 0.35 whereas the corresponding compound from deoxyparomomycin has an Rf value of 0.45.

c) Electrophoresis of product B from the methanolysis of paromomycin and deoxyparomomycin

Product B from the methanolysis of deoxyparomomycin behaved in a similar way, under various conditions of electrophoresis, to product B from paromomycin which is known to be a mixture of methylneobiosaminides (Table 3.9). From this information and that obtained from chromatography it seems probable that products B from paromomycin and deoxyparomomycin are identical and are methylneobiosaminides, these being produced from neobiosamine (see Scheme 3.2).

d) The hydrolysis of deoxyparomamine

Product A from deoxyparomomycin methanolysis was shown to be deoxyparomamine by the following experiment.

Deoxyparomamine (6mg) was N-acetylated (Section 2.19) and hydrolysed in 6N HCl (Section 2.20). The hydrolysate was subjected to paper chromatography together with standards. Chromatography of the hydrolysate produced only one spot (Table 3.10) which has an Rf value similar to 2,4-dideoxystreptamine and D-glucosamine. It was concluded that subunits I and II were probably D-glucosamine and 2,4-dideoxystreptamine respectively.

e) Antibacterial potency of deoxyparomomycin

Using the procedure described in Section 2.11 it was shown that deoxyparomomycin was a less active antibiotic than paromomycin (Table 3.11).

Table 3.8 Chromatographic properties of the methanolysis products from the paromomycins

Antibiotics were subject to methanolysis as described (Section 3.4b). The residue produced when the methanol and HCl were removed was applied to paper and developed for 4.5 hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). Compounds were detected using the staining technique (Section 2.9).

Starting material	Rf value
paromomycin	0.25
deoxyparomomycin	0.30

Methanolysis products		Rf value
from paromomycin;	product A (paromamine)	0.35
	product B (methylneobiosaminides)	0.6
from deoxyparomomycin;	product A	0.45
	product B	0.6

Table 3.9 Electrophoresis of product B from the methanolysis of paromomycin and deoxyparomomycin

The products B from the methanolysis described in Section 3.4b were purified on paper using the solvent MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). The compound with an Rf of 0.6 was eluted and subjected to paper electrophoresis in two different buffer systems (Section 2.18). Compounds were detected by staining (Section 2.9).

a) Electrophoresis was carried out using 4,000 volts for 20 minutes in buffer pH3.5.

Compound	Distance migrated (cm)
product B from paromomycin	17.0 towards cathode
product B from deoxyparomomycin	17.5

b) Electrophoresis was carried out using 4,000 volts for 20 minutes in buffer pH10.

Compound	Distance migrated (cm)
product B from paromomycin	4.5 towards cathode
product B from deoxyparomomycin	4.0

The experiment described in b) above showed a non-migrating spot from both compounds.

Table 3.10 Chromatography of the hydrolysis products from deoxyparomamine

N-acetyl deoxyparomamine was hydrolysed as described in the text and the hydrolysate chromatogrammed for 4.5 hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). Compounds were detected using the staining technique (Section 2.9).

Compound		Rf value
deoxyparomamine hydrolysate		0.63
D-Glucosamine	(ring I)	0.64
2,4-dideoxystreptamine	(ring II)	0.63

Table 3.12 Chromatographic properties of dideoxyneamine

The antibiotic produced by S. fradiae in the presence of 2,4,5-trideoxystreptamine (Section 3.5) was chromatogrammed on paper for 4.5 hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). The antibiotic was detected using the staining technique (Section 2.9) and using a bioautogram (Section 2.10).

Compound	Rf value
2,4,5-trideoxystreptamine	0.65
antibiotic (dideoxyneamine)	0.45
deoxyneamine	0.35

Table 3.11 Antibacterial potency of deoxyparomomycin

The minimum inhibitory concentrations (M.I.C.) of the deoxyparomomycin components and the paromomycin components (prepared from the mixture using paper chromatography as described in Table 3.7a) against the organisms listed below were determined using the technique described in Section 2.11.

Organism	M.I.C. (μg/ml)			
	paromomycin		deoxyparomomycin	
	I	II	I	II*
E. coli PT1	2.5	20	20	>40
E. coli PT2	>80	>80	>80	>40
E. coli W3110	1.25	10	10	40
P. mirabilis PT3	0.65	2.5	5	10
S. aureus PT4	>80	>80	>80	>40
S. aureus PT5	>80	>80	80	>40
S. aureus PT6	2.5	20	>80	>40
P. aeruginosa PT7	>80	>80	>80	>40
S. sonnei C631-978	>80	>80	>80	>40
S. typhimurium LT2	10	40	40	>40
E. coli K-12 ML1629	>80	>80	>80	>40
E. coli K-12 ML1410	>80	>80	>80	>40
E. coli JR66/W699	>80	>80	>80	>40
P. aeruginosa H9	>80	>80	>80	>40

*Deoxyparomomycin II was tested only up to a concentration of 40μg/ml.

3.5 The biosynthesis of dideoxyneamine

If 2,4,5-trideoxystreptamine is incorporated into a neamine-type antibiotic such that it replaces 2-deoxystreptamine, the compound produced will have the structure shown in Figure 6.3 Insufficient starting material was available to be able to produce enough antibiotic to characterize it to the same degree as the other deoxyaminoglycosides.

a) Biosynthesis and isolation of dideoxyneamine

A sample of the antibiotic synthesized by the 2-deoxystreptamine-idiotroph of S.fradiae in the presence of 2,4,5-trideoxystreptamine (250μg/ml) was isolated and purified as described in Sections 2.4, 2.6 and 2.7. Chromatographic properties of the antibiotic are given in Table 3.12.

b) Antibacterial potency of dideoxyneamine

The antibacterial potency of the compound was determined using the technique described in Section 2.11. The results are presented in Table 3.13. As can be seen the compound is not such a potent antibiotic as neamine, which is itself inferior to neomycin, and it has a similar activity spectrum to that of neamine.

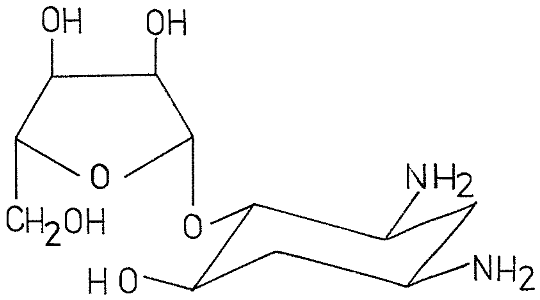
Table 3.13 Antibacterial potency of deoxyneamine and dideoxyneamine

The minimum inhibitory concentrations (M.I.C.) of neamine and deoxyneamine (prepared as described in Section 2.14), and dideoxyneamine (prepared as described in Section 3.5a) were determined using the technique described in Section 2.11.

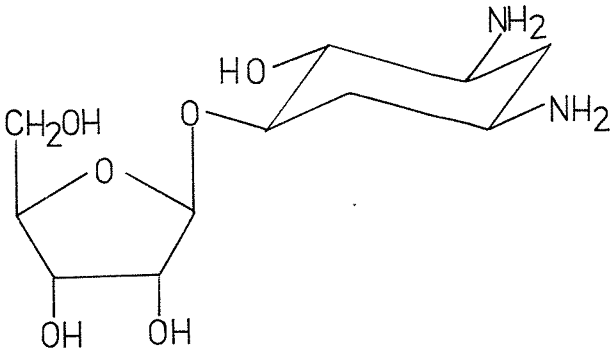
Organism	M.I.C. (µg/ml)		
	neamine	deoxyneamine	dideoxyneamine
E. coli PT1	40	80	80
E. coli PT2	>80	>80	>80
E. coli W3110	40	80	80
P. mirabilis PT3	20	40	40
S. aureus PT4	>80	>80	>80
S. aureus PT5	>80	>80	>80
S. aureus PT6	20	>80	80
P. aeruginosa PT7	>80	>80	>80
S. sonnei C631-978	80	80	>80
S. typhimurium LT2	20	>80	N.D.
E. coli K-12 ML1629	>80	N.D.	>80
E. coli K-12 ML1410	>80	N.D.	>80
E. coli JR66/W699	>80	N.D.	>80
P. aeruginosa H9	>80	N.D.	>80

N.D. represents not determined.

Figure 4.1 The structures of 4- and 5-O-β-ribosyl-2,6-dideoxystreptamine



4-O-β-ribosyl-2,6-dideoxystreptamine



5-O-β-ribosyl-2,6-dideoxystreptamine

Chapter 4

The conversion of 2,6-dideoxystreptamine ribosides into antibiotics

4.1 Introduction

The biosynthesis of neomycin by S. fradiae may involve an intermediate consisting of rings II-III (see Section 1.7). This chapter is an account of an attempt to determine the sequence of subunit assembly of neomycins using an analogue of the possible intermediate consisting of rings II-III. This was examined for its ability to support the biosynthesis of an active antibiotic by 2-deoxystreptamine-idiotrophs of aminoglycoside-producing organisms.

It has been suggested that aminocyclitol-glycosides (consisting of rings I-II of an aminoglycoside antibiotic) may be converted into antibiotic by 2-deoxystreptamine-idiotrophs via hydrolysis, releasing aminocyclitol which can then be incorporated into antibiotic (Rinehart & Stroshane, 1976). This chapter is also an account of a demonstration of antibiotic biosynthesis by such a mechanism.

4.2 Antibiotic production in the presence of 2,6-dideoxystreptamine-ribosides

From Table 4.1 it can be seen that while 2-deoxystreptamine-idiotrophs of both neomycin- and paromomycin-producing organisms produced antibiotic when grown in the presence of 4-O-β-ribosyl-2,6-dideoxystreptamine (Fig. 4.1), neither organism is capable of producing antibiotic in the presence of 5-O-β-ribosyl-2,6-dideoxystreptamine (Fig. 4.1). It was also found (Table 4.1) that 2-deoxystreptamine-idiotrophs of neomycin- and paromomycin-producing organisms produced compounds with antibacterial activity when grown in the presence of 2-deoxystreptamine and 5-O-β-ribosyl-2,6-dideoxystreptamine. This demonstrates that the latter compound does not inhibit antibiotic production. The 2-deoxystreptamine-idiotroph of the paromomycin-producing organism was also capable of converting deoxyneamine into antibiotic.

