Review Article

BIOSYNTHESIS OF AMINOCYCLITOL ANTIBIOTICS

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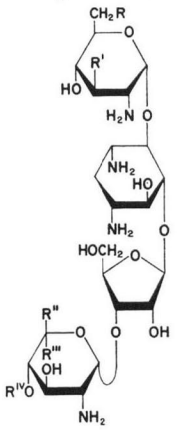
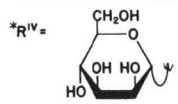
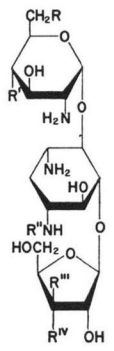
Aminocyclitol antibiotics[1~4] have been used clinically since streptomycin and neomycin first appeared. These two antibiotics were joined somewhat later in the clinic by kanamycin and paromomycin, and in recent years numerous other compounds have joined the ranks of the medically useful aminocyclitols. Most prominent among the more recent arrivals is gentamicin,[5,6] but other aminocyclitols, *i.e.*, ribostamycin,[7] tobramycin,[8] spectinomycin,[9] amikacin (BB-K8),[10a] and dibekacin[10b] the last two semi-synthetic aminocyclitol antibiotics, are also now in clinical use or seem destined soon to be used in medicine. Still others, *e.g.* the validamycins,[11] are used in treating plant diseases. In view of the comprehensive recent reviews on the chemistry[3,4,12] and mode of action,[13~16] of these antibiotics and the mechanism of resistance

Fig. 1. The 4,5-disubstituted deoxystreptamine antibiotics



	R	R'	R''	R'''	R''
NEOMYCIN B	NH_2	OH	H	CH_2NH_2	H
NEOMYCIN C	NH_2	OH	CH_2NH_2	H	H
PAROMOMYCIN I	OH	OH	H	CH_2NH_2	H
PAROMOMYCIN II	OH	OH	CH_2NH_2	H	H
LIVIDOMYCIN A	OH	H	H	CH_2NH_2	*
LIVIDOMYCIN B	OH	H	H	CH_2NH_2	H
2230-C	OH	OH	H	CH_2NH_2	*

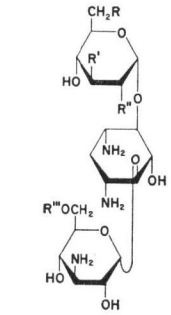




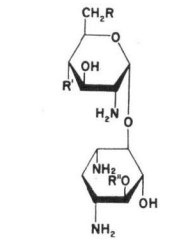
	R	R'	R''	R'''	R''
RIBOSTAMYCIN	NH_2	OH	H	H	OH
XYLOSTASIN	NH_2	OH	H	OH	H
BUTIROSIN A	NH_2	OH	*	OH	H
BUTIROSIN B	NH_2	OH	*	H	OH
4'-DEOXYBUTIROSIN A	NH_2	H	*	OH	H
4'-DEOXYBUTIROSIN B	NH_2	H	*	H	OH
LL BM408α	OH	OH	H	H	OH
BU-1709 E_1	OH	OH	*	OH	H
BU-1709 E_2	OH	OH	*	H	OH

*R'' = $-COCHOHCH_2CH_2NH_2$

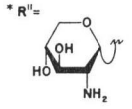
Fig. 2. The 4,6-disubstituted deoxystreptamine antibiotics

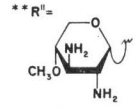


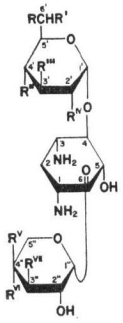
	R	R'	R''	R'''
KANAMYCIN A	NH_2	OH	OH	H
KANAMYCIN B	NH_2	OH	NH_2	H
KANAMYCIN C	OH	OH	NH_2	H
TOBRAMYCIN	NH_2	H	NH_2	H
NEBRAMYCIN FACTOR 4	NH_2	OH	NH_2	$CONH_2$
NEBRAMYCIN FACTOR 5'	NH_2	H	NH_2	$CONH_2$



	R	R'	R''
SELDOMYCIN FACTOR 1	OH	OH	*
" " 2	NH_2	H	H
" " 3	NH_2	OH	*
" " 4	NH_2	H	**

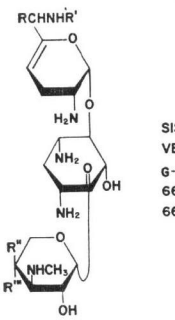






	R	R'	R''	R'''	R''	R'	R''	R'''
GENTAMICIN A	H	OH	OH	OH	NH_2	H	OH	$NHCH_3$
A_1	H	OH	OH	OH	NH_2	OH	H	$NHCH_3$
A_2	H	OH	OH	OH	NH_2	H	OH	OH
A_3	H	NH_2	OH	OH	OH	OH	H	$NHCH_3$
A_4	H	OH	OH	OH	NH_2	H	OH	$N(CHO)CH_3$
X_2	H	OH	OH	OH	NH_2	OH	CH_3	$NHCH_3$
B	H	NH_2	OH	OH	OH	OH	CH_3	$NHCH_3$
B_1	CH_3	NH_2	OH	OH	NH_2	OH	CH_3	$NHCH_3$
C_1	CH_3	$NHCH_3$	H	H	NH_2	OH	CH_3	$NHCH_3$
C_{1a}	H	NH_2	H	H	NH_2	OH	CH_3	$NHCH_3$
C_2	CH_3	NH_2	H	H	NH_2	OH	CH_3	$NHCH_3$
C_{2a}*	CH_3	NH_2	H	H	NH_2	OH	CH_3	$NHCH_3$
C_{2-III}†	H	NH_2	H	H	NH_2	OH	CH_3	$NHCH_3$
C_{2b}‡ (C_{2a}")	H	$NHCH_3$	H	H	NH_2	OH	CH_3	$NHCH_3$
G-418	CH_3	OH	OH	OH	NH_2	OH	CH_3	$NHCH_3$
JI-20A (GENTAMICIN X_3)	H	NH_2	OH	OH	NH_2	OH	CH_3	$NHCH_3$
JI-20B	CH_3	NH_2	OH	OH	NH_2	OH	CH_3	$NHCH_3$

*STEREOISOMER OF C_2 at C-6'
†STEREOISOMER OF C_{1a} at C-5'
‡SAGAMICIN



	R	R'	R''	R'''
SISOMICIN	H	H	OH	CH_3
VERDAMICIN	CH_3	H	OH	CH_3
G-52 (Sch 17726)	H	CH_3	OH	CH_3
66-40B	H	H	H	OH
66-40D	H	H	OH	H

to them,[17~19] we shall only show here their structures in order to indicate the diversity of the aminocyclitol antibiotics. We specifically limit our list to those antibiotics containing at least one aminocyclohexanol (aminocyclitol) ring.

The presently known aminocyclitol antibiotics can be divided into a number of distinct groups according to the aminocyclitol present. The largest is that containing deoxystreptamine and this group can be further divided into those antibiotics in which deoxystreptamine is substituted on two adjacent hydroxyls (4,5-disubstituted deoxystreptamine group), those in which deoxystreptamine is substituted on two non-adjacent hydroxyls (4,6-disubstituted deoxystreptamines), and those in which deoxystreptamine is monosubstituted. The 4,5-disubstituted deoxystreptamine antibiotics are shown in Fig. 1. These include the neomycins,[2] the paromomycins,[2] the lividomycins,[20] ribostamycin,[7] xylostasin,[21] and the butirosins[22] (including BU-1709E_1 and E_2[23] and LL BM-408α[24]). The 4,6-disubstituted deoxystreptamine antibiotics (Fig. 2) include the kanamycins,[1~4,12] tobramycin and its relatives the nebramycins,[8] the seldomycins,[25] and the gentamicins[5,6,26~30] and related compounds such as JI-20A and B,[31a] G-418[31b] sisomicin,[32] verdamicin,[33] 66-40B and D,[34] and G-52.[35] Antibiotics which are monosubstituted on deoxystrep-

Fig. 3. The monosubstituted deoxystreptamine antibiotics

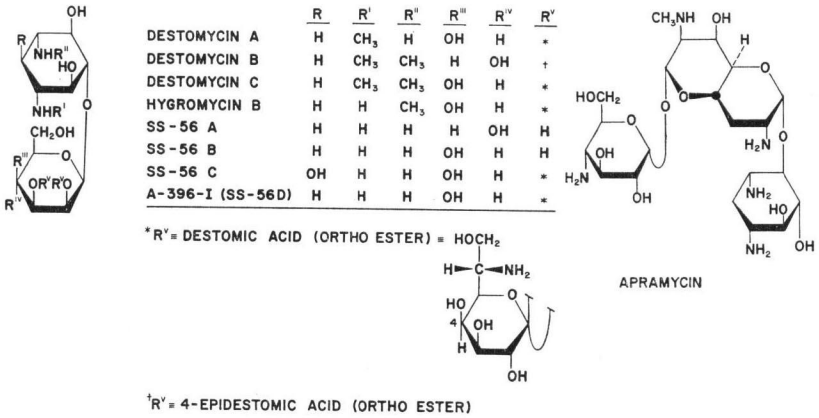


Fig. 4. Aminocyclitol antibiotics derived from streptidine, bluensidine, and actinamine

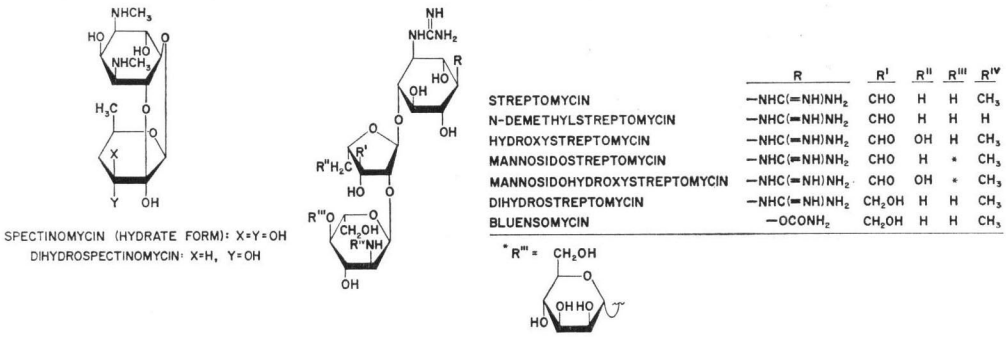
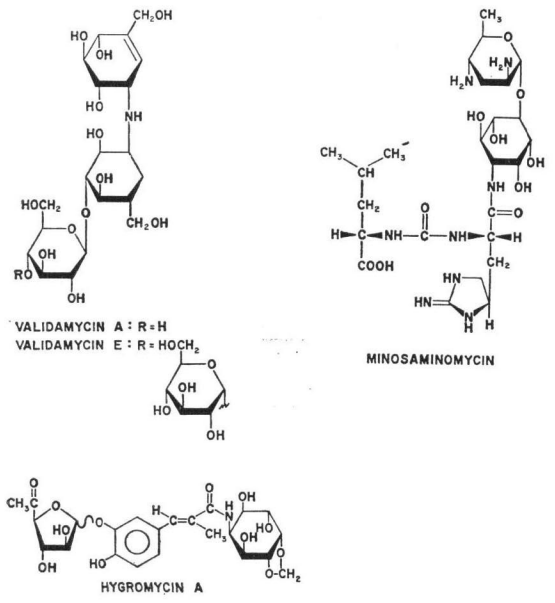


Fig. 5. Monoaminocyclitol antibiotics



tamine (Fig. 3) include the destomycins[36] and hygromycin B,[37] which are N-methylated and 5-substituted on deoxystreptamine, and others which lack the N-methyl group (SS-56A~D),[38] as well as the remarkable apramycin (4-substituted on deoxystreptamine).[39]

Among aminocyclitol antibiotics which do not contain deoxystreptamine the antibiotics derived from streptidine or its relative bluensidine (the streptomycins)[1~4] and from actinamine (the spectinomycins)[9,40] are the most important. This small group is shown in Fig. 4. Other antibiotics of known structures contain monoaminocyclitols. This group includes validamycins A and E,[11] with two aminocyclitol rings which bear both C-alkyl and N-alkyl substituents, hygromycin A,[41] and minosaminomycin[42] (Fig. 5).

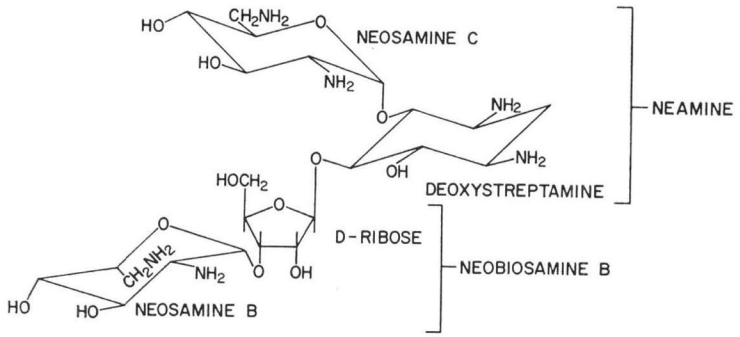
Antibiotics Containing Deoxystreptamine

Neomycin.

