NEOMYCIN BIOSYNTHESIS: THE INCORPORATION OF D-6-DEOXY-GLUCOSE DERIVATIVES AND VARIOUSLY LABELLED GLUCOSE INTO THE 2-DEOXYSTREPTAMINE RING

POSTULATED INVOLVEMENT OF 2-DEOXYINOSOSE SYNTHASE IN THE BIOSYNTHESIS

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(Received for publication November 25, 1991)

D-[6-$^3H_3$]6-Deoxy-5-ketoglucose (**10**) and D-[5,6-$^3H_2$]6-deoxyglucose (**11**) were incorporated into neomycins B and C using a growing culture of *Streptomyces fradiae*. D-[6-^{3}H]6-Deoxy-5-ketoglucose was incorporated into neomycin, as efficiently as the well established precursor D-glucose, and was found to label exclusively the 2-deoxystreptamine ring of the antibiotic. The results strengthened the previous proposals that in the formation of 2-deoxystreptamine the C-6 hydroxyl group of D-glucose is removed prior to the cyclisation reaction. Studies using the incorporation of D-[3-^{3}H]glucose, D-[3,4-$^3H_2$]glucose and D-[5-^{3}H]glucose into neomycin followed by the degradation of the latter established that in the biosynthesis of the 2-deoxystreptamine ring the C-4 and C-5 hydrogen atoms of glucose are removed. The loss of the C-4 hydrogen atom of the glucose is attributed to the formation of a 4-keto derivative which facilitates the removal of the C-5 hydrogen atom thus setting the stage for the expulsion of the C-6 hydroxyl group. The 5,6-olefinic intermediate formed in the process then undergoes cyclisation eventually releasing 2-deoxyinosose. The enzyme systems which participate in the conversion of D-glucose equivalent into 2-deoxyinosose may be described as 2-deoxyinosose synthase that in broad mechanistic terms resembles dehydroquinate synthase.

A large group of clinically useful antibiotics, to which streptomycin and neomycin belong, are classified as aminoglycoside-aminocyclitol antibiotics. The name highlights the fact that these antibiotics, in addition to an array of variously modified amino-sugars, contain an unusual cyclohexane derivative which can either be hexa-substituted as the streptamine unit (**1**) of streptomycin or penta-substituted as the 2-deoxystreptamine ring (**2**). The latter unit is present in neomycin (**3**) and more than fifty other related antibiotics. It is now known that the carbon skeletons of both types of aminocyclitols (**1** and **2**) arise from D-glucose[1,2] and the elegant studies of WALKER and WALKER[3] have established that, in the formation of the streptamine ring system of streptomycin, the key event is the inositol synthase catalysed cyclisation of glucose 6-phosphate into inositol 1-phosphate, Scheme 2. The latter, through a multistage process, is then converted into streptidine (this is a diguanidinated derivative of **1**). The knowledge of the latter biosynthesis provided the stimulus to consider the possibility that a cyclisation process similar to that shown in Scheme 2, followed by the reductive removal of the unwanted hydroxyl group, may also be involved in the formation of 2-deoxystreptamine. Gradually however observations were made which questioned this hypothesis and suggested that the two aminocyclitols (**1** and **2**) may be synthesised by two entirely different mechanisms. For example, the patterns for the incorporation of the C-skeleton of D-glucose