Table 4.1 Antibiotic production in the presence of 2,4-dideoxystreptamine derivatives

The following compounds were tested for their ability to support antibiotic production (Section 2.5).

Compound	Antibiotic production by 2-deoxystreptamine-idiotrophs (zone of inhibition; mm)	
	S. fradiae	*S. rimosus* forma *paromomycinus*
4-*O*-β-ribosyl-2,6-dideoxystreptamine (250μg/ml)	23	25
5-*O*-β-ribosyl-2,6-dideoxystreptamine (250μg/ml)	0	0
2-deoxystreptamine (125μg/ml)	43	40
2-deoxystreptamine (125μg/ml) plus 5-*O*-β-ribosyl-2,6-dideoxystreptamine (75μg/ml)	40	37
deoxyneamine (250μg/ml)	0	25

4.3 Antibiotic biosynthesis by a 2-deoxystreptamine-idiotroph of S. fradiae during growth with 4-O-β-ribosyl-2,6-dideoxystreptamine

When the 2-deoxystreptamine-idiotroph of S. fradiae was grown in Sebek complex medium (Section 2.3) supplemented with 4-O-β-ribosyl-2,6-dideoxystreptamine (125μg/ml), antibiotic was produced, the final activity being approximately equal to 80μg deoxyneomycin/ml (Section 2.4).

The following experiments were performed to characterize the antibiotic.

4.4 Chromatographic properties of the antibiotic

The antibiotic (produced as described in Section 2.4) was extracted from the culture (Section 2.6) and purified using paper chromatography (Section 2.7). The antibiotic was detected using the staining technique (Section 2.9) and using the bioautogram (Section 2.10). The Rf value was similar to that of deoxyneomycin (Table 4.2a).

Paper chromatography of the antibiotic developed with t-butanol/butan-2-one/methanol/6.5M-NH_3 (3:16:1:6, by vol.) demonstrated that the antibiotic contained at least two components (for Rf values see Table 4.2b), both possessing antibacterial activities and corresponding to the positions of deoxyneomycin components C and B. Furthermore purified antibiotic B components from deoxyneomycin and from the antibiotic derived from 4-O-β-ribosyl-2,6-dideoxystreptamine migrated together when re-chromatogrammed (Table 4.3), as detected using the bioautogram and staining method. Both antibiotics have equal potency (Section 4.8) therefore if the chromatography had separated the B components there should be two peaks of antibacterial activity of similar magnitude. The small peak of antibiotic present in the bioautogram at 5cm from the origin is probably due to the presence of small quantities of component C.

4.5 Electrophoretic properties of the antibiotic

Electrophoresis of the antibiotic in pH3.9 buffer showed that the antibiotic derived from 4-O-β-ribosyl-2,6-dideoxystreptamine migrated with deoxyneomycin (Table 4.4).

Table 4.2 Chromatographic properties of the antibiotic

a) Purified antibiotics (250µg) were chromatogrammed for 4 hours on paper using MeOH/NH_3-solution (sp. gr. 0.88). Compounds were detected by staining (Section 2.9).

Compound	Rf value
deoxyneomycin	0.2
antibiotic produced from 4-O-β-ribosyl-2,6-dideoxystreptamine	0.2

b) Purified antibiotics (250µg) were chromatogramed on paper for 60 hours using t-butanol/butan-2-one/methanol/6.5M-NH_3 solution (3:16:1:6, by vol.). Compounds were detected using the staining method (Section 2.9). The two components from both antibiotics were shown to be active using a bioautogram (Section 2.10).

Compound		Distance migrated (cm)
deoxyneomycin	A	13
	B	21
antibiotic produced from 4-O-β-ribosyl-2,6-dideoxystreptamine;	A	13
	B	24

Table 4.3 Co-chromatography of antibiotic B components

The faster migrating component (B) of deoxyneomycin and the antibiotic from the precursor 4-O-β-ribosyl-2,6-dideoxystreptamine were eluted from a paper chromatogram which had been developed as described in Table 4.2b. 250μg aliquots of each antibiotic component were applied to the same spot on a paper chromatogram which was developed as described in Table 4.2b for 72 hours.

a) Compounds detected by staining (Section 2.9)

Compound	Distance migrated (cm)
deoxyneomycin B	10.5
B component of antibiotic derived from 4-O-β-ribosyl-2,6-dideoxystreptamine	10.5
mixture of the two antibiotics above	10.5

b) Bioautogram of chromatogrammed mixture (Section 2.10)

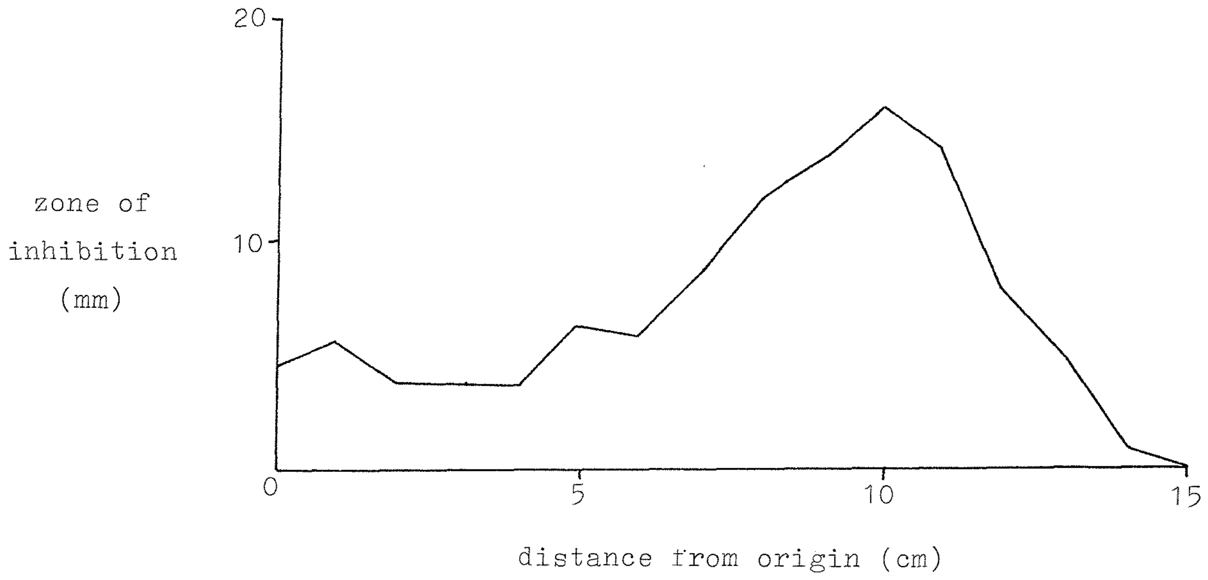


Table 4.4 Electrophoretic properties of the antibiotic

Antibiotics (250µg) were subjected to paper electrophoresis for 45 minutes using 2,000 volts in pH3.9 buffer (Section 2.18). Compounds were detected using the staining method (Section 2.9).

Compound	Distance migrated
deoxyneomycin	11.5cm towards cathode
antibiotic derived from 4-*O*-β-ribosyl-2,6-dideoxystreptamine	11.5cm towards cathode

4.6 Ribose content of the antibiotic

Antibiotic (0.5ml) in aqueous solution was assayed for ribose using the technique of Dutcher *et al*. (Section 2.13). In terms of ribose concentration a solution containing 10mg of deoxyneomycin/ml was equivalent to a solution containing 5mg of neomycin /ml, and a solution containing 10mg of antibiotic derived from 4-*O*-β-ribosyl-2,6-dideoxystreptamine was equivalent to a solution containing 4.8mg of neomycin/ml. Thus the antibiotic derived from 4-*O*-β-ribosyl-2,6-dideoxystreptamine contains 98% of the ribose content of deoxyneomycin.

4.7 Methanolysis of the antibiotic

The antibiotic was subjected to methanolysis followed by chromatography (Table 4.5). It was demonstrated that the Rf values of the methanolysis products from the antibiotic are the same as those obtained from the methanolysis products of deoxyneomycin.

4.8 Antibacterial potency of the antibiotic

Using the technique described in Section 2.11 the antibiotic potency of the B components of the compound derived from 4-*O*-β-ribosyl-2,6-dideoxystreptamine was shown to be very similar to the antibiotic potency of the corresponding deoxyneomycin component (Table 4.6), differing in only two cases. This last observation could be due to slight impurities in the components from the former antibiotic.

Table 4.5 Chromatography of methanolysis products from the antibiotic

Antibiotic derived from 4-O-β-ribosyl-2,6-dideoxystreptamine (1mg) was subject to methanolysis (Section 2.14) followed by paper chromatography of the products for four hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). Compounds were detected using the staining method (Section 2.9).

Methanolysis products	Rf value
from deoxyneomycin; A	0.4
B	0.7
from antibiotic derived from 4-O-β-ribosyl-2,6-dideoxystreptamine; A	0.4
B	0.7

Table 4.6 Antibacterial potency of the antibiotic

Using the technique described in Section 2.11, the antibacterial potencies (Minimum inhibitory concentration, M.I.C.) of deoxyneomycin B and the corresponding component of the antibiotic produced from 4-O-β-ribosyl-2,6-dideoxystreptamine were determined.