The deoxystreptamine-containing antibiotics constitute the largest group of aminocyclitol antibiotics and we shall discuss their biosynthesis first. The bulk of the effort in this class has been directed toward neomycin, whose biosynthesis was last surveyed in 1967.[43] The structure of neomycin B is shown in Fig. 6, with the four subunits identified. In addition to deoxystreptamine, there are two diaminohexoses, 2,6-diamino-2,6-dideoxy-D-glucose (neosamine C) and 2,6-diamino-2,6-dideoxy-L-idose (neosamine B), plus D-ribose. Studies prior to 1967[44,45] demonstrated that both [1-^{14}C]glucose and [6-^{14}C]glucose (Table 1) are well incorporated into neomycin and that all subunits of the antibiotic are labeled approximately equally, except ribose, which is labeled to a somewhat lesser extent. A current version of the degradation scheme giving the subunits is shown in Fig. 7. A milder hydrolysis of diacetylneobiosamine B allows isolation of ribose.

The specificity of labeling of the subunits by ^{14}C-labeled glucose has also been investigated,[44,45] following the periodate degradation schemes shown in Fig. 8. D-Glucose labels the neosamines in the corresponding positions (Fig. 9), *i.e.*, C-1 of glucose labels C-1 of the neosamines, C-6 of glucose labels C-6 of the neosamines.[46] A reasonable pathway for conversion of glucose to neosamines B and C, involving glucosamine as an intermediate (see below), is

Fig. 6. Structure of neomycin B with subunits labeled



NEOMYCIN B

Table 1. Distribution of label in neomycin subunits from labeled precursors

Precursor	% Incorporation into neomycin	Percent of neomycin radioactivity in subunit				Ref.
		Neosamine C	Deoxystreptamine	Neosamine B	Ribose	
[U-^{14}C]glucose	3.5	28.3	29.2	23.2	19.3	44
[1-^{14}C]glucose	3.6	30.2	30.1	24.1	15.6	44
[6-^{14}C]glucose	1.4	32.2	32.9	26.2	8.6	44
[1-^{14}C]glucosamine	14.4	24.0	29.6	44.8	1.6	44
[1-^{14}C]ribose	6.4	23	30	15	32	45
[1-^{14}C]deoxystreptamine	52.3	8.1	85.7	3.8	2.4	44

Fig. 7. Degradation scheme for neomycin B

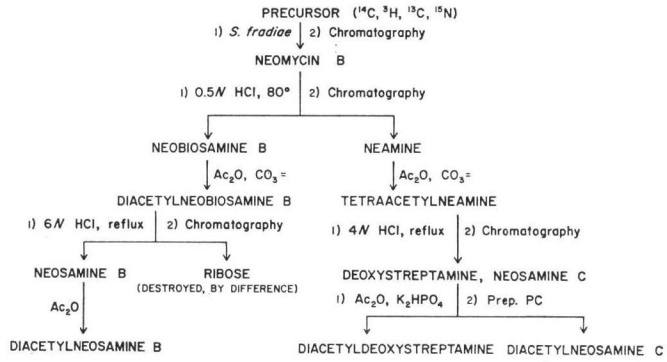
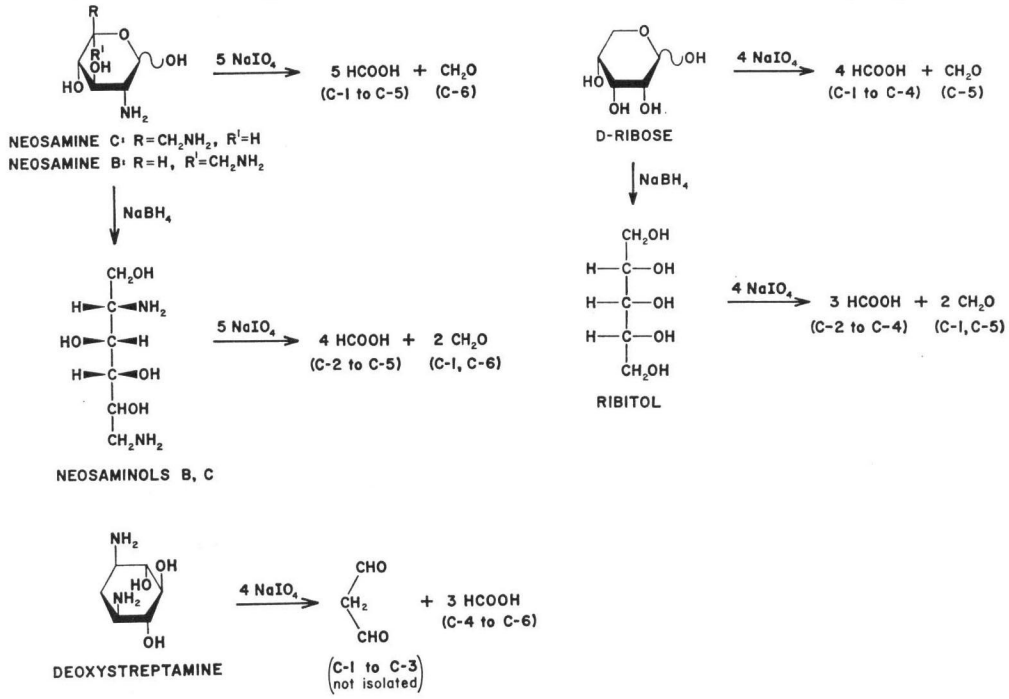


Fig. 8. Periodate degradations of neosamines B and C, D-ribose, and deoxystreptamine



shown in Fig. 10. The labeling pattern in ribose is more interesting and implies that two pathways must be followed, since part of the label from [1-^{14}C]glucose is lost and part labels C-1 of ribose,[44] while part of the label from [6-^{14}C]glucose is lost and part labels C-5 of ribose.[44] Retention of C-6 may be *via* the hexose monophosphate pathway[47] and retention of C-1 of glucose *via* the glucuronate pathway[48] (Fig. 11). The degradation scheme for deoxystreptamine in Fig. 8 is unsatisfactory since it can only localize the label to one or the other half of the molecule. Nevertheless, label from both [1-^{14}C]- and [6-^{14}C]glucose was found in the half of deoxystreptamine consisting of C-1 through C-3.[44]

In view of the unsatisfactory degradation scheme for ^{14}C-labeled deoxystreptamine, ^{13}C magnetic resonance (CMR) spectroscopy was enthusiastically embraced as a tool for studying aminocyclitol biosynthesis and [6-^{13}C]glucose was administered to *Streptomyces fradiae*.[49] The

Fig. 9. Labeling of neomycin subunits by D-glucose and D-glucosamine

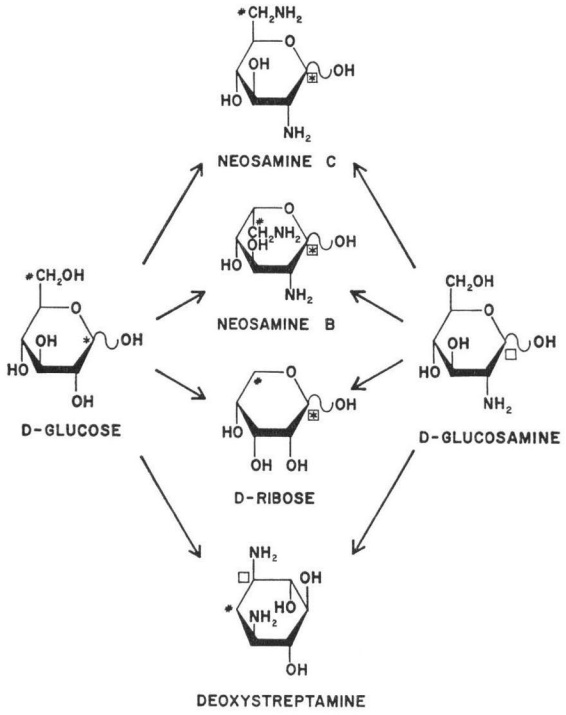


Fig. 10. Biosynthetic route from D-glucose to neosamines B and C

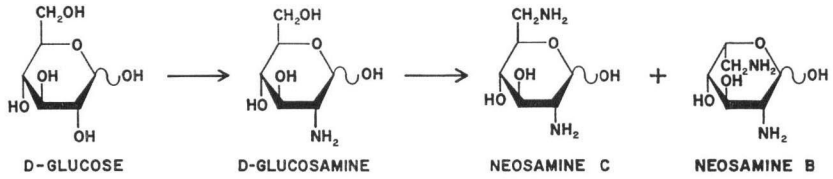


Fig. 11. Possible routes for biosynthesis of ribose from glucose

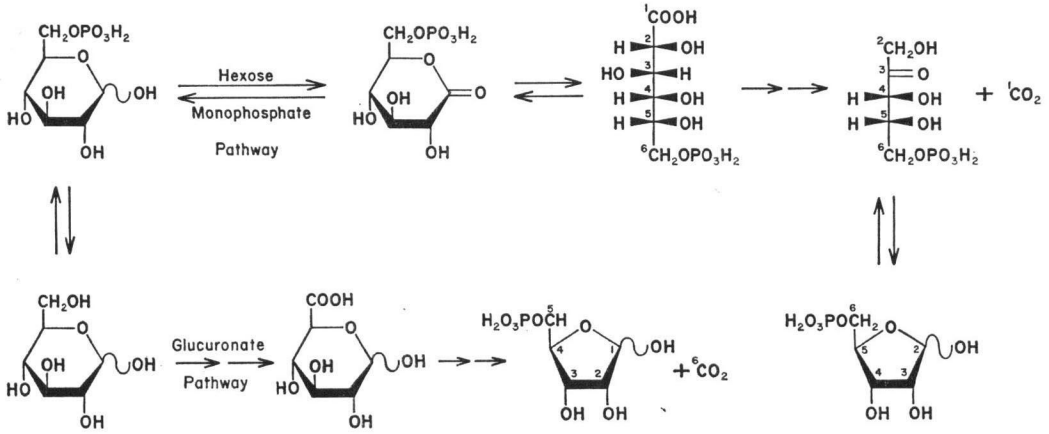
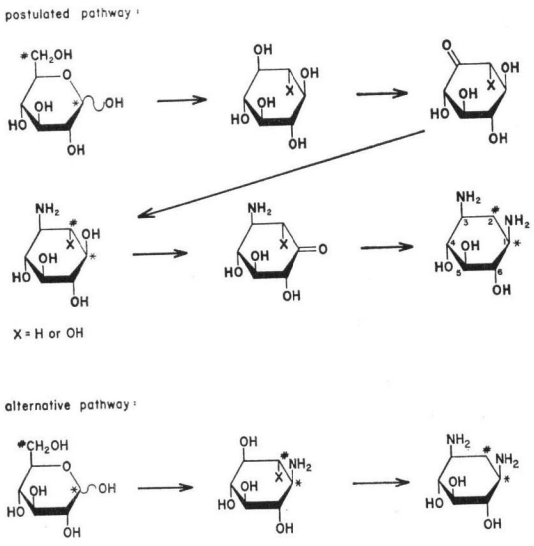


Table 2. Enrichment of carbons in neomycin B from ^{13}C-labeled precursors[49]

Carbon	Enrichment from	
	[6-^{13}C]glucose	[1-^{13}C]glucosamine
C-1	0.78	→7.07
C-2	1.00	0.86
C-3	1.30	1.25
C-4	1.30	1.25
C-5	1.30	1.25
C-6	→1·94	0.74
D-1	1.21	→2.17
D-2	→3.72	1.26
D-3	0.96	1.23
D-4	0.93	0.98
D-5	0.74	0.96
D-6	0.92	0.98
R-1	0.83	→1.60
R-2	0.92	0.98
R-3	0.87	0.94
R-4	0.88	0.99
R-5	→2.28	1.03
B-1	0.76	→6.32
B-2	1.14	1.03
B-3	1.15	1.14
B-4	0.86	1.07
B-5	1.07	1.13
B-6	→1.94	0.74

Fig. 12. Possible biosynthetic routes from D-glucose to deoxystreptamine



neomycin isolated was N-acetylated and its CMR spectrum was observed. The results (Table 2) demonstrated independently, without degradation, that C-6 of glucose labels the neosamine subunits at C-6 and labels ribose at C-5, as had been determined earlier in the carbon-14 studies. In addition, it was possible by CMR spectroscopy to locate the label at a single carbon atom, C-2, of deoxystreptamine. A postulated biosynthetic route from glucose to deoxystreptamine which would give this result is shown in Fig. 12. The substituent X may be either a hydroxyl group or a hydrogen atom. This route, involving initial oxidation and amination at C-3 of the aminocyclitol ring (which arises from C-5 of glucose), followed by similar reactions at C-1 of the aminocyclitol ring (from C-1 of glucose), is favored by analogy to results with streptidine to be discussed below. However, an alternative route (also shown in Fig. 12), involving the reverse sequence—oxidation-amination at the ring's C-1 first, followed by oxidation-amination at its C-3—cannot be ruled out by evidence obtained thus far.