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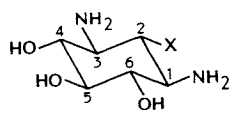
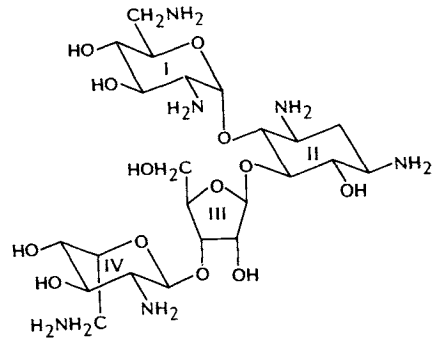
into the two aminocyclitols were found to be different, the C-6 of glucose occupied position 2 in deoxystreptamine[4] but position 6 in the streptamine ring[5,6]. Furthermore, the studies of KAKINUMA *et al.* on ribostamycin[7] and our work on neomycin[8] revealed that the 2-deoxystreptamine ring of these antibiotics was formed from D-glucose with the retention of both of its C-6 hydrogen atoms. Since the mechanism of the type shown in Scheme 2 requires the loss of one of the C-6 hydrogen atoms of glucose[9] during the condensation reaction, the results[7,8] suggested that the pathways for streptamine and 2-deoxystreptamine biosynthesis diverged prior to the cyclisation stage and led to the proposal of two closely related mechanisms[7,8] (Paths a and b, Scheme 3) for the construction of the deoxy centre of the aminocyclitol (**2**). The common feature in both the mechanisms is the involvement of the enolic intermediate (**7**, Scheme 3) which participates in nucleophilic attack at the C-1 carbonyl producing a deoxycarbocyclic ring. The difference in the two mechanisms, however, is that whereas in Path a, Scheme 3, the enolic species is produced *via* a direct elimination reaction necessitating the removal of a non-activated C-5 hydrogen, this feature is avoided in the alternative mechanism (Path b) where hydrogen removal is facilitated by the generation of a carbonyl function. These features have been studied using two interlocking approaches. Firstly, two deoxy sugars, 6-deoxyglucose (**11**) and 6-deoxy-5-ketoglucose (**10**), labelled with

Scheme 1. Structures of streptamine (**1**), 2-deoxystreptamine (**2**) and neomycin B (**3**).



1 X=OH
2 X=H



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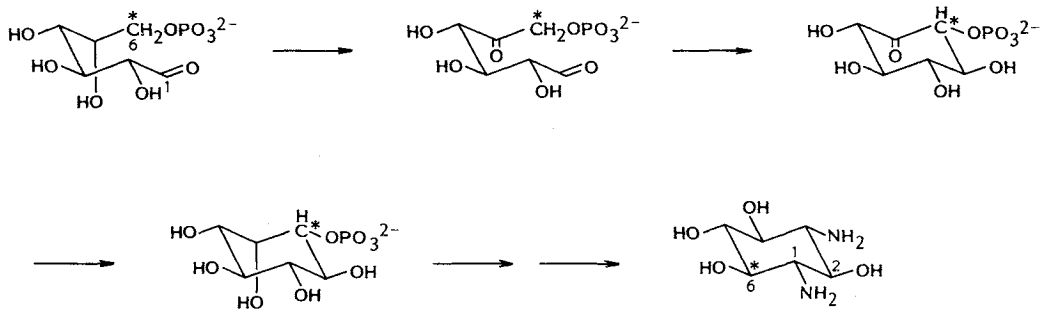
Neomycin C has the aminomethyl group in Ring IV in the equatorial orientation.