Test organism	M.I.C. (μg/ml)	
	deoxyneomycin B	component B from antibiotic produced from 4-O-β-ribosyl-2,6-dideoxystreptamine
E. coli PT1	2.5	2.5
E. coli PT2	> 80	> 80
P. mirabilis PT3	5	10
S. aureus PT4	> 80	> 80
S. aureus PT5	> 80	> 80
S. aureus PT6	2.5	2.5
P. aeruginosa PT7	> 80	> 80
S. typhimurium LT2	5	10
E. coli K-12 ML 1629	> 80	> 80
E. coli K-12 ML 1410	> 80	> 80
E. coli JR66/W677	> 80	> 80
P. aeruginosa H9	> 80	> 80

Chapter 5

The conversion of neamine into neomycin by a Streptomyces species

5.1 Introduction

It is probable that 2-deoxystreptamine (ring II of neomycin) exists as a free intermediate during neomycin biosynthesis (see Section 1.7) and that this is then glycosylated at positions 4 and 5 by rings I and rings III or III-IV respectively. There is little evidence in favour of either mechanism although Rinehart (1964) and Falkner (1969) have both proposed routes for neomycin biosynthesis involving the intermediacy of neamine (see Section 1.7).

This chapter is an account of the conversion of neamine intact into an antibiotic by _Streptomyces rimosus_ forma _paromomycinus_. This antibiotic was subsequently shown to be neomycin but in earlier experiments is referred to as antibiotic.

5.2 The biosynthesis of antibiotic from neamine

a) Biosynthesis of antibiotic during growth on solid medium

When a 2-deoxystreptamine-idiotroph of _S. rimosus_ forma _paromomycinus_ was grown on nutrient agar in the presence of neamine, antibiotic was produced (Table 5.1). By contrast, 2-deoxystreptamine-idiotrophs of _S. fradiae_ and _S. kanamyceticus_ both failed to produce antibiotic under similar conditions. The reason for this is not obvious and is discussed later (Section 6.5). The growth of all three _Streptomyces_ was inhibited by 100μg of neamine/ml of the incubation medium.

b) Biosynthesis of antibiotic during growth in liquid medium

Table 5.2 shows the result of an experiment in which the 2-deoxystreptamine-idiotroph of _S. rimosus_ forma _paromomycinus_ was grown in liquid medium in the presence of neamine. It is seen that neamine itself at concentrations of 100μg/ml shows antibiotic activity. While there was no initial antibiotic activity in the broth with 50μg/ml or less of neamine, after 5 days incubation antibacterial activity was present, antibiotic production being optimal using 50μg neamine/ml medium. The results presented in Table 5.3 demonstrate that antibiotic activity was at a maximum after 5 days incubation and decreased after 6 days incubation.

Table 5.1 The biosynthesis of antibiotic from neamine during growth on solid medium

The conversion of neamine into antibiotic by various 2-deoxystreptamine-idiotrophs was examined using the techniques described in Section 2.5. The organisms are described in Section 2.2.

Compound added	Zone of growth inhibition produced (mm)		
	S.fradiae	S.rimosus forma paromomycinus	S.kanamyceticus
none	0	0	0
deoxystreptamine (250μg/ml)	41	33	37
neamine (50μg/ml)	0	31	0
neamine (100μg/ml)	no growth	no growth	no growth

Table 5.2 The biosynthesis of antibiotic from neamine during growth in liquid medium

The 2-deoxystreptamine-idiotroph of the paromycin-producing organism, S. rimosus forma paromomycinus, was grown in liquid medium (Section 2.3) for three days. 2.5ml of this culture was used to inoculate 50ml of similar medium containing neamine in a 500ml Erlenmeyer flask. This was incubated as described in Section 2.3. Samples of the culture were removed at the intervals indicated and the antibiotic activity assayed using the procedure described in Section 2.12.

Neamine (μg/ml)	Antibiotic activity*		Increase in antibiotic activity
	Initial	Final (after 5 days)	
0	0	0	0
10	0	0-4	0-4
20	0	4	4
30	0	7.5	7.5
40	0	11	11
50	0	14	14
100	9	11	2
250	20	20	0

*antibiotic activity is expressed in terms of activity equivalent to μg of neomycin/ml medium.

Table 5.3 Time course of antibiotic production from neamine

The 2-deoxystreptamine-idiotroph of *S. rimosus* forma *paromomycinus* was grown for seven days in the presence of neamine hydrochloride (50μg/ml medium). Samples were removed from the culture daily and assayed for antibiotic. Details of methods are in Section 2.4 and 2.11.

Incubation period (days)	Antibiotic (equivalent to μg neomycin/ml)
0	0
1	5
2	10
3	12
4	15.5
5	20
6	20
7	15.5

The reason for this decrease is not known but it is a common occurrence in antibiotic-producing cultures (for example see Wagman et al., 1973)

5.3 Isolation of antibiotic and its separation into two active components

The 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus was grown in the presence of neamine (50µg/ml) (Section 2.4) and the resulting antibiotic was extracted and purified as described in Sections 2.6 and 2.7. The purified antibiotic was rechromatogrammed with standard neomycin. The results presented in Table 5.4 show that the biosynthesized antibiotic migrated the same distance as the standard neomycin.

A sample of the antibiotic was separated by further chromatography into two components; these corresponded to neomycins B and C as detected by staining and antibacterial activity (Table 5.5). The antibiotic activity associated with component C in the mixture was less in the case of the antibiotic derived from neamine than in the commercial sample of neomycin.

These results suggest the antibiotic synthesized from neamine is neomycin. The following experiment confirms this.

5.4 Methanolysis of the antibiotic

A sample of the antibiotic produced by the 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus supplemented with neamine (50µg/ml) was subjected to methanolysis followed by paper chromatography (Table 5.6). The results demonstrate that both of the methanolysis products from the antibiotic have similar chromatographic properties to the corresponding methanolysis products from standard neomycin, but that neither product migrates with paromamine which is one of the methanolysis products obtained from paromomycin.

It is concluded that the antibiotic produced from neamine in the experiments above is a mixture of neomycin B and C.

It is possible, however, that neomycin was synthesized from neamine via a hydrolysis mechanism in which neamine was cleaved to produce free 2-deoxystreptamine which was then incorporated into a neomycin-type antibiotic. Alternatively neamine may be cleaved and

Table 5.4 The chromatographic properties of the antibiotic produced from neamine by *S. rimosus* forma *paromomycinus*

The antibiotic produced by the 2-deoxystreptamine-idiotroph of *S. rimosus* forma *paromomycinus* in the presence of neamine (50μg/ml) (described in Section 2.4) was extracted and purified as described in Section 2.6 and 2.7. The purified antibiotic was eluted from the first chromatogram with water and subjected to paper chromatography with standard neomycin using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V) as solvent and developing the chromatogram for 7 hours. Approximately 250μg of each compound was applied to the paper. The antibiotics were detected as described in Section 2.9.

Compound	Distance migrated relative to neamine
neamine	1
neomycin	0.55
paromomycin	0.77
antibiotic synthesized from neamine	0.52

Table 5.5 The separation into components of the antibiotic produced from neamine by the 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus

250µg aliquots of the purified antibiotic, synthesized from neamine as described in Table 5.4, and neomycin standards were subjected to paper chromatography developed for 72 hours using the solvent t-butanol/butan-2-one/6.5M-NH_3 solution/methanol (3:16:6:1, by vol.).

The antibiotics were detected using the staining method (Section 2.9) and by bioautography (Section 2.10).

a) antibiotic detected by staining

Compound	Distance migrated (cm)
neomycin B	11
neomycin C	6
antibiotic derived from neamine 1)	10
2)	6

b) antibiotic detected using a bioautogram

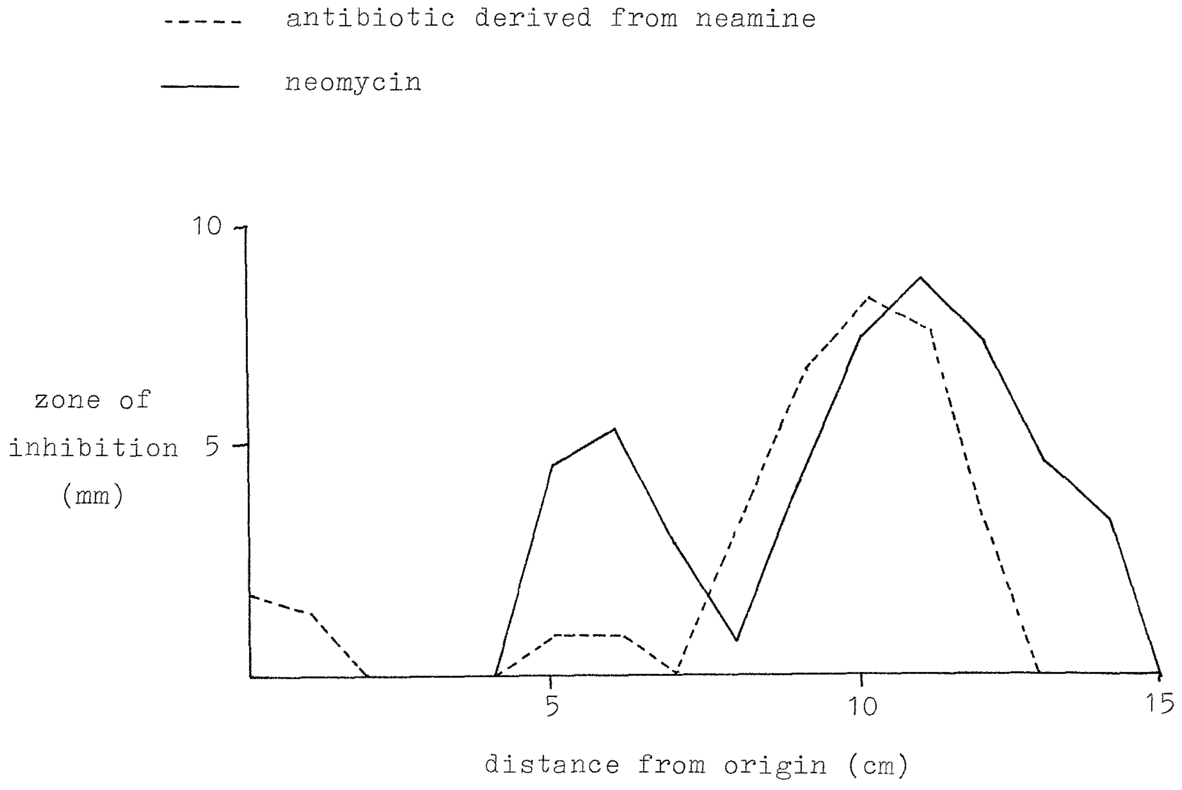


Table 5.6 Methanolysis of the antibiotic

Purified antibiotic synthesized from neamine by the 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus (see Table 5.4) was subjected to methanolysis (Section 2.14). The products of methanolysis were subjected to paper chromatography developed for 4 hours using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V). Compounds were detected using the staining method (Section 2.9).