A number of potential intermediates between glucose and the subunits of neomycin have been investigated. It was learned early that [1-^{14}C]glucosamine was better incorporated into neomycin than [1-^{14}C]glucose (Table 1).[44] Degradation of the labeled neomycin by the scheme in Figs. 7 and 8 showed that, as with glucose, the incorporation was specific,[44] with only C-1 of neosamines being labeled. Similarly, when [1-^{13}C]glucosamine was administered, the labeling pattern was as expected (Table 2),[49] with C-1 of the neosamines, ribose and deoxystreptamine being labeled. The neosamines are labeled at a much higher level by glucosamine than by glucose, which argues that glucosamine is further along the biosynthetic pathway to the neosamines than

is glucose, *i.e.*, that glucosamine is an intermediate between glucose and the neosamines, as shown in Fig. 10. This conversion of glucose to glucosamine is ubiquitous in biological systems.[50]

On the other hand, ribose, of course, lacks nitrogen and is presumably formed from glucosamine *via* glucose, the reverse of the reaction in Fig. 10. A glucosamine-6-phosphate deaminase, which can accomplish this transformation, has been isolated from *Escherichia coli*,[51,52] and this enzyme or a similar one could convert glucosamine to glucose in *S. fradiae*. Both ribose and deoxystreptamine are less labeled (relative to the neosamines) by glucosamine than by glucose. This and the similar level of ^{13}C label in ribose and deoxystreptamine from both [1-^{13}C]glucosamine and [6-^{13}C]glucose suggest that deoxystreptamine, like ribose, arises from glucose directly and from glucosamine *via* glucose, again by the reverse of the reaction in Fig. 10. That the nitrogen of glucosamine is indeed lost in its conversion to deoxystreptamine has been confirmed using [^{15}N]glucosamine.[53,54] The neomycin produced was degraded (Fig. 13) and the results (Table 3) show that the labeled nitrogen atoms are found only in the neosamine subunits, *i.e.*, that the deoxystreptamine nitrogen atoms are unlabeled. Deoxystreptamine is proposed to arise from glucose by the pathway shown in Fig. 12, *via* an inositol-type intermediate similar to that reported for streptomycin.[55] *Myo*-inositol has been found not to be an intermediate in the neomycin biosynthetic pathway,[46] arguing for a deoxyinositol or deoxyinosose intermediate (X=H) in Fig. 12.

Fig. 13. Labeling of neomycin and its subunits by [^{15}N]glucosamine

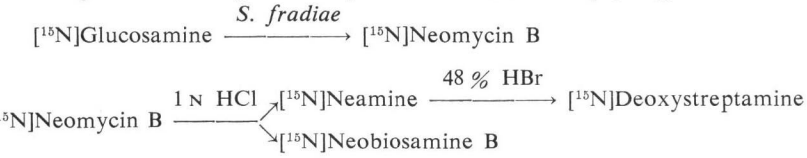


Table 3. Distribution of ^{15}N in neomycin B from [^{15}N]glucosamine[53]

Compound	Atom % enriched	^{15}N/mole
Neomycin B	4.113	0.2468
Neamine	3.214	0.1286
Deoxystreptamine	0.283	0.0057
Neobiosamine B	5.109	0.1022
Neosamine C*	2.931	0.1229

*By difference

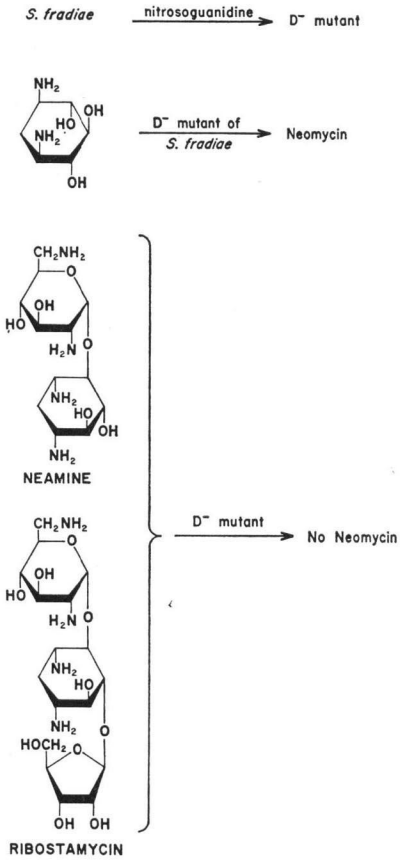
In addition to labeled glucose and glucosamine, the labeled subunits themselves (except neosamine B) have been administered to *S. fradiae* to determine whether they are biosynthetic intermediates. [1-^{14}C]Ribose labeled all the subunits (Table 1), presumably *via* glucose, but it labeled the ribose subunit most heavily and it was directly incorporated, since the label remained at the C-1 position of ribose in neomycin.[45] [1-^{14}C]Deoxystreptamine (isolated from neomycin derived from a [1-^{14}C]glucose feeding) was also incorporated (Table 1) into the deoxystreptamine moiety of neomycin[44] and only into that moiety. On the other hand, [1-^{14}C]-neosamine C was found to be not incorporated into neomycin,[46] probably because the intermediates between glucosamine and the neosamine moieties of neomycin are enzyme-bound.

A second procedure has also been employed, in addition to feeding labeled deoxystreptamine, to demonstrate direct incorporation of deoxystreptamine into neomycin. Mutant strains of *S. fradiae* have been developed[56~58] which produce neomycin only in the presence of added deoxystreptamine, thus confirming that it appears underivatized as a biosynthetic intermediate

(Fig. 14).

The same mutants have also been employed in studying larger subunits as potential intermediates between deoxystreptamine and neomycin (Fig. 14). Neamine and ribostamycin were tested for bioconversion to neomycin, but no neomycin production was observed.[59,60] The results were somewhat ambiguous since the potential precursors are themselves antibiotics and at high concentration neamine killed the producing microorganism.

Fig. 14. Incorporation by D⁻ mutant of precursors into neomycin



A mutant requiring deoxystreptamine for synthesis of an antibiotic can accept a deoxystreptamine derivative which is further along the biosynthetic pathway only if certain conditions are met. If, for example, a deoxystreptamine-requiring mutant of *S. fradiae* produces neomycin when neamine is added to the fermentation medium, this positive result demonstrates that neamine appears underivatized on the biosynthetic pathway to neomycin. However, failure to obtain bioconversion to neomycin (negative results) can be explained in a number of ways:

(1) The order of subunit assembly may be one in which a subunit other than neosamine C (ribose or neobiosamine B) is joined to deoxystreptamine first, *i.e.*, neamine may not be formed as such *en route* to neomycin.

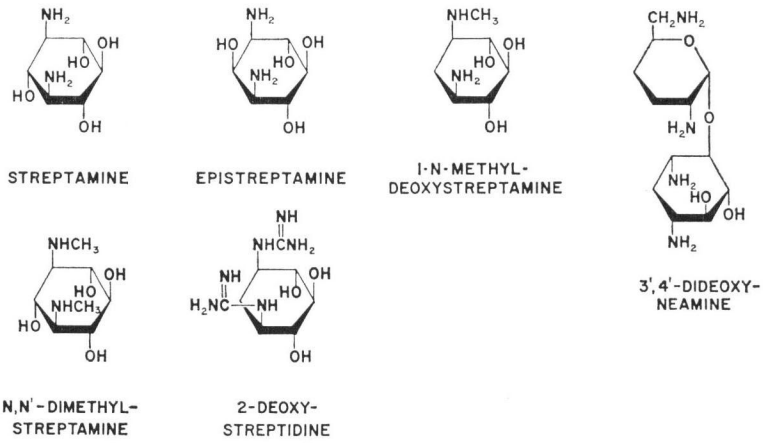
(2) Deoxystreptamine may be converted to a derivative before a second subunit (neosamine C) is attached. This would give a derivative of the pseudodisaccharide subunit (neamine) and if no enzyme existed to convert the added pseudodisaccharide (neamine) to that derivative, the pseudodisaccharide's bioconversion to neomycin could not occur.

(3) The pseudodisaccharide intermediate (*e.g.* neamine) may be required to be enzyme-bound and not be taken up from the medium.

(4) The disaccharide intermediate (neamine) may not pass through the cell wall. [This is difficult to accept, since neamine was toxic to the producing cells, as noted above.]

Although details of the pseudodisaccharide and pseudotrisaccharide stages are still uncertain, some information exists about the final stages of neomycin biosynthesis.[61~63] Derivatives of neomycin assigned phosphoamide structures have been reported to enhance antibiotic yields when added to the production media. In addition, an alkaline phosphatase which can convert these compounds to neomycin has been observed in the producing organism, and the *de novo* synthesis of this enzyme has been directly correlated to neomycin biosynthesis.[64] Thus, cleavage of a neomycin phosphoamide or phosphate has been postulated to be the last step in the biosynthesis of neomycin. Unfortunately, the position of the phosphorylation has not been determined.

Fig. 15. Aminocyclitols incorporated, using the mutasynthetic approach, into one or more modified aminocyclitol antibiotics



As a practical outgrowth of the neomycin biosynthetic study, the same mutants used to study neomycin biosynthesis have been employed to prepare antibiotics related to neomycin by the incorporation of related aminocyclitols. Of many compounds administered which were related to deoxystreptamine, only streptamine and epistreptamine (Fig. 15) were observed to be incorporated in early studies.[56~58,65,66] We refer to this procedure for preparing antibiotics as mutasynthesis, the antibiotics as mutasynthetics, and the aminocyclitols introduced as mutasynthons.

Paromomycin.

Paromomycins (Fig. 1) are the product of *S. rimosus* forma *paromomycinus*. No studies have been reported on the biosynthesis of paromomycin employing labeled precursors. However, *S. fradiae*, which produces neomycin as the major product, has been shown to produce trace quantities of paromomycins as well, and the biosyntheses of the two antibiotics are, thus, presumably parallel. A mutant strain of *S. rimosus* forma *paromomycinus* has been developed which requires exogenous deoxystreptamine for antibiotic production (Fig. 16);[58,59] thus, deoxystreptamine appears underivatized on the biosynthetic pathway to paromomycin as well. On the other hand, feeding the plausible pseudodisaccharide intermediate paromamine to the mutant of *S. rimosus* forma *paromomycinus* proved unsuccessful in producing paromomycin.[59] Thus, the biosynthesis of paromomycin clearly mimics the neomycin pattern, as expected. A number of deoxystreptamine analogs were also administered, with results similar to those with the *S. fradiae* mutants; in this case the only analog incorporated was streptamine (Fig. 15).

Ribostamycin.