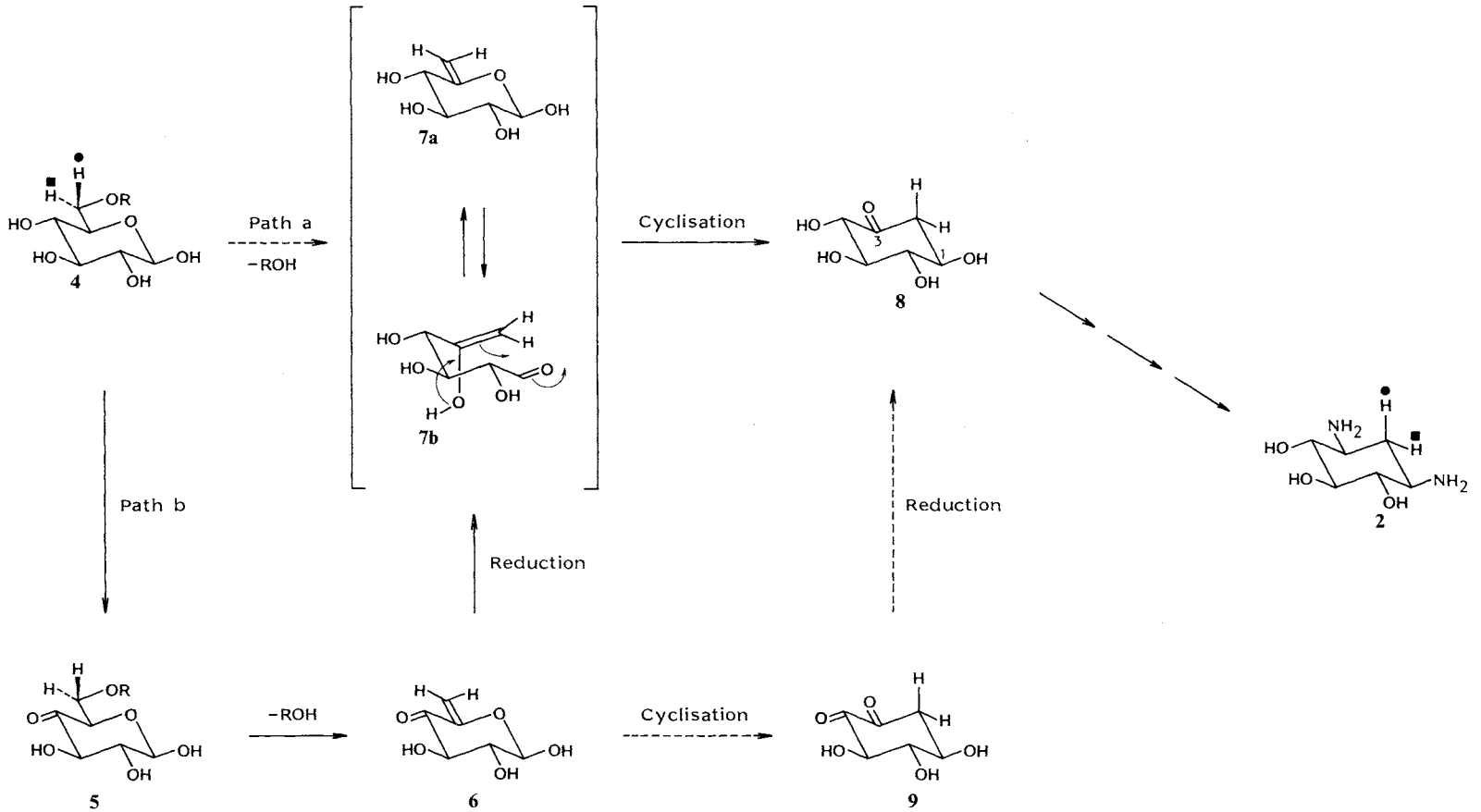
Scheme 2. The mechanism of *myo*-inositol 1-phosphate synthase reaction.



myo-Inositol 1-phosphate **1**

The scheme highlights that one of the C-6 hydrogen atoms of glucose 6-phosphate is removed in the condensation process and that the C-6 of glucose occupies position 6 in streptamine (**1**).

Scheme 3. The postulated mechanism for the formation of a deoxycyclitol ring from glucose.



R in **4** is a leaving group presumably a phosphate. The scheme shows the retention of both the C-6 hydrogen atoms of glucose in the predicted cyclised product (**8**). The further conversion of **8** into **2** occurs *via* a multistage process involving transamination at C-3 and oxidation followed by transamination at C-1. The solid arrows show the preferred reaction sequence. That the H_{Re} ($H^{\bullet}$) and H_{Si} ($^{\blacksquare}H$) in the precursor (**4**) occupy axial and equatorial orientations, respectively in the product (**2**) is shown in refs 7 and 13.