Compound		Rf value
antibiotic derived from neamine		0.2
neomycin		0.2
methanolysis products from antibiotic derived from neamine	1)	0.25
	2)	0.7
neamine		0.25
methyl neobiosaminide		0.7
paromamine		0.4

then resynthesized prior to conversion into neomycin.

The following radiochemical experiments were designed to confirm that neamine is converted into neomycin intact.

5.5 The conversion of radioactive neamine into neomycins

The experiment described in Section 5.3 was repeated using neamine containing ^{14}C and ^{3}H in ring I, and ^{3}H only in ring II. This latter neamine is designated [ring I-^{14}C,^{3}H; ring II-^{3}H] neamine (for details of the preparation of this compound see Section 2.16).

The double labelled neamine was incubated with the 2-deoxystreptamine-idiotroph of the paromomycin-producing organism for 5 days in liquid culture, and at the end of this period the antibiotics were extracted from the broth and separated using paper chromatography. The areas corresponding to neamine, neomycin B and neomycin C were eluted and the radioactivity and antibiotic activity determined in each sample. The results in Table 5.7 show that neamine, and the compounds corresponding to neomycin B and neomycin C all have similar ^{3}H:^{14}C ratios, suggesting that neamine is incorporated intact into the neomycins.

Neomycin was biosynthesized from a mixture containing equal amounts of the double-labelled neamine and non-radioactive 2-deoxystreptamine. Once again the ^{3}H:^{14}C ratios of the precursor neamine and the antibiotic produced were almost identical (Table 5.8) confirming that neamine is incorporated intact and not via a hydrolysis-resynthesis mechanism.

5.6 The distribution of radioactivity in neomycin biosynthesized from radioactive neamine

To show that neamine was incorporated into rings I-II of neomycin the following experiment was performed.

The double-labelled neamine was incubated with the 2-deoxystreptamine-idiotroph of the paromomycin-producing organism and the neomycin produced was isolated and purified. A sample of the latter was subjected to methanolysis followed by chromatography. The majority of the ^{14}C and ^{3}H isolated from the chromatogram was associated with neamine (Table 5.9). During this biosynthesis and degradation procedure no change in the ^{3}H:^{14}C ratio of the precursor neamine and that obtained from the degradation of neomycin was observed (Table 5.10).

Table 5.7 The conversion of radioactive neamine into neomycin

2.5mg of [ring I-^{14}C, ^{3}H; ring II-^{3}H] neamine (see Section 2.16) was incubated for five days in a 50ml culture of the 2-deoxystreptamine-idiotroph of *S. rimosus* forma *paromomycinus* as described in Section 2.4. Synthesized antibiotic and unchanged neamine were isolated from the culture as described in Section 2.6 and purified as described in Section 2.7 excepting that the chromatogram was developed for 72 hours using the solvent system t-butanol/butan-2-one/methanol/6.5M-NH_3 solution (3:16:1:6, by vol.). For the determination of specific radioactivity, neomycin and neamine were assayed by the procedure described in Section 2.12; purified neomycin B was used as a standard.

Radioactivity was measured in 10ml tritoscint II using a Philips liquid scintilation analyser (Section 2.17).

Compound isolated	dpm		^{3}H: ^{14}C ratio	Specific Radioactivity (dpm/μmol)	
	^{3}H	^{14}C		^{3}H	^{14}C
neamine	9290	510	18.2	8930	490
neomycin B	4790	300	16	8300	520
neomycin C	2220	125	17.7	-	-

Table 5.8 The conversion of radioactive neamine into neomycin in the presence of 2-deoxystreptamine

The experiment described in Table 5.7 was carried out with the modification that 2.5mg non-radioactive 2-deoxystreptamine was added to the culture medium together with the radioactive neamine.

Compound isolated	Radioactivity (dpm)		$^{3}H:^{14}C$ ratio
	^{3}H	^{14}C	
neamine	7290	530	14
neomycin B	9920	620	16
neomycin C	3160	270	12

Table 5.9 The distribution of radioactivity in neomycin biosynthesized from radioactive neamine

Neomycin was synthesized from radioactive neamine as described in Table 5.7. The neomycins were extracted and purified (Sections 2.6 and 2.7) and subjected to methanolysis (Section 2.14) together with 200mg of neomycin sulphate carrier. The products were subjected to paper chromatography developed using MeOH/NH_3 solution (sp. gr. 0.88) (4:1, V/V) for 4 hours and the chromatogram divided into five sections. Each section was eluted with water and the radioactivity determined.

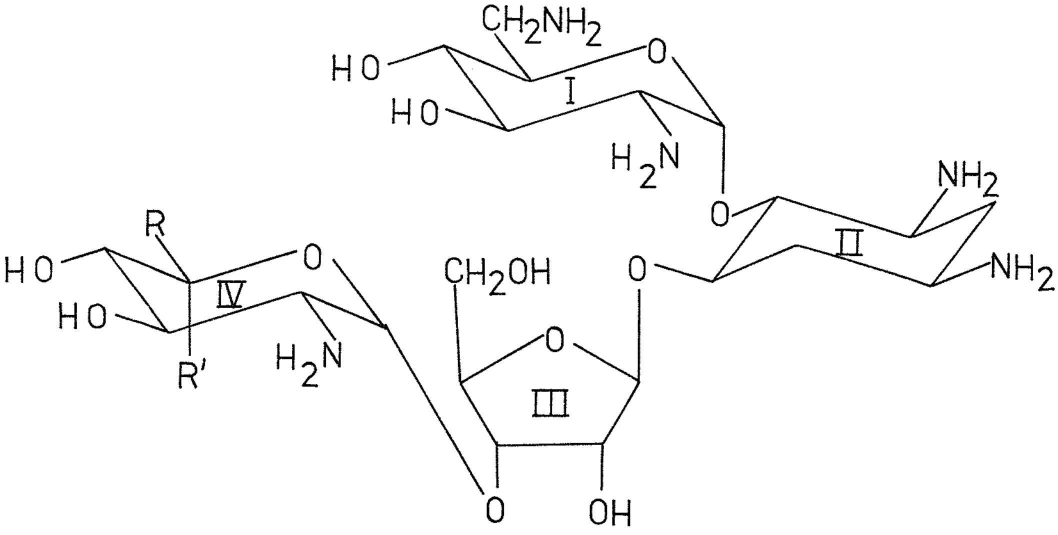
Isotope	Radioactivity (dpm) in section eluted				
	1	2	3	4	5
^{3}H	830	4600	380	510	30
^{14}C	660	3900	400	620	310
Section corresponds to	-	ring I-II	-	ring III-IV	-

Table 5.10 Radioactivity associated with aminoglycosides during a synthesis and degradation cycle

The radioactivity associated with i) a sample of the neomycin biosynthesized in the experiment described in Table 5.9, ii) neamine produced from the methanolysis of this biosynthesized neomycin, and iii) unchanged neamine isolated from the culture broth in the same experiment, was determined as described in Section 2.17.

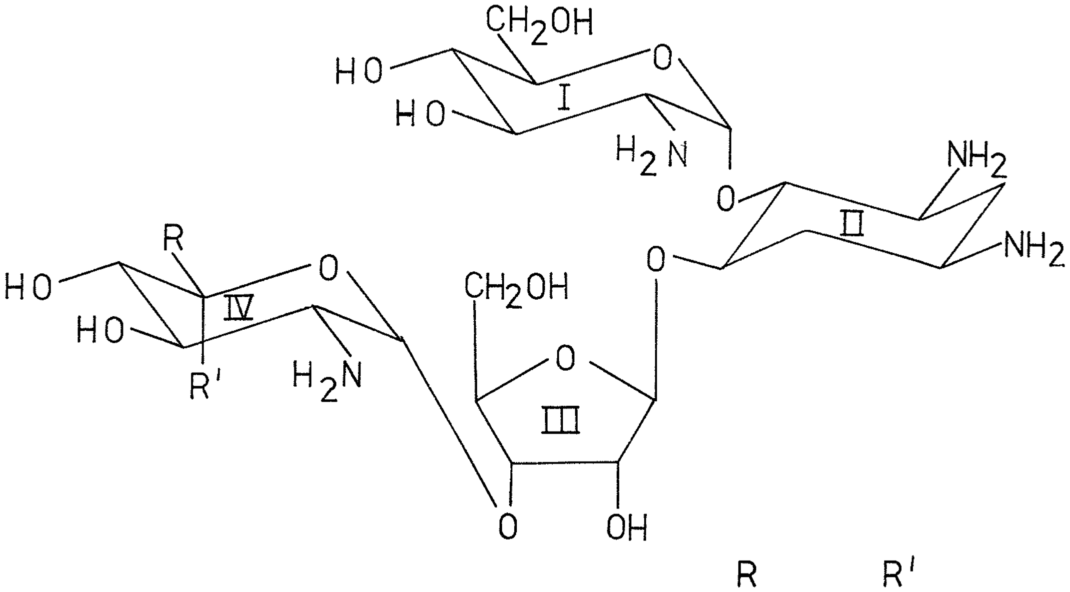
Compound	Radioactivity (dpm)		^{3}H: ^{14}C ratio
	^{3}H	^{14}C	
neomycin isolated from culture	1520	1400	1.08
neamine isolated from culture	4560	3820	1.19
neamine produced by the methanolysis of the isolated neomycin	4600	3900	1.18

Figure 6.1 The structure of deoxyneomycin



	R	R'
deoxyneomycin B	H	CH_2NH_2
deoxyneomycin C	CH_2NH_2	H

Figure 6.2 The structure of deoxyparomomycin



	R	R'
deoxyparomomycin I	H	CH_2NH_2
deoxyparomomycin II	CH_2NH_2	H

Chapter 6
Discussion

This discussion is concerned with the biosynthesis of neomycins and of novel antibiotics, and with the influence of the aminocyclitol moiety on the biological activity of these compounds.