No studies with labeled precursors have been reported employing *S. ribosidificus*, which produces ribostamycin (Fig. 1). However, the biosynthesis must parallel that of neomycin since ribostamycin is a part of the latter antibiotic. Mutant strains of *S. ribosidificus* have been developed[67] which, like the *S. fradiae* and *S. rimosus* mutants, lack the ability to biosynthesize deoxystreptamine. When exogenous deoxystreptamine was added to the culture medium, ribostamycin was produced (Fig. 17). Unlike the *S. fradiae* and *S. rimosus* mutants this mutant also bioconverted neamine to ribostamycin (Fig. 17), which suggests that ribose is the last

Fig. 16. Studies of paromomycin biosynthesis employing a D⁻ mutant

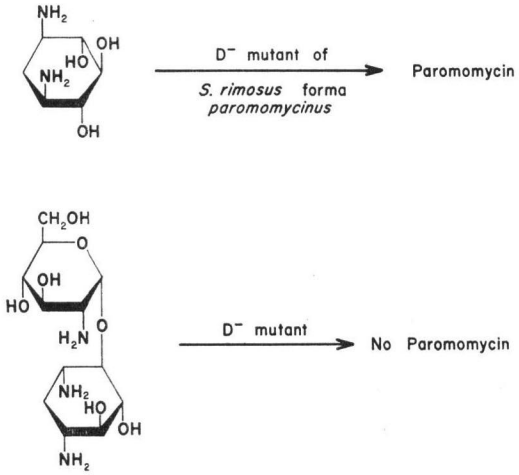


Fig. 17. Incorporation of intermediates into ribostamycin by a D⁻ mutant

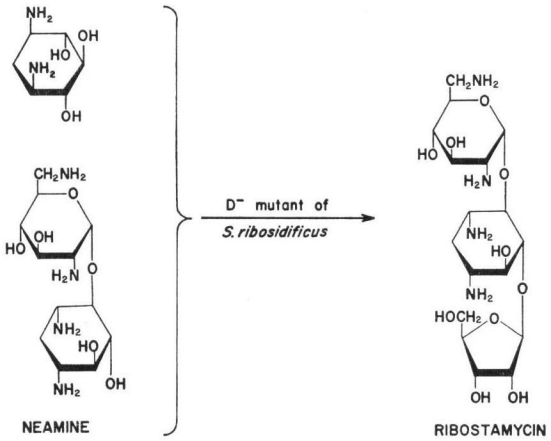
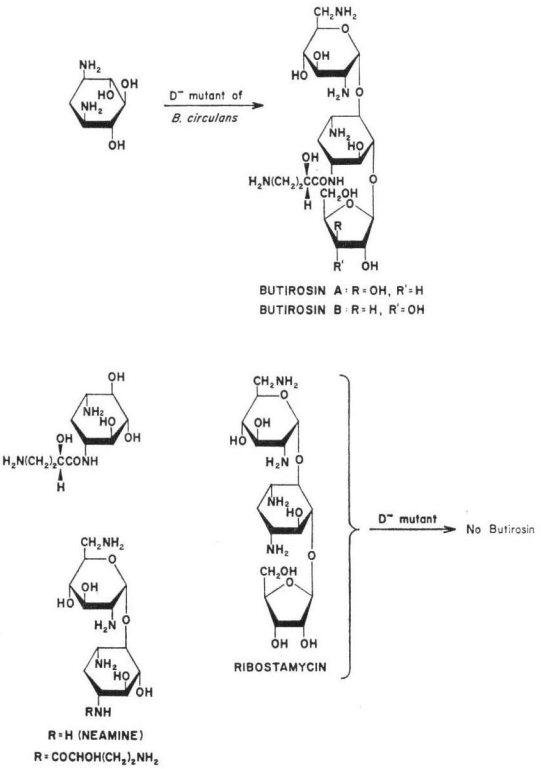


Fig. 18. Bioconversion and lack of bioconversion of subunits to butirosins



subunit added in the biosynthesis of ribostamycin. Because of the similarity between ribostamycin and neomycin, it seems likely that the biosynthesis of these molecules should parallel one another and that neamine should be incorporated into neomycin as well. The apparent discrepancy between neomycin and ribostamycin biosynthesis deserves further investigation.

The *S. ribosidificus* mutant was also somewhat less selective than the *S. fradiae* mutant in its acceptance of deoxystreptamine analogs. It utilized 1-N-methyldeoxystreptamine and 3′,4′-dideoxyneamine (Fig. 15) as well as streptamine and epi-streptamine in producing ribostamycin analogs.

Butirosin.

The butirosins (Fig. 1) are aminocyclitol antibiotics produced by *Bacillus circulans* which are composed of a neamine moiety substituted at C-1 with L(−)-γ-amino-α-hydroxybutyric acid and at C-5 with either xylose (butirosin A) or ribose (butirosin B). Recent work[68] using a deoxystreptamine-requiring mutant of *B. circulans* (Fig. 18) has shown that the biosynthetic pattern reflects those seen above in neomycin and paromomycin (and below in kanamycin) mutants,[65] *i.e.*, deoxystreptamine serves as a substrate for biosynthesis, but neamine does not. It is significant that in butirosin both amino groups are attached to the aminocyclitol before

derivatization of the 1-amino group occurs. This pattern differs from that for streptidine biosynthesis[55] (see below), where guanylation of the 1-amino group occurs before the second amino group is attached, and streptamine does not serve as a substrate in streptidine-negative mutants of *S. griseus*.[69]

Fig. 19. Subunits of kanamycins

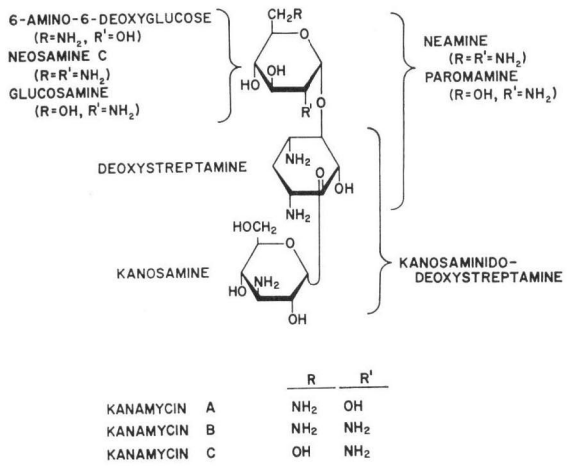
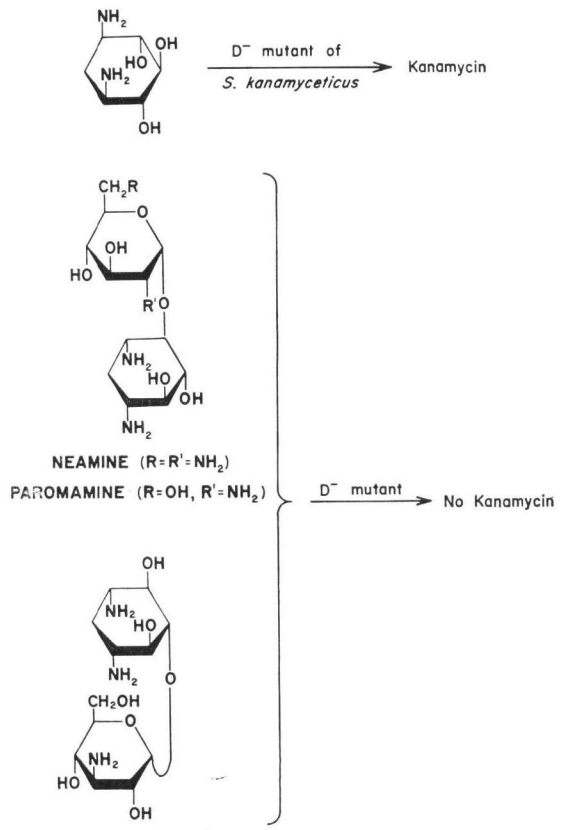


Fig. 20. Mutant studies of kanamycin biosynthesis



Additionally, 1-N-(γ-amino-α-hydroxybutyryl)deoxystreptamine, des(aminohydroxybutyryl)butirosin B (ribostamycin), which lacks the amino acid, and des(pentofuranosyl)butirosin [1-N-(γ-amino-α-hydroxybutyryl)neamine], which lacks the pentose, were administered to the *B. circulans* mutant without incorporation (Fig. 18). These results, like those with neomycin, are disappointing in that they do not allow one to determine the sequence of formation of glycosidic bonds in the biosynthesis of butirosin.

Kanamycin.

The kanamycins (Fig. 19) are aminocyclitol antibiotics which contain deoxystreptamine substituted at C-6 by kanosamine (3-amino-3-deoxy-D-glucose) and at C-4 by an aminoglucose (6-amino-6-deoxy-D-glucose in kanamycin A, neosamine C in kanamycin B, D-glucosamine in kanamycin C). Labeling studies on kanamycins B and C have not been reported, but labeling of kanamycin A by D-[1-^{14}C]glucose[70] was similar to that found for neomycin in that all three subunits were approximately equally labeled; however, the position of labeling within the subunits was not determined. When D-[1-^{14}C]glucosamine was fed, only the 6-amino-6-deoxy-D-glucose moiety of kanamycin A was reported to be labeled. This is a confusing result, since glucosamine probably labels 6-aminoglucose by prior conversion to glucose, and the other units are all known to be labeled by glucose, too. Although glucose was best incorporated when administered after 24 hours' incubation (as in the neomycin feedings), glucosamine was best incorporated after 96 hours. This suggests that the formation of 6-amino-6-deoxy-D-glucose from glucosamine (*via* glucose)

occurs relatively late in the biosynthesis of kanamycin, perhaps after kanosamine and deoxystreptamine have been formed. The higher incorporation of glucosamine (33 %) relative to glucose (3.8 %) is probably a result of the later feeding, since less labeled material would be utilized by the organisms in primary metabolism. Paromamine (Fig. 19, a disaccharide component of kanamycin C) was also isolated and was shown to be labeled by both glucose and glucosamine, although the latter compound labeled only the glucosamine subunit of paromamine.

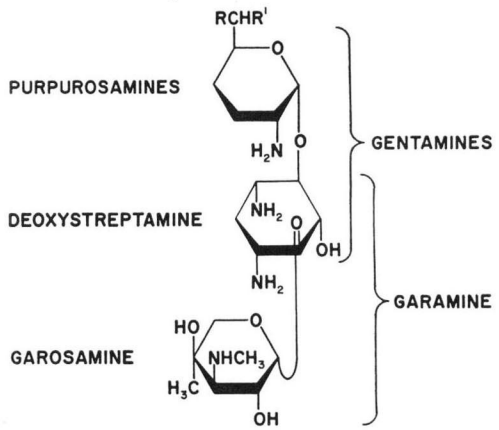
Feeding[71] of the biosynthetically labeled subunits of kanamycin A—6-amino-6-deoxy-D-glucose, kanosamine, and deoxystreptamine, as well as paromamine and kanamycin B—indicated labeling of kanamycin A by only deoxystreptamine. However, the results are difficult to interpret in that the deoxystreptamine administered was 100-fold more radioactive than the other percursors; moreover, one cannot determine the percent incorporation from the data reported. Label from the incorporated deoxystreptamine was located, as expected, in the deoxystreptamine portion of the kanamycin A molecule.

It has been observed that the addition of deoxystreptamine to the fermentation medium of *S. kanamyceticus* improved the antibiotic yield.[72] In addition, the biosynthesis of kanamycin has been investigated by two groups[58,59,67] using deoxystreptamine-requiring mutant strains of *S. kanamyceticus*. The results (Fig. 20) were similar to those for neomycin, paromomycin and ribostamycin in that deoxystreptamine allowed the formation of kanamycin but neamine and paromamine (components of kanamycins B and C) and kanosaminidodeoxystreptamine (a component of all three kanamycins) did not allow the production of kanamycin.[59] In attempts to produce kanamycin analogs 2-epistreptamine and 1-N-methyldeoxystreptamine (Fig. 15) were bioconverted to antibiotics,[57,58,67] but many other aminocyclitols were not.

Gentamicins.

As shown above in Fig. 2, gentamicin is a complex of numerous deoxystreptamine antibiotics produced by *Micromonospora purpurea*. Commercial gentamicin consists mainly of the gentamicin C components, whose subunits are labeled in Fig. 21. These compounds differ from the neomycins, kanamycins, and paromomycins in that they contain both C-methyl and N-methyl substituents. D-[U-^{14}C]-Glucose is incorporated[73] into gentamicin as it is into the other aminocyclitol antibiotics, but no degradations have been carried out to establish which of the various subunits are labeled. D-[1-^{14}C]Glucosamine was better in-

Fig. 21. Components and subunits of the gentamicin C complex.



		R	R'
GENTAMICIN	C_1	CH_3	$NHCH_3$
	C_{1a}	H	NH_2
	C_2	CH_3	NH_2
	$C_{2\text{-}a}$*	CH_3	NH_2
	$C_{2\text{-}a}$†	H	NH_2
	C_{2b}("C_{2a}")†	H	$NHCH_3$

*STEREOISOMER OF C_2 at C-6'
†STEREOISOMER OF C_{1a} at C-5'
†SAGAMICIN

Table 4. Incorporation of [$^{14}CH_3$]methionine into gentamicin components[73]

Time of addition (hour)	% Radioactivity in component			% Incorporation
	C_{1a}	C_2	C_1	
0	3.99	15.35	62.17	10.8
0, 24, 48*	2.88	18.05	59.55	17.3

* Added in three equal portions.

corporated[73] (2.6 % versus 0.9 % for glucose), but, again, no degradations have been reported.

The source of the methyl groups has been the cause of some confusion. Although both [2-^{14}C]glycine and DL-[3-^{14}C]serine were well incorporated[73] (3.5 % and 2.8 %, respectively), by far the best incorporation (11.3 %) was obtained by feeding L-[methyl-^{14}C]methionine to the growing microorganism in successive equivolume aliquots over a period of 48 hours.[73] The distribution of radioactivity among the components of the complex was determined by radioscanning paper chromatograms, which separated the major components, gentamicins C_{1a}, C_2 and C_1. The results (Table 4) indicated that gentamicin C_1 (which contains two C-methyl and two N-methyl groups) was more heavily labeled than gentamicin C_2 (which contains one N-methyl group and two C-methyl groups), which in turn was more heavily labeled than gentamicin C_{1a} (which contains one C-methyl and one N-methyl group). These results suggest both C- and N-methylation by methionine, but the ratios of the level of labeling (which should be 4 : 3 : 2 instead of the 60 : 18 : 3 found) do not support this argument quantitatively.