6.1 The role of the aminocyclitol moiety of neomycins in determining biological activity

It has been demonstrated that modification of the aminocyclitol moiety of aminoglycosides can have a profound effect on the biological properties of the antibiotic.

The effect on the biological activity of neomycin in which 2-deoxystreptamine is replaced with streptamine or epi-streptamine has been examined (Davies, 1970). It has been shown that the streptamine-containing analogue, hybrimycin A_1 (Fig. 1.34), is approximately as active as neomycin B (Fig. 1.4) as an antibiotic, as an inhibitor of RNA-directed protein synthesis and in its ability to produce misreading during poly-uridine-directed peptide biosynthesis. The streptamine-containing neomycin C analogue, hybrimycin A_2 (Fig. 1.34), was more effective in both of the latter experiments than the parent molecule, neomycin C (Fig. 1.4).

However the replacement of the 2-deoxystreptamine-moiety of neomycin with epi-streptamine produces the antibiotics hybrimycin B_1 and B_2 (Fig. 1.34), neither of which is as potent as neomycin B or C in any of the above experiments.

Hybrimycins A and B are both more effective substrates, than neomycin, for the neomycin-inactivating enzymes aminoglycoside phosphotransferase (3') and aminoglycoside acetyltransferase (Davies, 1970).

An alteration of the aminocyclitol moiety of neomycin therefore produces an antibiotic different from neomycin in a number of respects.

6.2 Neomycin analogues containing 2,4-dideoxystreptamine

From the results of the experiments described in Section 3.3 and 3.4, it is concluded that 2,4-dideoxystreptamine was incorporated into deoxyneomycin (Fig. 6.1) by the 2-deoxystreptamine-idiotroph of

S. fradiae, and into deoxyparomomycin (Fig. 6.2) by the 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus. The reactions involved in the elucidation of the structures are outlined in Schemes. 3.1 and 3.2. Methanolysis of the antibiotics produced methylneobiosaminides and either deoxyneamine or deoxyparomamine. Hydrolysis of deoxyneamine or deoxyparomamine produced 2,4-dideoxystreptamine and either neosamine C or D-glucosamine.

That deoxyneomycin and deoxyparomomycin are antibiotics proves that the free hydroxyl of 2-deoxystreptamine is not essential for biological activity.

Deoxyneomycin C (Fig. 6.1) was shown to be a more active antibiotic than neomycin C. This is similar to the observation that hybrimycin A_2 (Fig. 1.34), the streptamine-containing analogue of neomycin C, was a more potent protein synthesis inhibitor than neomycin C. Whether or not there is a common mechanism for the higher activity of both neomycin C analogues is difficult to ascertain.

Neither deoxyneomycin nor deoxyparomomycin is active against organisms resistant to the parent antibiotics, including those organisms whose resistance is due to the presence of the antibiotic inactivating enzymes, aminoglycoside phosphotransferase (3') I or II, or aminoglycoside nucleotidyl transferase (3) (Section 1.10).

6.3 The conversion of 2,4,5-trideoxystreptamine into an antibiotic

The synthesis of an antibiotic, dideoxyneamine, by the 2-deoxystreptamine-idiotroph of S. fradiae in the presence of 2,4,5-trideoxystreptamine is reported in Section 3.5. Although detailed characterization of the antibacterial compound was not performed, its mobility on paper chromatography, in which the compound, like deoxyneamine, migrated further than neamine, is consistent with the compound having the structure shown in Fig. 6.3. That this compound is an active antibiotic shows that no hydroxyl group on the 2-deoxystreptamine-moiety of neamine is required for biological activity. Organisms which were resistant to neamine were also resistant to dideoxyneamine.

Figure 6.3 The structure of dideoxyneomycin

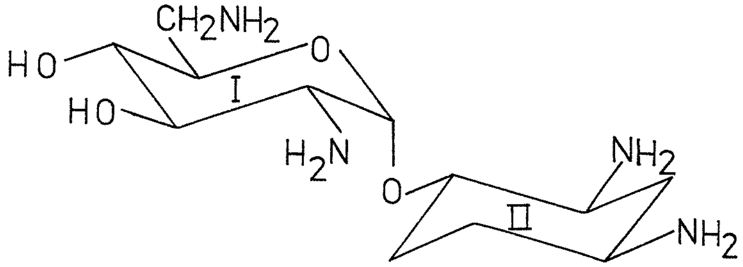
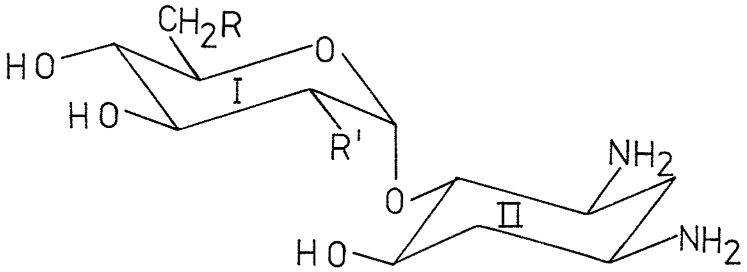
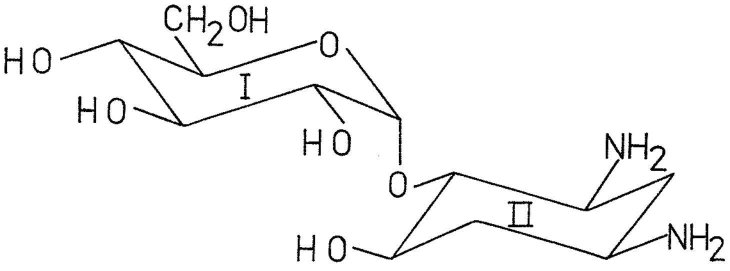


Figure 6.4 The structure of three deoxykanamine analogues



deoxy-analogue of rings I-II of ;	R	R
kanamycin A	NH_2	OH
kanamycin B	NH_2	NH_2
kanamycin C	OH	NH_2

Figure 6.5 The structure of 3-O-β -glucosyl-2,6-dideoxystreptamine



6.4 The failure of S. kanamyceticus to convert 2,4-dideoxystreptamine into antibiotic

Although 2,4-dideoxystreptamine lacks the hydroxyl-group of which ring III of kanamycin is normally attached, the 2-deoxystreptamine-idiotroph of *S. kanamyceticus* might be expected to synthesize deoxyneamine-type analogues (Fig. 6.4) in the presence of 2,4-dideoxystreptamine. One of these compounds, deoxyneamine (the deoxy-analogue of rings I-II of kanamycin B), has been shown to be an antibiotic (Section 3.3), and it is reasonable to suppose that if it were produced it would be detected. Since the medium from the incubation of the 2-deoxystreptamine-idiotroph of *S. kanamyceticus* with 2,4-dideoxystreptamine did not contain antibiotic activity it is concluded that deoxyneamine was not produced.

It has been demonstrated that when a 2-deoxystreptamine-idiotroph of *S. kanamyceticus* was incubated with 2-deoxystreptamine-analogues, kanamycin type antibiotics were produced which contained D-glucose as ring I (Kojima & Satoh, 1973; Fig. 1.36). It is possible that the 2-deoxystreptamine-idiotroph of *S. kanamyceticus* incubated with 2,4-dideoxystreptamine (Section 3.2) produced a compound of the structure shown in Fig. 6.5, which also contains D-glucose as ring I. It is reasonable to presume that such a compound will not be an active antibiotic (Benveniste & Davies, 1973) and would not therefore be detected using the technique described in Section 3.2.

6.5 The failure of 2-deoxystreptamine analogues to support antibiotic production

There are a number of possible reasons why the majority of aminocyclitols and cyclitols tested (Fig. 3.2) did not support antibiotic production. It is possible that not all of the compounds entered the organisms, or that they were modified but the compound produced lacked antibacterial activity, or that the compounds were not modified.

6.6 The effect of myo-inosose-2 on neomycin

Myo-inosose-2 might have been incorporated into antibiotic although none was actually detected in the experiments described in Section 3.2. This is because *myo*-inosose-2 has been shown to inactivate neomycin produced by either the wild-type S. fradiae or the 2-deoxystreptamine-idiotroph of the same organism (see Appendix). Thus if an antibiotic of the neomycin-type had been produced it would probably have been inactivated.

6.7 The conversion of 4-0-β-ribosyl-2,6-dideoxystreptamine into an antibiotic

Antibiotics were produced by the 2-deoxystreptamine-idiotrophs of S. fradiae and S. rimosus forma paromomycinus grown in the presence of 4-O-β-ribosyl-2,6-dideoxystreptamine (Chapter 4).

If the 2-deoxystreptamine-idiotroph of S. fradiae had incorporated 4-O-β-ribosyl-2,6-dideoxystreptamine intact into an antibiotic, then a compound with the structure given in Fig. 6.6 would be expected. This antibiotic should differ from deoxyneomycin (Fig. 6.1) in the following ways. The ribose content would be double that of deoxyneomycin. On electrophoresis in acid buffer, when amino-groups would be protonated, the antibiotic with 4-amino groups would be expected to migrate less than deoxyneomycin with 6-amino groups.

It has been suggested that the amino-groups on ring I have a profound effect on the antimicrobial activity of the aminoglycosides (Benveniste & Davies, 1973). It would be reasonable to expect therefore that replacement of the neosamine C moiety of deoxyneomycin with ribose would have some effect on the antibiotic potency of the compound.

The results reported in Section 4 are not consistent with the responses expected if 4-O-β-ribosyl-2,6-dideoxystreptamine was incorporated into an antibiotic as an intact unit (Fig. 6.6). Moreover the results suggest that the antibiotic produced by the 2-deoxystreptamine-idiotroph of S. fradiae supplemented with 4-O-β-ribosyl-2,6-dideoxystreptamine is identical to deoxyneomycin. The antibiotic produced by the organism supplemented with 4-O-β-ribosyl-2,6-dideoxystreptamine must therefore arise via a hydrolysis mechanism (Fig. 6.7).