In a later study[74] using L-[methyl-^{14}C]methionine, the gentamicin C components were separated by column chromatography and hydrolyzed into their subunits, which were separated from polar contaminants by paper chromatography and analyzed for relative radioactivity by radioscanning. The results (Table 5) showed that deoxystreptamine and C_{1a}-purpurosamine

Table 5. Distribution of ^{14}C-label in gentamicin components[74]

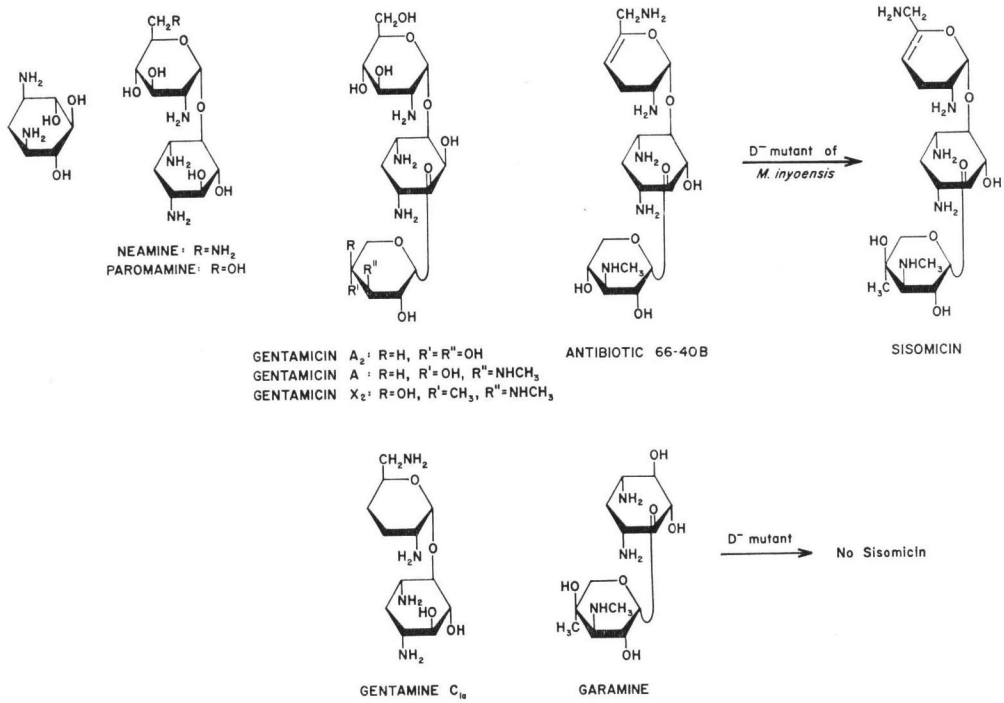
Component	Specific activity (μCi/mg)	Radioactivity of subunit (μCi)		
		Purpurosamine	Deoxystreptamine	Garosamine
C_1	1.54	225.95	0	226.73
C_2	1.09	16.43	0	18.54
C_{1a}	0.92	0	0	66.52

were unlabeled; this was expected, since neither unit contains C- or N-methyl groups. The gentamicin C_1 results (equal labeling of garosamine and C_1-purpurosamine) would be explained if methionine labels both C- and N-methyl groups or if only C- or only N-methyl labeling occurs, because both garosamine and C_1-purpurosamine contain both C- and N-methyl groups. The gentamicin C_2 results argue for only C-methylation by methionine, as garosamine (which contains one N-methyl and one C-methyl group) and C_2-purpurosamine (which contains a C-methyl but no N-methyl group) are about equally labeled. However, an unpublished result cited by LEE, *et al.*,[74] states that labeling experiments using L-[methyl-^{13}C]methionine show that all of the methyl carbons in the gentamicin complex are methionine derived. Clearly this contradiction needs to be resolved.

Sisomicin.

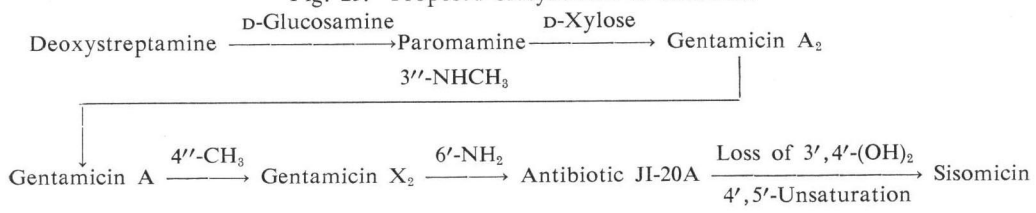
Sisomicin is an analog of gentamicin which is characterized by an unsaturated aminosugar

Fig. 22. Formation of sisomicin by a D⁻ mutant in the presence of deoxystreptamine, pseudodisaccharides, and pseudotrisaccharides



(Fig. 2). No results have been reported with labeled compounds, but a mutant of *Micromonospora inyoensis* has been recently reported[75] which requires exogenous deoxystreptamine for production of sisomicin. A number of pseudodisaccharides and pseudotrisaccharides (Fig. 22) containing deoxystreptamine were also administered[76] to the mutant. Two pseudodisaccharides were reported to be bioconverted to sisomicin and, of these, paromamine was a better substrate than neamine, suggesting either that neamine is first deaminated to paromamine or that the enzymes are somewhat non-selective and a parallel pathway exists to sisomicin. Sisomicin was not produced by the mutant in the presence of two other potential pseudodisaccharide precursors, gentamine C_{1a} (3′, 4′-dideoxyneamine) and garamine. Four pseudotrisaccharides (gentamicins A, A_2, X_2 and antibiotic 66-40B, Fig.22) were reported to allow production of sisomicin by the D⁻ mutant, although only in the case of gentamicin A feeding was sufficient antibiotic produced to allow its chemical characterization as sisomicin. With the other three precursors, the antibiotic obtained was judged by thin-layer chromatography and paper chromatography to be sisomicin.

Fig. 23. Proposed biosynthesis of sisomicin



Based on these results, TESTA and TILLEY[76] have suggested a biosynthetic scheme for sisomicin (Fig. 23) in which the role of neamine is unspecified. There are a number of problems with this proposed biosynthesis. First, the sisomicin reported to be produced by these pseudotrisaccharides should be identified by chemical rather than chromatographic techniques. Second, one would, *a priori*, expect bioconversions in the hexose portions of sisomicin to take place on a nucleotide-bound substrate, as in the formation of dihydrostreptose discussed below. Consequently, the conversions of the pseudodisaccharides and pseudotrisaccharides may involve degradation to deoxystreptamine and its incorporation into sisomicin. TESTA and TILLEY argue that, since two other pseudodisaccharides and six other pseudotrisaccharides containing deoxystreptamine did not allow the formation of sisomicin, it was unlikely that the compounds tested were first degraded to deoxystreptamine and reincorporated into sisomicin. However, a selective enzymatic degradation of the successfully incorporated pseudodisaccharides and pseudotrisaccharides is possible. Their argument would be strengthened if the disaccharides and trisaccharides could be shown, perhaps by double labeling, to be incorporated as a unit.

As with the other D^- mutants described above, new antibiotics were obtained through bioconversion of deoxystreptamine—in this case, streptamine, epi-streptamine, and 2, 5-dideoxystreptamine.

Antibiotics Containing Actinamine

Spectinomycin.

Spectinomycin and dihydrospectinomycin (Fig. 4) are the only antibiotics containing actinamine. Glucose serves as the source of the carbon skeleton, as it does for the other aminocyclitol antibiotics. When D-[6-^{3}H]glucose was administered[77] to *S. flavopersicus*, 3.5 % of the label was incorporated into spectinomycin. Degradation of the compound (Fig. 24) located the label at C-6′ of actinospectose (Table 6) but failed to locate it at a specific carbon of the aminocyclitol. L-Methionine was shown to be the source of the N-methyl groups. Recent results of feeding D-[6-^{13}C]glucose to *S. spectabilis* (Table 7)[78] confirm the earlier finding of label at C-6′. In addition, a lesser amount of label was found at the N-methyl carbons (Fig.

Fig. 24. Degradative pathway employed by MITSCHER, *et al.*,[77] for locating label in spectinomycin

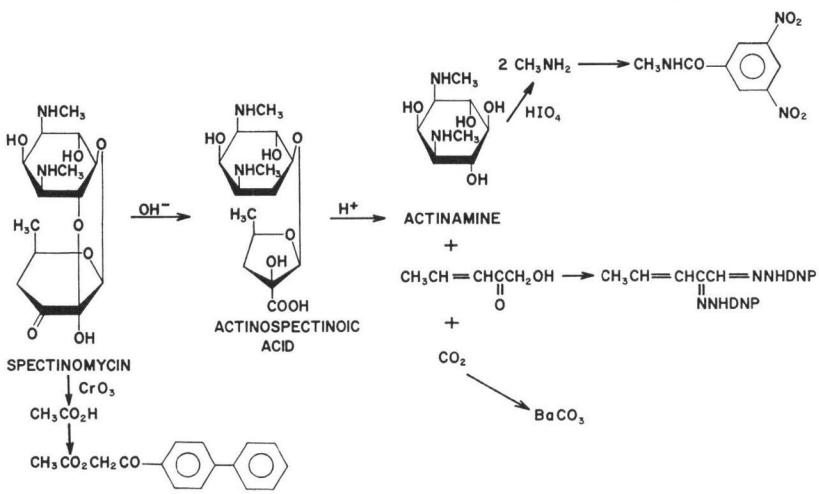


Table 6. Distribution of label within spectinomycin from various precursors[77]

Precursor	% Incorporation	% Total activity		
		Actinamine	N-CH_3	C-6′
[Methyl-^{14}C]methionine	38.8	100	98	0
[6-^{3}H]D-glucose	3.5	57	a	43
[2-^{14}C]*Myo*-inositol	47.0	100	a	0
[2-^{14}C]Actinamine	6.6	a	a	a

a: Not reported

Table 7. Distribution of label within spectinomycin from D-[6-^{13}C]glucose[75]

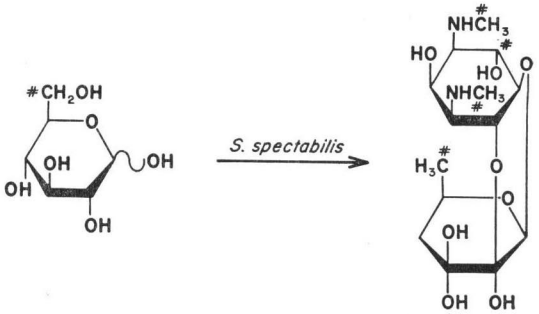
Atom	Enrichment	Atom	Enrichment
C-1	0.84	C-1′	1.00*
C-2	0.92	C-2′	1.00*
C-3	0.88	C-3′	1.00
C-4	1.06	C-4′	0.87
C-5	1.28	C-5′	0.96
C-6	3.14	C-6′	3.40
1-N-CH_3	1.67		
3-N-CH_3			

* By definition

25), presumably introduced *via* the following route:[79] [6-^{13}C]glucose→[3-^{13}C]glycerate→[3-^{13}C]-serine→[methylene-^{13}C]tetrahydrofolate→[methyl-^{13}C]methionine.

Of greater interest is the location of label within the aminocyclitol, found at C-6. MITSCHER[77] had postulated that the C-1 and/or C-3 positions of actinamine would be labeled by C-6 of glucose, but no degradations were carried out to locate the labeled positions in the actinamine moiety. The carbon-13 data place the label specifically at C-6 of actinamine, which is similar to the results of the streptomycin carbon-13 studies described below. The biosynthesis of actinamine apparently proceeds by a pathway related to that postulated for deoxystreptamine in Fig. 12, but for actinamine the second amination occurs on the β-carbon (with respect to the first aminated carbon) in the counterclockwise direction (Fig. 26, path a), while for deoxystreptamine it occurs in the clockwise direction (Fig. 26, path b). Of course, the alternative path of Fig. 12 could also explain the deoxystreptamine results, in which case the initial carbon aminated would differ.