This is the first report of an aminocyclitol-glycoside being metabolised by an antibiotic-producing organism using this mechanism, although it has been previously suggested that it may occur (Rinehart & Stroshane, 1976).

Figure 6.6 The structure of a deoxyneomycin B analogue containing -ribose as ring I

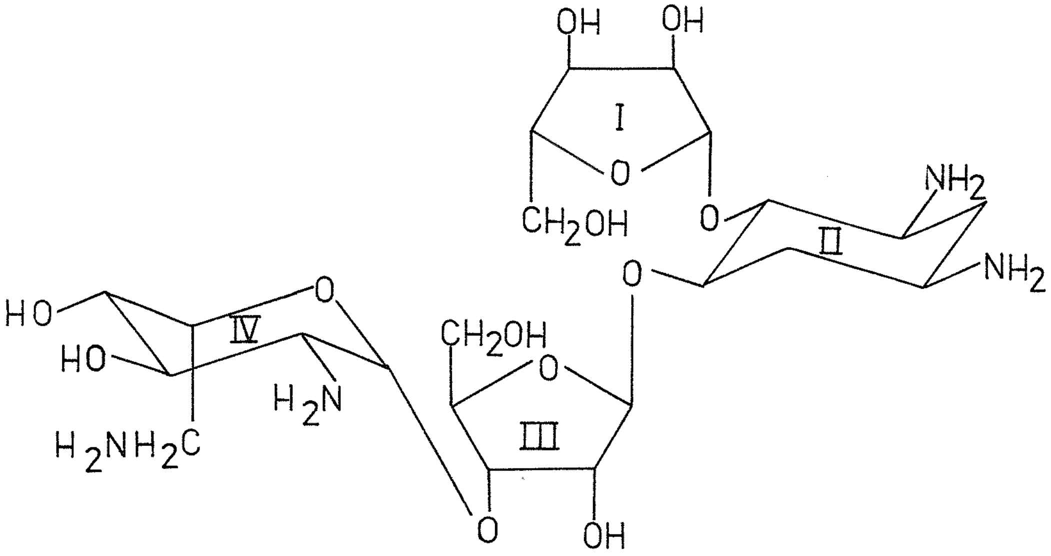
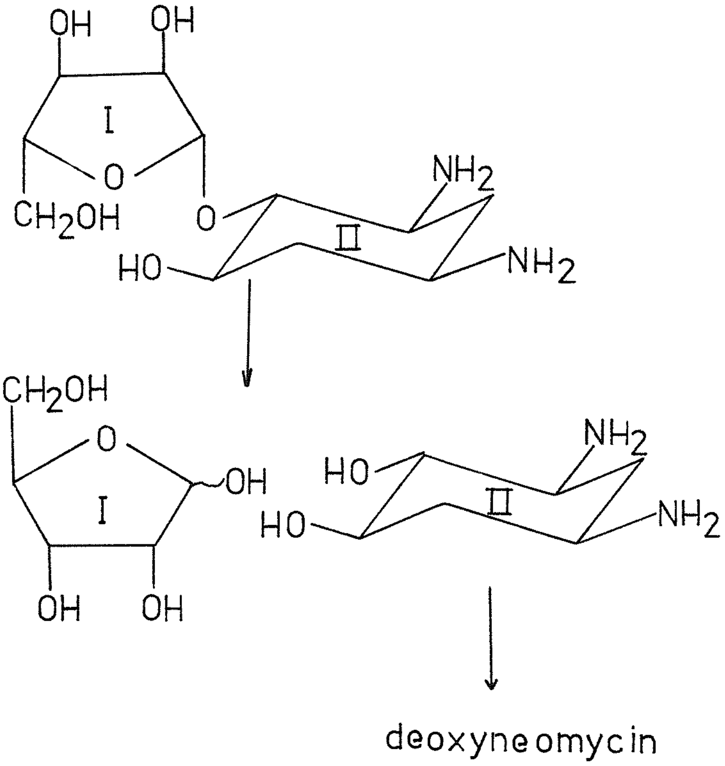


Figure 6.7 The conversion of 4-O- -ribosyl-2,6-dideoxystreptamine into antibiotic



It is assumed that the 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus metabolises 4-O-β-ribosyl-2,6-dideoxystreptamine in a way similar to S. fradiae and that it produces deoxyparomomycin.

6.8 The conversion of neamine into neomycin

The results from the experiments reported in Chapter 5 demonstrate that neamine is converted into neomycin by the 2-deoxystreptamine-idiotroph of S. rimosus forma paromomycinus.

Although supplementing cultures of this organism with neamine might be expected to lead to neomycin-production, the observation that these cultures produced antibiotic activity does not necessarily mean neamine was incorporated intact into neomycin. Evidence to suggest this may occur is provided by the demonstration that 4-O-β-ribosyl-2,6-dideoxystreptamine was hydrolysed by the 2-deoxystreptamine-idiotroph of S. fradiae prior to conversion into antibiotic (Section 6.7).

Thus it was considered possible that the antibacterial activity detected in the experiments described in Section 5.2 was not due to neomycin but to an antibiotic formed by the incorporation of the 2-deoxystreptamine moiety from hydrolysed neamine into a paromomycin-related compound.

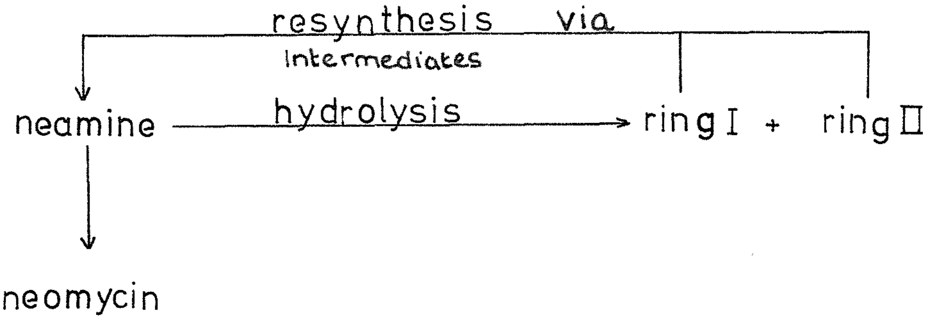
The observation that the antibiotic isolated from cultures of the 2-deoxystreptamine-idiotroph of the paromomycin-producing organism supplemented with neamine has characteristics similar to neomycin, rather than paromomycin, with respect to chromatographic properties (Section 5.3) and from the products of methanolysis (Section 5.4), strongly suggests that neamine was converted intact into the antibiotic neomycin. The radiochemical experiments were designed to confirm this.

6.9 The conversion of radioactive neamine into neomycin

If the antibiotic produced from neamine was a result of the hydrolysis of neamine and the released 2-deoxystreptamine being incorporated into antibiotic, the loss of ring I from neamine and its replacement with a subunit synthesized by the organism may occur. Antibiotic synthesized from [ring I-^{14}C,^{3}H; ring II-^{3}H] neamine by such a mechanism should therefore contain no ^{14}C and the ^{3}H:^{14}C ratio of the antibiotic produced should approach infinity. However the results in Section 5.5 show that the ^{3}H:^{14}C ratio of the starting neamine and the isolated antibiotics were the same, indicating that the precursor neamine was incorporated intact.

The possibility that the species of neamine incorporated into neomycin had been formed by the hydrolysis-resynthesis cycle (Fig. 6.8) was eliminated by growing the 2-deoxystreptamine-idiotroph in the presence of [ring I-^{14}C,^{3}H; ring II-^{3}H] neamine and non-radioactive 2-deoxystreptamine. If a biosynthetic pathway involved hydrolysis of neamine then the ^{3}H associated with the 2-deoxystreptamine produced would be diluted with non-radioactive 2-deoxystreptamine. Consequently the ^{3}H:^{14}C ratio of the antibiotic produced under these circumstances should decrease. However the results in Section 5.5 show that the ^{3}H:^{14}C ratios of the original neamine and the biosynthesized neomycin were identical, thus suggesting that the neamine incorporated into antibiotic is the original species added to the culture.

Figure 6.8 The conversion of neamine into neomycin via a hydrolysis/resynthesis mechanism



The observation that neomycin synthesized from [ring I-^{14}C,^{3}H; ring II-^{3}H] neamine contains radioactivity associated with the neamine part of the molecule only (Section 5.6) is also consistent with the conclusion that added neamine was incorporated intact into neomycin.

6.10 The role of neamine in neomycin biosynthesis

There are two possible routes for the assembly of neomycin from preformed subunits (Fig. 6.9). In route a) neamine (rings I-II) is an intermediate and in route b) either the compound comprising rings II-III or that comprising rings II-III-IV are intermediates.

Figure 6.9 Two alternative pathways for neomycin biosynthesis from preformed subunits

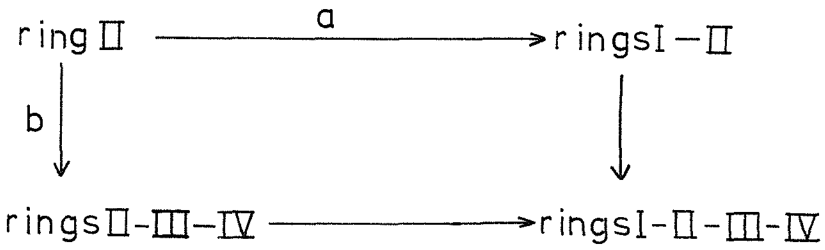
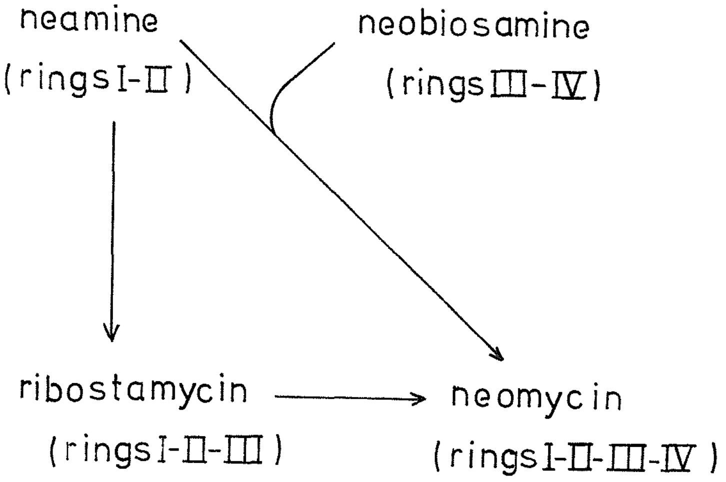


Figure 6.10 The conversion of neamine into neomycin



Although in the past a choice between these two pathways has been difficult the work reported in Chapter 5 suggests that neamine is involved in neomycin biosynthesis (Fig. 6.9a). This excludes route b) of Fig. 6.9 as a biosynthetic pathway for neomycin production.