Fig. 25. Carbon atoms of spectinomycin labeled by D-[6-^{13}C]glucose



Of potential intermediates between glucose and spectinomycin (Table 6), *myo*-[2-^{14}C]inositol was quite well incorporated into spectinomycin (47 %), as was actinamine (6.6 %).[77] This result caused the earlier authors to argue that the lower level of actinamine labeling indicates either its incorporation is *via* a minor pathway or that it is first deaminated into *myo*-inositol

Fig. 26. Two pathways for conversion of a monoaminocyclitol to a substituted diaminocyclitol

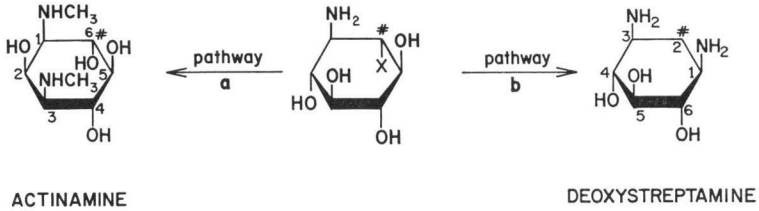
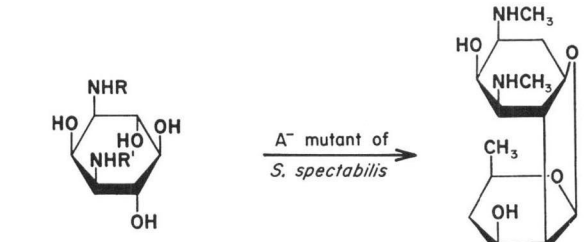


Fig. 27. Production of spectinomycin by an A⁻ mutant



before bioconversion. This argument was refuted, however, in a recent mutant study. SLECHTA and COATS[80] have isolated a mutant of *S. spectabilis* which is unable to produce spectinomycin in the absence of exogenous actinamine (Fig. 27). The mutant also produced spectinomycin in the presence of N-demethylactinamine (N-methyl-*epi*-streptamine) or *epi*-streptamine (*myo*-inosadiamine, N, N′-didemethylactinamine). When unlabeled actinamine and methyl-labeled methionine were added to the mutant culture, no radioactively-labeled spectinomycin was produced. This result demonstrates that actinamine is directly incorporated into spectinomycin without prior degradation and, thus, that methylation takes place on the aminocyclitol before attachment of the sugar moiety. The methyl groups appear to be added as a last step in the biosynthesis of actinamine since, when unlabeled *epi*-streptamine and methyl-labeled methionine were given to the mutant, three radioactive materials in addition to methionine were observed at the end of fermentation—actinamine, spectinomycin, and actinospectinoic acid, a rearranged product whose structure is found in Fig. 24. A control without *epi*-streptamine showed only labeled methionine present. N-Methyl-*epi*-streptamine also served as a precursor, again demonstrating the role of methionine as methylating agent.

Similar results (three radioactive spots) were obtained using the all-equatorial isomer of the aminocyclitol (N,N′-dimethylstreptamine, Fig. 15), but there was no biological activity associated with the radioactive spots.

Antibiotics Containing Streptidine and Bluensidine

Streptomycin.

The streptidine-containing antibiotics constitute an important group of compounds which were among the first antibiotics discovered. The structures of the streptomycin group were given in Fig. 4 and that of streptomycin is repeated in Fig. 28 with the subunits identified.

Fig. 28. Streptomycin, with subunits identified

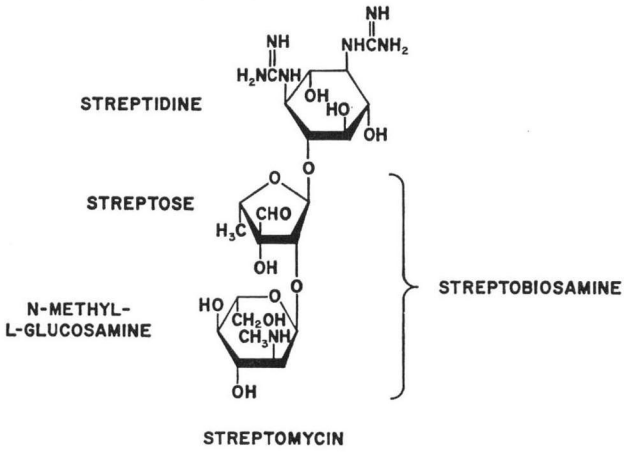


Fig. 29. Carbons of streptomycin labeled by specifically labeled D-glucose

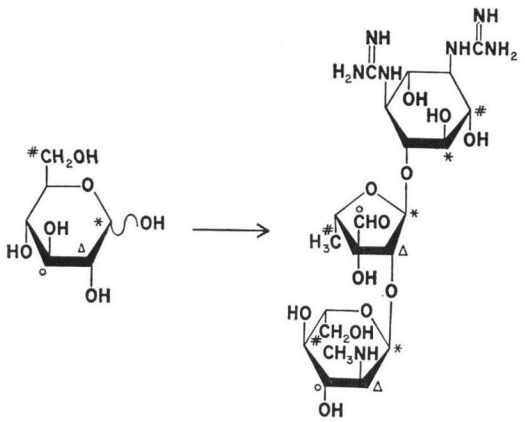


Table 8. Distribution of radioactivity in streptomycin from D-[U-^{14}C]glucose[84]

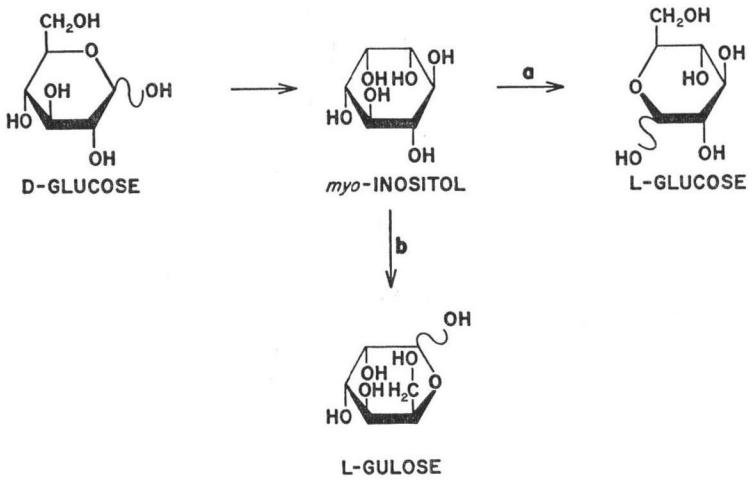
Compound	% Radioactivity
Streptomycin	100
Streptidine	34
streptamine	30
guanidine carbons	3
Streptose (by difference)	32
N-Methyl-L-glucosamine	34

The biosynthesis of streptomycin has been the subject of a number of recent reviews.[55,81,82] Most of the information on streptomycin biosynthesis was derived from initial studies on the incorporation of radioactive precursors and from later enzymatic studies. At present no information is available on the order of formation of the glycosidic linkages which join the three moieties.

Early investigations[83] using D-[^{14}C]glucose demonstrated that, as in all the other aminocyclitol antibiotics, glucose is the precursor to the skeletal carbon atoms of the streptomycin molecule. HUNTER and HOCKENHULL[84] fed uniformly labeled D-glucose to *S. griseus*, achieving 5 % incorporation into the isolated streptomycin. Subsequent hydrolysis showed that all three subunits were approximately equally labeled (Table 8).

Specifically labeled glucose has been used to study the biosynthesis of all of the subunits of streptomycin (Fig. 29) and results will now be discussed for the individual subunits. A number of hypothetical biosynthetic routes are possible for the formation of N-methyl-L-glucosamine from D-glucose, in which the principal point of interest is the inversion of stereochemistry. One possibility would involve *de novo* reassembly of the aminosugar from pieces derived from catabolism of D-glucose. Another possibility would involve intermediate cyclization to an inositol, in a process similar to that which gives rise to actinamine (preceding section) and to streptidine (see below), followed by cleavage at a bond different from that

Fig. 30. Hypothetical routes to L-hexoses from D-glucose *via* *myo*-inositol



formed during cyclization. For example, cleavage of *myo*-inositol at a point diametrically opposite the bond formed by cyclization would give rise to L-glucose (Fig. 30, path, **a**). A more complicated scheme would allow L-glucose to arise from a similar cyclization with cleavage at the same point as formation but with inversion of the aldehyde and primary hydroxyl positions (Fig. 30, path **b**) to give L-gulose, which could be epimerized at C-3 and C-4 to give L-glucose. Finally, the N-methyl-L-glucosamine might arise from D-glucose *via* multiple epimerizations similar to those suggested by VOLK[85] for the formation of D-arabinose from L-arabinose.

All mechanisms but the last were eliminated in the early work of SILVERMAN and RIEDER,[86] who fed specifically labeled D-glucose to *S. griseus*. The isolated streptomycin was degraded and the labeled positions located (Fig. 31, Table 9). Their scheme determined label at C-1 and C-6 and distinguished C-2 and C-3 from C-4 and C-5. Later, BRUTON, *et al.*,[87] used essentially

Fig. 31. Degradation scheme for N-methyl-L-glucosamine

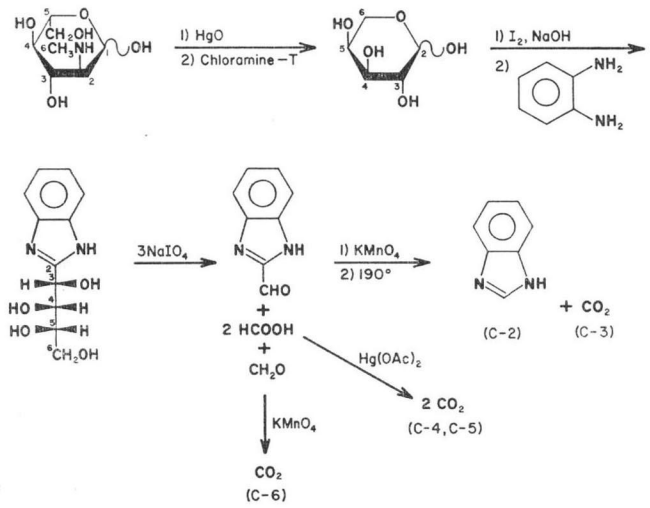


Table 9. Distribution of ^{14}C in N-methyl-L-glucosamine moiety from D-glucose and D-glucosamine labeled at various positions

Carbon atom	% Radioactivity in carbon atoms of N-methyl-L-glucosamine from labeled D-glucoses and D-glucosamine						
	D-glucose						D-[1-^{14}C] glucosamine
	[1-^{14}C]		[2-^{14}C]	[3,4-^{14}C]	[6-^{14}C]		
C-1	75	(62)[a]	2	3	1	(2)	68
2	6	(0)	76	3	10	(0)	10
3		(3)	2	33			
4,5	7	(2)	8	36	4	(2)	4
6	2	(11)	2	6	72	(75)	7
N-CH_3	10[b]	(21)[b]	9[b]	18[b]	12[b]	(20)[b]	10[b]

[a] Values in parentheses from Ref. 86, others from Ref. 87.
[b] By difference

the same scheme and employed other specifically labeled precursors. The results of these two groups (Table 9) suggest that D-glucose is incorporated as a unit into N-methyl-L-glucosamine, with carbons of the hexose becoming corresponding carbons of the hexosamine, although some catabolism is suggested by the enrichment of C-6 from C-1, as was also reported by AKAMATSU and ARAI.[88] This has been explained[86] as resulting from the transaldolase-transketolase pathway, by which label at C-1 of glucose will label significantly C-1 and C-6 of N-methyl-L-glucosamine, whereas label from C-6 of glucose will significantly label only C-6 of N-methyl-L-glucosamine.

The positions labeled rule out intermediate cyclitol formation; this would lead to specific incorporation, but different carbon atoms would be labeled (see Fig. 30).

Glucose labeled specifically at a number of positions has also been used to determine the labeling pattern in streptose.[89~91,93] Streptomycin isolated from cultures of *S. griseus* was hydrolyzed to streptidine and streptobiosamine and the latter was further degraded to locate the position of label in streptose (Fig. 32). The results (Table 10) show specific labeling of the streptose moiety: C-1 of glucose labels streptose at C-1, glucose C-3 labels streptose at C-3′ (the formyl group), and glucose C-6 labels streptose at C-5. The results with specifically labeled glucose confirmed earlier studies, which argued that C-formylation is not a biosynthetic reaction in the formation of streptose. Possible formyl precursors, including L-[methyl-^{14}C]-methionine and sodium [^{14}C]-formate, were administered without incorporation into the formyl group,[89,92] while L-[3-^{14}C]serine was significantly but not specifically incorporated.[93]

The labeling pattern in streptidine from feeding ^{14}C-labeled glucose to *S. griseus* was reported by BRUCE, *et al.*,[94] and by HORNER and RUSS.[95] Their results obtained from degradation of streptidine (Fig. 33) showed that a majority (68 % and 83 %, respectively) of the label from D-[1-^{14}C]glucose was found at C-5 of streptidine, while a majority of the label was retained in the other five carbons when [2-^{14}C]-, [3,4-^{14}C]-, and [6-^{14}C]D-glucose were administered.