The observation that neamine is produced by S. fradiae in certain conditions (Perlman & O'Brien, 1953) is explained by the organism being unable to complete the subunit assembly. This may be due to the inhibition of the biosynthesis of rings III and IV or the inhibition of the formation of the glycosidic-link between neamine and the next intermediate of neomycin biosynthesis.

The reason that 5-O-β-ribosyl-2,6-dideoxystreptamine (rings II-III of deoxyneomycin) does not, but deoxyneamine does, support antibiotic production (Section 4.2) is explained by the involvement of a neamine-type compound as an obligatory intermediate of subunit assembly and that the enzymes involved in this pathway cannot accept a compound consisting of rings II-III.

The synthesis of dideoxyneamine from 2,4,5-trideoxystreptamine argues against the involvement of compounds of the rings II-III or rings II-III-IV type in neomycin biosynthesis. Thus if rings II-III or rings II-III-IV had to be synthesized prior to the addition of ring I, then 2,4,5-trideoxystreptamine, which lacks the hydroxyl group to which ring III is linked, could not be converted into an antibiotic of the neamine type.

While this work was in progress Testa & Tilley (1975) demonstrated that neamine and paromamine were precursors for sisomicin biosynthesis. Although they omitted to demonstrate that these compounds were incorporated into sisomicin intact, a pathway for the biosynthesis of this antibiotic was proposed which involved an intermediate of the rings I-II type. Further evidence that an intermediate of the rings I-II type is involved in the biosynthesis of other aminoglycosides is provided by the demonstration that a 2-deoxystreptamine-idiotroph of S. ribosidificus can convert neamine into ribostamycin (Kojima & Satoh, 1973), and by the isolation of a mutant of the gentamicin-producing organism, M. purpurea, which produces only paromamine (Testa & Tilley, 1976).

6.11 The biosynthesis of neomycin from neamine

There are two possible pathways for the conversion of neamine into neomycin (Fig. 6.10). In pathway a) rings III-IV are added as one unit. In pathway b) ribostamycin is synthesized from neamine, and this is then converted into neomycin.

Although neobiosamines, rings III-IV, have never been isolated their involvement in neomycin and biosynthesis has been proposed by Falkner (1969). The evidence for this suggestion is that rings III-IV of neomycin synthesized by S. fradiae in the presence of radioactive precursors were labelled less than rings I-II (Foght, 1963; Schimbor, 1966; Falkner, 1969). As the radioactive precursors were added after incubation of the culture had started, it was suggested that a proportion of rings III-IV(or rings III and IV) had been synthesized prior to the addition of labelled compound and therefore contained less radioactivity than rings I-II, which were suggested to be synthesized subsequent to the addition of label.

In contrast ribostamycin, which consists of rings I-II-III of neomycin, is produced by S. ribosidificus. If ribostamycin were an intermediate of neomycin biosynthesis it would exclude pathway a) of Fig. 6.10.

An unambiguous choice between the two routes in Fig. 6.10 is not possible.

6.12 Biosynthesis of the glycosidic bonds of neomycins

Neomycin is elaborated by the formation of glycosidic-linkages between the subunits. Many naturally occurring glycosylation reactions involve activated-sugar intermediates which are usually nucleoside diphosphate (NDP) derivatives (Glaser, 1964). These activated intermediates react with an acceptor (R-OH) in the following way (Ferrier & Collins, 1972);

$$\text{NDP-sugar} + \text{ROH} \longrightarrow \text{Glycosyl-OR} + \text{NDP}$$

It is possible that the glycosidic bonds of neomycin are synthesized by a similar mechanism. Neamine for example could be produced by a route involving NDP-neosamine C as the activated-intermediate and 2-deoxystreptamine as the acceptor-molecule. NDP-neosamine C could be synthesized from nucleoside triphosphate and neosamine C 1-phosphate, or via an NDP-derivative of a neosamine C precursor which is further modified to produce NDP-neosamine C. Rinehart (1964) has proposed that neosamine C and neosamine B are synthesized by a similar pathway which diverges to produce either epimer (Section 1.5). As epimerization reactions have also been shown to involve NDP-intermediates (Glaser, 1972), it is possible that UDP-neosamine C and UDP-neosamine B are produced from a UDP-derivative of a common precursor.

Appendix

The effect of myo-inosose-2 on neomycin-producing *S. fradiae* cultures

A.1 Introduction

During experiments designed to elucidate the precursors involved in the biosynthesis of the carbon skeleton of streptomycin *myo*-inosose-2 was incubated with *S. griseus* cultures (Hunter & Hockenhull, 1955). It was observed that *myo*-inosose-2 (1mg/ml incubation medium) significantly reduced the final streptomycin activity. It was suggested that *myo*-inosose-2 inhibits the production of streptomycin by inhibiting either the biosynthesis of *N*-methyl-L-glucosamine or the incorporation of streptidine into streptomycin.

In experiments performed on a neomycin-producing culture it was found that the presence of *myo*-inosose-2 decreased the final neomycin activity. This appendix is an account of the effect of *myo*-inosose-2 on neomycin producing cultures.

A.2 The reduction of neomycin activity in *S. fradiae* cultures by myo-inosose-2

The effect of *myo*-inosose-2 on neomycin activity produced in cultures of *S. fradiae* in two different media is shown in Table A.1. In a complex medium neomycin activity after five days incubation was halved using 0.5mg *myo*-inosose-2/ml and in a defined medium neomycin activity was halved by 0.1mg *myo*-inosose-2/ml.

A 2-deoxystreptamine-idiotroph grown in the presence of 2-deoxystreptamine and *myo*-inosose-2 produces less antibacterial activity than when it is grown in the presence of only 2-deoxystreptamine (Table A.2).

In cultures of both organism it is possible by adding sufficient *myo*-inosose-2 to completely abolish the antibacterial activity.

It was considered that *myo*-inosose-2 might react with neomycin to produce an inactive derivative. To investigate this possibility *myo*-inosose-2 and neomycin were incubated together in fresh growth medium and the antibiotic activity measured. The results obtained (Table A.3) demonstrate a slight decrease in antibiotic activity after 5 days incubation. This decrease in antibiotic activity was not dependent on *myo*-inosose-2.

However when neomycin was incubated together with *myo*-inosose-2 in an *S. fradiae* culture the antibiotic activity decreased (Table A.4). It is concluded that *S. fradiae* produces conditions where *myo*-inosose-2 dependent neomycin inactivation is catalysed. The factor responsible for this catalysis is produced only by cultures older than 3 days (Table A.5).

It has previously been demonstrated by Perlman *et al*. (1974) and Perlman & Cowan (1974) that aminoglycosides can be chemically modified in the presence of aldo-sugars. The *N*-glycosides thus produced are less active than the parent antibiotic. For example neomycin B glucoside was prepared which was between 5 and 33% as potent an antibiotic as the parent compound (Perlman & Cowan, 1974).

It is possible that neomycin is modified by *myo*-inosose-2 by a mechanism similar to that proposed for the modification of aminoglycosides by aldo-sugars.

Table A.1 The reduction of neomycin activity in *S. fradiae* (wild type) cultures by myo-inosose-2

a) Sebek complex medium (50mls; Section 2.3) in a 500ml baffled flask was inoculated with 2.5ml of a 2-day old culture of *S. fradiae* grown in similar medium. *Myo*-inosose-2 solution (which had been sterilized at 103KPa for 15 minutes in a steam autoclave) was added at the start of the incubation period and neomycin assayed after 5 days (Section 2.12).

myo-inosose-2 (mg/ml)	neomycin (μg/ml)
0	600
0.5	320
2.0	120
5.0	0

b) Majumdar and Majumdar medium (10ml; Section 2.3) in a 125ml conical flask was inoculated with 1ml of a 3-day old culture of S. fradiae grown in similar medium. *Myo*-inosose-2 was added to the medium as in A.1a and the culture incubated for 5 days. Neomycin was then assayed (Section 2.12).

myo-inosose-2 (mg/ml)	neomycin (μg/ml)
0	320
0.1	140
0.2	40
0.5	10
1.25	0

Table A.2 The reduction of neomycin activity in S. fradiae (2-deoxystreptamine-idiotroph) cultures by myo-inosose-2

The 2-deoxystreptamine-idiotroph of S. fradiae was grown for 2 days in Sebek complex medium (Section 2.3). 1ml of this was added to 10ml of similar medium to which had been added 2-deoxystreptamine (5mg) and myo-inosose-2. Neomycin activity was assayed after 5 days incubation (Section 2.12).

myo-inosose-2 (mg/ml)	neomycin (μg/ml)
0	220
0.25	170
0.5	150
1	120
5	0

Table A.3 The effect of myo-inosose-2 on neomycin in sterile culture medium

Neomycin (500 g/ml) and myo-inosose-2 were incubated in sterile Sebek complex medium (Section 2.3) for 5 days at 30°C and neomycin assayed (Section 2.12).

myo-inosose-2 (mg/ml)	neomycin (μg/ml)
0	380
5	380

Table A.4 Neomycin inactivation by myo inosose-2

A 10% inoculum of a 2-day old culture of the 2-deoxystreptamine-idiotroph of *S. fradiae* was added to Sebek complex medium (10ml; Section 2.3). *Myo*-inosose-2 was then added as in Table A.1a. After 2 days incubation neomycin was added to the cultures and after a further 3 days neomycin was assayed.

myo-inosose-2 (mg/ml)	neomycin (µg/ml)
0	400
5	72

Table A.5 The effect of the time of addition of myo-inosose-2 on neomycin production by S. fradiae

A 10% inoculum of a 2-day old culture of *S. fradiae* was added to 10ml of Sebek complex medium (Section 2.3). *Myo*-inosose-2 (50mg) was added at different times during incubation and samples were removed from the cultures for neomycin assay (Section 2.12).