The results of the ^{14}C-labeled glucose feedings have been confirmed by ^{13}C results in a recent paper.[96] D-[6-^{13}C]Glucose was administered to *S. griseus* and the resulting streptomycin was analyzed by CMR to obtain the incorporation pattern directly without degradation. D-[6-^{13}C]Glucose was found to label N-methyl-L-glucosamine at C-6″ and streptose at C-5′. In

Fig. 32. Degradation schemes used to locate labeled carbons in streptose

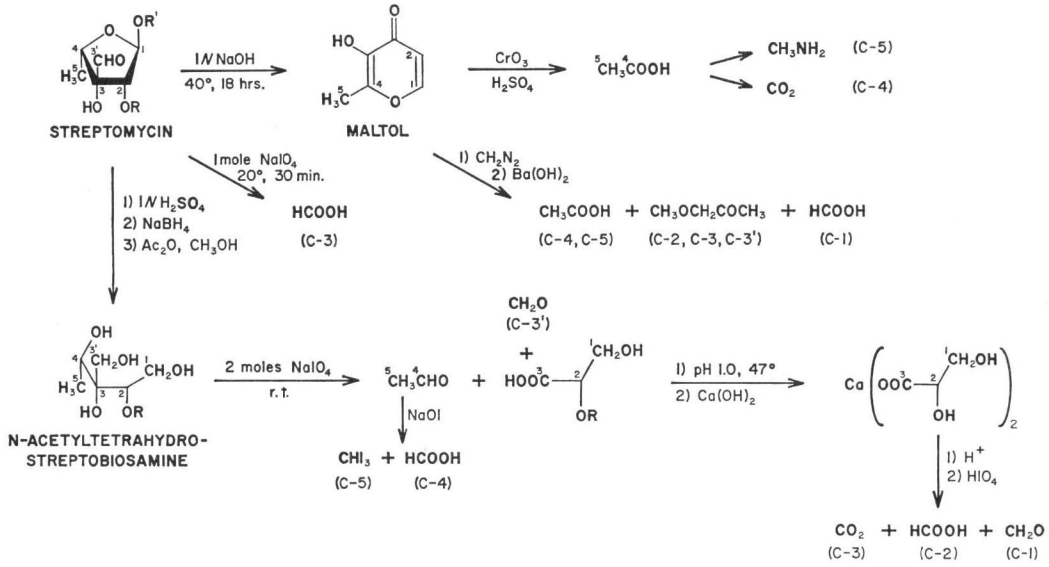
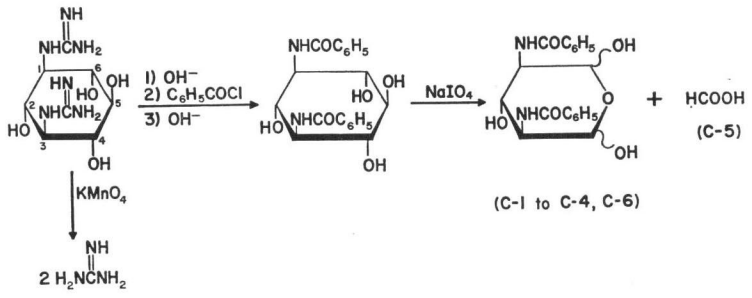


Table 10. Distribution of ^{14}C in streptose moiety from D-glucose labeled at various positions

Carbon atom	% Radioactivity in carbon atoms of streptose from labeled D-glucose					
	1-^{14}C[a]	1,3-^{14}C[b]	2-^{14}C[c]	3,4-^{14}C[c]	3-^{14}C[d]	6-^{14}C[c]
1	67.5	—	7	0	↑	↑
2	↑	—	73	0	11	12
3	14.2	—	—	50	↓	↓
3′	↓	39.6	0	50	86	9
4	[				]	9[e]
5	19.4	—	7	0	3	64

[a]Ref. 89. [b]Ref. 90. [c]Ref. 93. [d]Ref. 91. [e]By difference

Fig. 33. Degradation scheme used to locate label at C-5 of streptidine



addition, more specific information was obtained regarding streptidine, since D-[6-^{13}C]glucose labeled C-6 of streptidine.

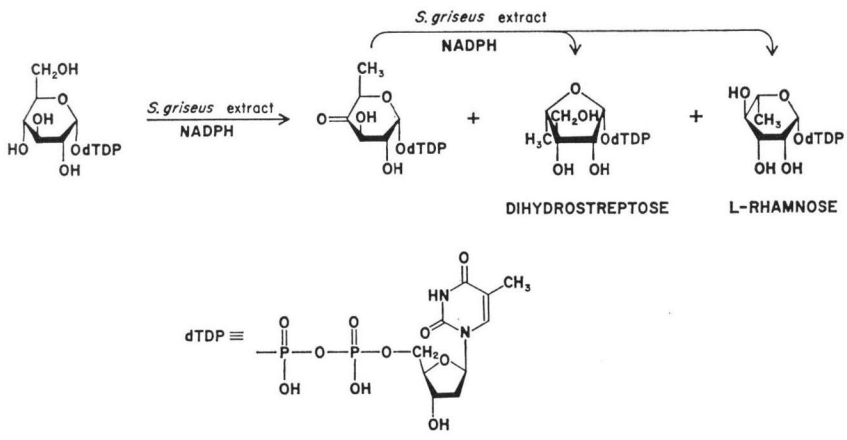
Primary precursors other than glucose are required for the C_1 units of N-methyl-L-glucosamine and streptidine. The methyl group of the former moiety has been shown[89] to arise

from transmethylation, with L-methionine as the methyl donor. A reductive process with formate as donor was ruled out by HORNER,[92] since sodium [^{14}C]formate was not incorporated. The guanidino groups of streptidine are labeled by [guanidino-^{14}C]arginine.[97~100] In addition, unlabeled arginine was found to dilute the [guanidino-^{14}C]streptomycin which resulted from incubation with [^{14}C]carbon dioxide.[101] Other potential precursors which labeled only the guanidino carbons[102] (presumably after conversion to C_1 units followed by incorporation into L-arginine, which would then serve as substrate for transamidination) included sodium [2-^{14}C]-acetate, [2-^{14}C]glycine and [^{14}C]carbon dioxide. Early workers[83] determined that the guanidino carbons of streptidine do not arise directly from glucose, although a small amount of label was found in the guanidino groups when labeled glucose was fed, presumably from prior conversion to carbon dioxide.

In addition to the primary precursors discussed above, numerous possible intermediates in the biosynthesis of streptomycin have been administered to *S. griseus*. BRUTON, *et al.*,[87] have shown that L-glucose is not incorporated into N-methyl-L-glucosamine or either of the other subunits of streptomycin. On the other hand, they found better incorporation into N-methyl-L-glucosamine of D-[1-^{14}C]glucosamine (59~64 %) than of D-[1-^{14}C]glucose (43 %). Most of the label (68 %) remained at C-1 of the N-methyl-L-glucosamine moiety in the glucosamine feeding (Table 9). Further, [^{15}N]glucosamine was found to label the streptobiosamine portion (and thus N-methyl-L-glucosamine) of streptomycin.[103] However, better incorporation of glucosamine was found in the streptidine moiety, which has since been shown to arise directly from glucose. Thus, the distribution of label in N-methyl-L-glucosamine suggests its formation from multiple stereochemical inversions of D-glucose *via* D-glucosamine.

The specific labeling pattern in streptose suggests a mechanism involving intramolecular carbon-carbon rearrangements of the glucose molecule. HORNER[81] suggested two possible mechanisms for the formation of streptose based on the labeling patterns from glucose. The first involved formation of dTDP-rhamnose from glucose, followed by rearrangement at C-3 and C-4 to form streptose. This possibility was based on reports[104] that dTDP-rhamnose was isolated from cultures of *S. griseus*. Additionally, cell-free extracts of *S. griseus* can synthesize

Fig. 34. Conversion of DTDP-D-glucose by *S. griseus* extracts to DTDP-dihydrostreptose and related compounds



dTDP-rhamnose from dTDP-glucose.[105] A second possible mechanism involves intramolecular rearrangement of glucose or a derivative prior to reduction at carbon-6. ORTMANN, *et al.*,[106] have shown that a third possibility is, however, the case (Fig. 34). A cell-free extract of *S. griseus* was incubated with dTDP-D-[U-^{14}C]glucose in the presence of NADPH. The radioactive products included dTDP-dihydrostreptose, dTDP-4-oxo-4,6-dideoxy-D-glucose, and dTDP-L-rhamnose. Preincubation of dTDP-D-[U-^{14}C]glucose with the enzyme extract from *S. griseus* or *S. aureofaciens* without NADPH gave labeled dTDP-4-oxo-4,6-dideoxy-D-glucose. When this compound was incubated with cell-free extracts of *S. griseus* in the presence of NADPH, dTDP-L-rhamnose and dTDP-dihydrostreptose were formed as labeled products. The latter compound easily decomposed to dihydrostreptose monophosphate, which further decomposed to dihydrostreptose.

In addition, these researchers studied the enzymatic activity of X-amidinotransferase and dTDP-dihydrostreptose synthase in relation to streptomycin formation. The enzymes were seen to parallel one another and preceded streptomycin production. dTDP-Rhamnose production does not, however, seem to be directly involved with streptomycin biosynthesis. The amount of dTDP-L-rhamnose formed from dTDP-4-oxo-4,6-dideoxy-D-glucose is small early in the fermentation (20 % at 47 hours) but increases toward the end (35 % at 186 hours), when dihydrostreptose is no longer formed.[106]

dTDP-Dihydrostreptose could be involved with streptomycin biosynthesis in a number of ways. It may be that this compound is the primary rearrangement product of glucose and can subsequently be oxidized to dTDP-streptose. Alternatively, it may be incorporated into dihydrostreptomycin, which may be a precursor of streptomycin. The enzyme extracts used in these did not produce dihydrostreptomycin in detectable amounts. Further work is needed to clarify the role of dTDP-dihydrostreptose in streptomycin biosynthesis.

An intermediate that has been studied for its conversion to streptidine is *myo*-inositol. Isotope dilution data[107] suggest that *myo*-inositol plays a role in the biosynthesis of streptidine from glucose. Labeled *myo*-inositol has also been shown to be well incorporated into streptidine; evidence has been presented above which rules out its initial conversion to glucose followed by incorporation. When *myo*-[2-^{14}C]inositol was fed, incorporations were tenfold the D-glucose value and radioactivity was present exclusively in the streptidine portion.[108] *Scyllo*-inositol also serves as a precursor to streptidine,[87] but it appears that this compound is first converted to *myo*-inositol, probably *via myo*-inosose-2, as has been demonstrated in rats[109] and plants.[110] BRUTON, *et al.*,[87] observed the same level of incorporation in parallel experiments using *scyllo*- and *myo*-inositol, with essentially all of the label incorporated into the streptidine subunit. Analysis of the mycelial hydrolyzate for both *scyllo*- and *myo*-inositols showed that in the case of the *myo*-inositol feeding, only *myo*-inositol was labeled in the mycelium. When labeled *scyllo*-inositol was fed, again the labeled compound was *myo*-inositol. When labeled glucose was fed, only *myo*-inositol was labeled in the mycelium. The data to date suggest that D-glucose is converted to *myo*-inositol,[107] probably by a mechanism similar to that reported by CHEN and CHARALAMPOUS[111] and KINDL, *et al.*[112] in yeasts.

Labeling of C-6 of streptidine by D-[6-^{13}C]glucose is in agreement with the conversion of glucose to streptidine *via myo*-inosose and pathway **a** of Fig. 35. That this pathway is followed in both streptidine and actinamine biosynthesis but not in deoxystreptamine biosynthesis

Fig. 35. Comparative labeling patterns of streptidine and deoxystreptamine

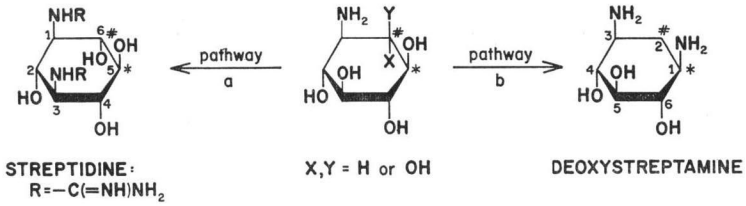
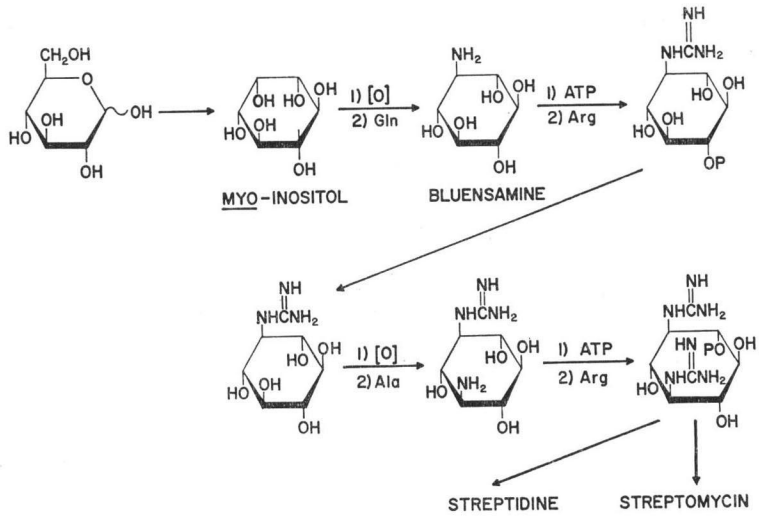


Fig. 36. Biosynthetic intermediates between D-glucose and streptidine



(pathway **b** of Fig. 35), argues that the direction of β-carbon amination in the aminocyclitol (counterclockwise *vs.* clockwise) is determined by the presence of a hydroxyl group (X=OH in Fig. 35) on the carbon arising from C-6 of glucose, rather than by the presence of a guanidino group on the carbon arising from C-5 of glucose.

A great deal has been reported, based on enzyme studies, regarding the intermediates between *myo*-inositol and streptidine. The current concept of the steps involved in streptidine biosynthesis is presented in Fig. 36. WALKER[113,114] has studied the enzymatic conversion of *myo*-inositol to bluensamine. The aminating enzyme, L-glutamine : keto-*scyllo*-inositol aminotransferase, is relatively non-specific. Amine donors can include *scyllo*-inosamine, *myo*-inosamine, streptamine and 2-deoxystreptamine, with *myo*-inosose-2 as amine acceptor. At high concentrations, L-alanine and L-glutamate can serve as donors and pyruvate and 2-ketoglutarate as acceptors. The corresponding D-amino acids are inactive.

Bluensamine is next activated by phosphorylation with ATP : inosamine phosphotransferase.[115] This enzyme may be responsible for phosphorylation of both bluensamine and 1-D-1-guanidino-3-amino-1,3-dideoxy-*scyllo*-inositol,[116] although 1-L-guanidino-3-amino-1,3-dideoxy-*scyllo*-inositol is not a substrate.[55] HORNER has also examined the mechanism of guanido group transfer, by comparing mycelial transamidinase activity with antibiotic production.[117] The processes were parallel, thus suggesting that the transamidinase enzyme was the active transfer agent. WALKER[118] has subsequently shown that the enzyme L-arginine: inosamine-phosphate

amidinotransferase probably carried out its task *via* an enzyme-S-C(=NH_2^+)-NH_2 intermediate. The enzyme is specific in both the L-arginine and inosamine phosphate sites, although high concentrations of glycylglycine, 1,4-diaminobutylphosphonic acid, ornithine analogs, and 4-aminocyclohexanol-1-phosphate can serve as substrates. In the aminocyclohexanols the presence of a phosphate group "*para*" to the amino group is obligatory.

Dephosphorylation of the 1-guanidino-1-deoxy-*scyllo*-inositol 4-phosphate prior to a second transamination is required. A guanidino group "*para*" to the phosphate also seems to be required for the dephosphorylation.[119]

Analogous reactions attach the second guanidino group.[55] The enzyme(s) responsible for dehydrogenation of the monoguanidino-*scyllo*-inositol have not yet been reported. Transamination of the product of this dehydrogenation (1-D-1-guanidino-3-keto-1-deoxy-*scyllo*-inositol) requires L-alanine, L-glutamate, or L-glutamine as amino donor. The enzyme has been separated from the first amino transferase enzyme.[114] Phosphorylation of the amino product[120] is evidently achieved using the same enzyme as that which phosphorylates bluensamine.[115] The position for phosphorylation is again "*para*" to the amino group.[55] Formation of streptidine-6 phosphate is then accomplished by an aminidinotransferase enzyme, again using arginine as guanidino donor.[121] This enzyme appears to differ from that which attaches the first guanido group.[122] A transamidinase has also been studied[123] in a strain of *S. griseus* which does not produce streptomycin. Streptidine was isolated from the cell wall of this non-producing strain. The enzyme activity increased upon addition of arginine and/or *myo*-inositol.

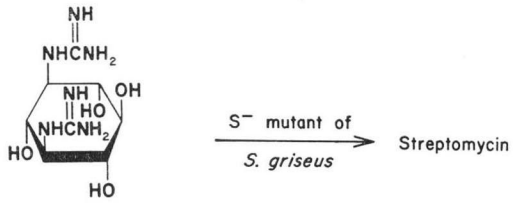
WALKER[124] has reported that analogous enzymatic pathways operate in *S. hygroscopicus* forma *glebosus* which produces bluensomycin. However, the point of introduction of the carbamoyl group remains uncertain.

The dephosphorylation of the streptidine moiety probably does not occur until after this group is attached to the rest of the streptomycin molecule. With high phosphate concentrations, streptomycin production is inhibited[123,125] and streptomycin-(*streptidino*-6)phosphate accumulates in the culture medium.[126] Additional evidence supporting the hypothesis that dephosphorylation of the streptidine-6 phosphate moiety is the last step in streptomycin biosynthesis can be found elsewhere. A strain of *S. griseus* produces streptomycin-(*streptidino*-6)-phosphate when grown in high concentrations of D-glucose.[127~129]

A phosphatase has been partially purified and appears to act preferentially on substrates which have phosphate esterified with free or combined streptidine.[119] These results suggest that either free streptidine is not a normal precursor or that it can be rephosphorylated after being glycosidically linked to the other subunits. This could be accomplished by ATP : streptomycin-(*streptidino*-6)-phosphotransferase, which can phosphorylate free streptidine, streptomycin, and presumably, intermediate compounds.[121,126,130]

The mutant approach has also been applied to streptomycin biosynthesis. In an early study,[131] a blocked mutant was shown to require a low molecular weight substance ("A-factor") in order to produce streptomycin. TOVAROVA, *et al.*,[131] reported that this "A-factor" was involved in streptidine biosynthesis but did not characterize this substance. More recently, NAGAOKA and DEMAIN[69] have isolated a mutant requiring streptidine for production of streptomycin (Fig. 37) thus demonstrating that streptidine is incorporated intact into streptomycin. This mutant did not produce streptomycin when streptamine (Fig. 15), a potential intermediate

Fig. 37. Incorporation of streptidine into streptomycin by an S⁻ mutant



in streptidine biosynthesis, was administered, thus arguing again that guanidylation of the first amino group precedes the introduction of the second amino group. In the presence of added 2-deoxystreptidine (Fig. 15) this mutant also produced an antibiotic, although it was not isolated. WALKER and WALKER[120] had found that both streptidine and 2-deoxystreptidine can serve as substrates for a phosphorylating enzyme, thus the incorporation of 2-deoxystreptidine by the *S. griseus* mutant is not surprising.

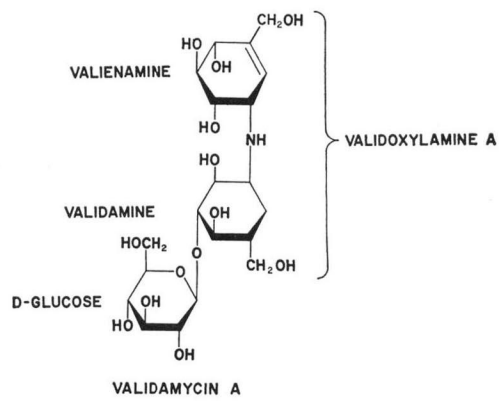
The only disagreement with dephosphorylation as the last step in streptomycin biosynthesis comes from HEDING and BAJPAI,[132] who suggested that methylation occurs last in streptomycin biosynthesis. They fed [methyl-^{14}C]methionine, along with N-demethyldihydrostreptomycin and with a mixture of N-demethylstreptomycin and N-demethyldihydrostreptomycin in separate experiments, to active cultures of *S. griseus*. In the first case, dihydrostreptomycin was the only labeled product formed. In the second, streptomycin was the product labeled more heavily. The strain of *S. griseus* used produced only streptomycin under control conditions. The authors concluded that both N-demethylstreptomycin and N-demethyldihydrostreptomycin are methylated by *S. griseus*, suggesting that the last step in biosynthesis is N-methylation. However, it must be pointed out that the level of added precursors was quite high (10 mg/ml) compared to the expected production in the control (2 mg/ml) and that the precursors were incubated for 28 hours, during which time both catabolism and anabolism might occur. A cell-free system employing N-demethylstreptomycin and L-methionine would be of help in testing this hypothesis. On balance, it appears likely that dephosphorylation occurs as the final step in normal streptomycin biosynthesis.

Antibiotics Containing Monoaminocyclitols

Validamycin.

A recent paper[133] has shown that D-[U-^{14}C]glucose labels equally the three subunits of validamycin A (Fig. 38), demonstrating that glucose serves as the carbon source for validamycin A. In addition, the pseudodisaccharide [^{14}C]-validoxylamine A, was shown to be incorporated into validamycin A, with virtually no label detected in the D-glucose moiety of validamycin A. This result argues that the glucose moiety is added last in the biosynthetic sequence, by glucosidation of validoxylamine A.

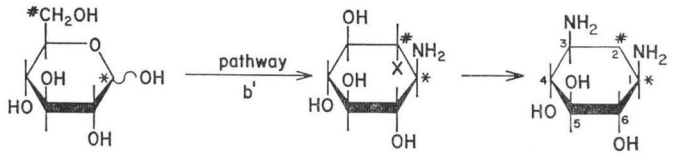
Fig. 38. Validamycin A, with subunits identified



Comparison of the Biosyntheses of Aminocyclitol Antibiotics

The biosyntheses of the aminocyclitol antibiotics are quite similar overall, but differ somewhat in details. While glucose provides the carbon backbone of all the subunits in

Fig. 39. Alternative pathway to deoxystreptamine from glucose



the aminocyclitol antibiotics studied thus far, the enzymatic systems which convert glucose to some very similar compounds appear to vary considerably.

It might be thought that streptidine (in streptomycin), actinamine (in spectinomycin) and deoxystreptamine (in neomycin) would be biosynthesized in a similar manner from glucose. It now appears that the addition of the second nitrogen to the monoaminocyclitol intermediate in neomycin biosynthesis[49] takes place in a direction opposite that found in the streptomycin[55,96] and spectinomycin[78] systems (Figs. 26 and 36), although an alternative pathway to deoxystreptamine is possible (Fig. 12). Additionally, *myo*-inositol is well incorporated into the streptidine moiety of streptomycin[108] and the actinamine moiety of spectinomycin,[77] whereas it is not incorporated into neomycin.[46]

Other variations are evident, even with more closely related antibiotics. Neamine can be bioconverted to ribostamycin and sisomicin by deoxystreptamine-requiring mutants of *S. ribosidificus*[67] and *M. inyoensis*,[76] respectively, but similar mutants of *S. fradiae*,[59] *S. kanamyceticus*,[59,67] *B. circulans*,[68] and *S. rimosus* forma *paromomycinus*[59] do not accept neamine (or paromamine) as a derivative of deoxystreptamine. The enzymes which convert glucosamine to glucose in *S. fradiae* (neomycin)[49] do not seem to be present in *S. kanamyceticus* (kanamycin), since glucosamine is well incorporated into all four neomycin subunits but this precursor labels kanamycin A in only the 6-amino-6-deoxy-D-glucose moiety.[70]

Obviously, no comparison of biosynthetic pathways is valid between dissimilar moieties, but glucosamine (paromomycin) and N-methyl-L-glucosamine (streptomycin) are enantiomers, except for the methyl group. The formation of glucosamine from glucose is a straightforward bioconversion,[50] whereas the formation of N-methyl-L-glucosamine requires considerable enzymatic alteration.[86,87,89]

Conclusion

A great deal about the biosynthesis of this important class of antibiotics remains unknown. Little is certain about the order in which the subunits are joined, although it can be inferred from certain mutant studies[67,75] that, at least in ribostamycin (and sisomicin) the order is neosamine C (or 2, 6-diamino-2, 3, 4, 6 tetradeoxy-D-glycero-hex-4-enose, dehydropurpurosamine) adding to deoxystreptamine, followed by addition of a third unit. The enzymatic steps of bioconversion of glucose to the subunits of the aminocyclitol antibiotics are well understood only for the streptidine moiety of streptomycin.[55] The intermediate compounds for the other subunits are only partially characterized.

Much has been accomplished since the discovery of these important compounds in the late 1940's. Clearly much additional research effort remains to be expended before the biosynthesis of the aminocyclitol antibiotics is completely understood.

Acknowledgement

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