Age of culture when myo-inosose-2 added (days)	neomycin (µg/ml) age of culture (days)				
	1	2	3	4	5
0	62	180	350	180	80
1	78*	220	220	100	62
2	-	220*	330	180	62
3	-	-	270*	180	70
4	-	-	-	400*	140
control	-	-	-	-	600

*neomycin assayed before *myo*-inosose-2 added to culture

References

Akita, E., Tsuruoka, T., Ezaki, N. & Nüda, T. (1970). J. Antibiot. 23, 173-183.

Bandyophadhyay, S.K. & Majumdar, S.K. (1974). Antimicrob. Ag. Chemother. 5, 431-434.

Benveniste, R. & Davies, J. (1973). Ann. Rev. Bioch. 42, 471-506.

Carter, H.E., Dyer, J.R., Shaw, P.D., Rinehart, K.L., Jr. & Hichens, M. (1961). J. Amer. Chem. Soc. 83, 3723-3724.

Claes, P.J., Compernolle, F., Vanderhaeghe, H. (1974). J. Antibiot. 27, 931-942.

Claridge, C.A., Bush, J.A., Defuria, M.D. & Price, K.E. (1974). Devel. Industr. Microbiol. 15, 101-113.

Cleophax, J., Gero, S.D., Leboul, J., Akhtar, M., Barnett, J.E.G. & Pearce, C.J. (1976). J. Amer. Chem. Soc. 98, 7110-7112.

Davies, J. (1970). Biochim. Biophys. Acta. 222, 674-676.

Dulaney, E.L. (1948). J. Bacteriol. 56, 305-313.

Dutcher, J.D. & Donin, M.N. (1952). J. Amer. Chem. Soc. 74, 3420-3422.

Dutcher, J.D., Hosansky, N. & Sherman, J.D. (1953). Antibiot. Chemother. 3, 534-536.

Falkner, F.C. (1969). Studies on the biosynthesis of neomycin. Ph.D. thesis, University of Illinois, Urbana, Illinois.

Ferrier, R.J. & Collins, P.M. (1972). In Monosaccharide Chemistry p271. Penguin Books Ltd., England.

Foght, J.L. (1963). Biosynthesis studies on the neomycins. Ph.D. thesis, University of Illinois, Urbana, Illinois.

Glaser, L. (1964). In Comprehensive Biochemistry (Florkin, M. & Stotz, E. eds.) vol. 15, Elsevior Publishing Company, Amsterdam.

Glaser, L. (1972). In The Enzymes (Boyer, P.D. ed.), 3rd edn., vol. VI., p.p.355-380, Academic Press, New York.

Heding, H. (1964). Science. 143, 953-954.

Heding, H. (1968). Acta. Chem. Scand. 22, 1649-1654.

Heding, H. & Bajpai, K. (1973). J. Antibiot. 26, 725-727.

Hessler, E.J.,Jahnke, H.K., Robertson, J.H.,Tsuji, K., Rinehart,K.L. Jr. & Shier, W.T. (1970). J. Antibiot. 23, 464-466.

Hunter, G.D. & Hockenhull, D.J.D. (1955). Biochem. J. 59, 268-272.

Kojima, M. & Satoh, A. (1973). J. Antibiot. 26, 784-786.

Lee, B.K., Testa, R.T., Wagman, G.H., Liu, C.M., McDaniel, L. & Schaffner, C. (1973). J. Antibiot. 26, 728-731.

Lee, B.K., Condon, R.G., Wagman, G.H., Byrne, K. & Schaffner, C. (1974). J. Antibiot. 27, 822-825.

Lee, B.K., Condon, R.G., Murauski, A. & Wagman, G.H. (1975). J. Antibiot. 28, 163-166.

Lee, B.K., Condon, R.G., Wagman, G.H. & Katz, E. (1976a). Antimicrob. Ag. Chemother. 9, 151-159.

Lee, B.K., Condon, R.G., Wagman, G.H., Weinstein, M.J. & Katz, E. (1976b). J. Antibiot. 29, 677-684.

Loewus, F.A. & Kelly, S. (1962). Biochem. Biophys. Res. Commun. 7, 204-208.

Majumdar, S.K. & Kutzner, H.J. (1962). Appl. Microbiol. 10,157-168.

Majumdar, M.K. & Majumdar, S.K. (1970). Biochem. J. 120, 271-278.

Majumdar, M.K. & Majumdar, S.K. (1971). Biochem. J. 122, 397-404.

Matsuhashi, Y., Yagisawa, M., Kondo, S., Takeuchi, T. & Umezawa, H. (1975). J. Antibiot. 28, 442-447.

Miller, A.L. & Walker, J.B. (1969). J. Bacteriol. 99, 401-405.

Nagaoka, K. & Demain, A.L. (1974). Abstracts, 74th Ann. Mtg. Amer. Soc. Microbiol., Chigago, p19.

Nagaoka, K. & Demain, A.L. (1975). J. Antibiot. 27, 627-635.

Nomi, R.,Nimi, O. & Kado, T. (1968). Agr. Biol. Chem. 32, 1256-1260.

Olesker, A., Mercier, D., Gero, S.D., Pearce, C.J. & Barnett, J.E.G. (1975). J. Antibiot. 28, 490-491.

Pan, S.C. & Dutcher, J.D. (1956). Anal. Chem. 28, 836-838.

Pearce, C.J., Akhtar, M., Anthony, C., Barnett, J.E.G. & Gero, S.D. (1976). Biochem. J. 159, 601-606.

Peck, R.L., Hoffhine, C.E. Jr., Gale, P. & Folkers, K. (1949). J. Amer. Chem. Soc. 71, 2590-2591.

Perlman, D. & Cowan, S.K. (1974). J. Antibiot. 27, 637-638.

Perlman, D. & O'Brien, E. (1953). Bact. Proc. p20.

Perlman, D., Endō, T., Hinz, R.S., Cowan, S.K. & Endō, S. (1974). J. Antibiot. 27, 525-528.

Price, K., Godfrey, J. & Kawaguchi, H. (1974). Advan. Appl. Microbiol. 18, 191-307.

Rinehart, K.L. Jr. (1964). The neomycins and related antibiotics, John Wiley & Sons, Inc., New York.

Rinehart, K.L. & Stroshane, R.M. (1976). J. Antibiot. 29, 319-353.

Rinehart, K.L. Jr., Malik, J.M., Nystrom, R.S., Stroshane, R.M., Truitt, S.T., Taniguchi, M., Rolls, J.P., Haak, W.J. & Ruff, B.A. (1974a). J. Amer. Chem. Soc. 96, 2263-2265.

Rinehart, K.L. Jr., Stroshane, R.M., Shier, W.T., Truitt, S.T., Taniguchi, M., Malik, J.M., Schaefer, P.C., Rolls, J.P. & Haak, W.J. (1974b). Abstracts, 168th Nat. Mtg. Amer. Chem. Soc., Atlantic City, N. J. Sept. 1974. MICR19.

Rolls, J.P., Ruff, B.D., Haak, W.J., Rinehart, K.L. Jr. & Stroshane, R.M. (1975). Abstracts 75th Ann. Mtg. Amer. Soc. Microbiol., New York. p95.

Schimbor, R.F. (1966). The microbiological incorporation of labelled intermediates into the neomycin antibiotics. Ph.D. thesis, University of Illinois, Urbana, Illinois.

Sebek, O. (1955). Arch. Biochem. Biophys., 57, 71-79.

Shier, W.T., Rinehart, K.L. & Gottlieb, D. (1969). Proc. Nat. Acad. Sci. U.S.A. 63, 198-204.

Shier, W.T., Ogawa, S., Hichens, M. & Rinehart, K.L. Jr. (1973). J. Antibiot. 26, 551-561.

Shier, W.T., Rinehart, K. & Gottlieb, D. (1972). U.S. Pat. 3669838, 1972.

Shier, W.T., Schaefer, P.C., Gottlieb, D. & Rinehart, K.L. Jr. (1974). Biochemistry. 13, 5073-5078.

Shomura, T., Ezaki, N., Tsuruoka, T., Niwa, T., Akita, E. & Nüda, T. (1970). J. Antibiot. 23, 155-161.

Taylor, H.D. & Schmitz, H. (1976). J. Antibiot. 29, 532-535.

Testa, R. & Tilley, B. (1975). J. Antibiot. 28, 573-579.

Testa, R.T. & Tilley, B.C. (1976). J. Antibiot. 29, 140-146.

Testa, R.T., Wagman, G.H., Wagman, P.J.L. & Weinstein, M.J. (1974). J. Antibiot. 27, 917-921.

Umezawa, H., Okanishi, M., Kondo, S., Hamana, K., Utahara, R., Maeda, K. & Mitsuhashi, S. (1967). Science. 157, 1559-1561.

Umezawa, H., Yamamoto, H., Yagisawa, M., Kondo, S., Takeuchi, T. & Chabbert, Y-A. (1973). J. Antibiot, 26, 407-411.

Umezawa, S., Tsuchiya, T., Muto, R., Nishimura, Y. & Umezawa, H. (1971). J. Antibiot. 24, 274-275.

Wagman, G.H., Marquez, J.A., Walkins, P.D., Bailey, J.V., Gentile, F. & Weinstein, M.J. (1973). J. Antibiot. 26, 732-736.

Walker, J.B. & Skorvaga, M. (1973). J. Biol. Chem. 248, 2435-2440.