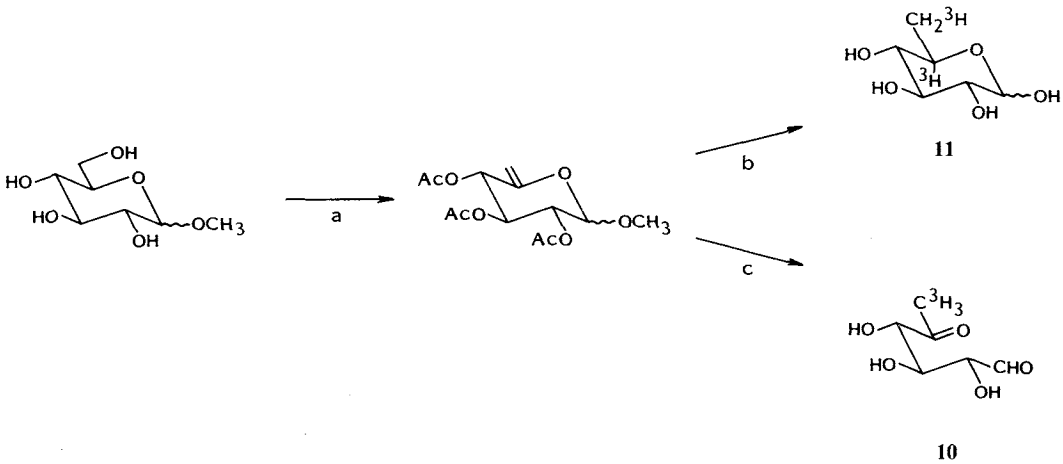
^3H were synthesised and their role as precursors of neomycin evaluated using *Streptomyces fradiae*. Secondly, the fate of the C-3, C-4, and C-5 hydrogen atoms of glucose during the incorporation of the latter into the 2-deoxystreptamine ring of the antibiotic was investigated with the aid of appropriately tritiated glucose samples. The paper extends the approaches used previously for the delineation of the mechanisms of the formation of neosamines B and C rings of neomycin[10] (these are Ring I and Ring IV, respectively in structure **3**) to the biosynthesis of 2-deoxystreptamine and gives a fuller account of studies originally published in a preliminary communication[11] and in a Ph. D. Thesis[12].

Results and Discussion

The fact that the two pro-chiral hydrogen atoms of glucose are stereospecifically[7] transferred to C-2 of 2-deoxystreptamine suggests that if an enol of the type (**7**) participates in the biosynthesis then this species is produced as a transient intermediate and remains bound to the active-site until the completion of the cyclisation process. We envisaged that 6-deoxy-5-ketoglucose (**10**, $^3H=H$) represents a stable tautomeric form of the enol intermediate and that such a compound following binding to the relevant enzyme may generate an enolic species suitable for cyclisation. To test this hypothesis 6-deoxy-5-ketoglucose containing ^3H at C-6 was synthesised according to the sequence of Scheme 4 as described in the Experimental section, and incubated with *S. fradiae*. Under the conditions used in this study *S. fradiae* produces two isomers of the antibiotic, neomycins B and C (Scheme 1) in the ratio of 9 : 1. Hereafter the mixture is referred to as neomycin and this was found to contain 0.3% of the ^3H label originally present in the precursor (Table 1) which by degradation was shown to be located only in the 2-deoxystreptamine ring of neomycin. The conclusion was confirmed and further evaluated by another experiment in which a mixture of [*U*-^{14}C]glucose and [6-^3H]6-deoxy-5-ketoglucose was fed to *S. fradiae* and a comprehensive degradation of the doubly labelled neomycin performed. The results are summarised in Table 2 and allow several conclusions to be drawn. The fact that the ^3H-^{14}C ratio of neomycin was 1/4 that of the original mixture of two precursors suggests that 6-deoxy-5-keto sugar is incorporated as well in the aminocyclitol ring as

Scheme 4. The sequence of reactions for the preparation of the deoxyhexoses **10** and **11**.

(a) CH_3SO_2Cl, $(CH_3CO)_2O$, NaI, pyridine; (b) $^3H_2$ and Pt separation of the two C-5 epimers, CH_3ONa, H^+; (c) ^3H_2O and CF_3CO_2H.



11

10

Table 1. Incorporation of single labelled 6-deoxy sugars into neomycins.

Precursor	Total amount of precursor added		% of original incorporation into neomycins
	Weight mg	dpm ^3H	
1a) [6-^{3}H]6-Deoxy-5-keto-D-glucose	7.9	165×10^6	0.28
1b) [6-^{3}H]6-Deoxy-5-keto-D-glucose	1.9	40×10^6	0.30
2) [5,6-^{3}H]6-Deoxy-glucose	1.9	40×10^6	0.03

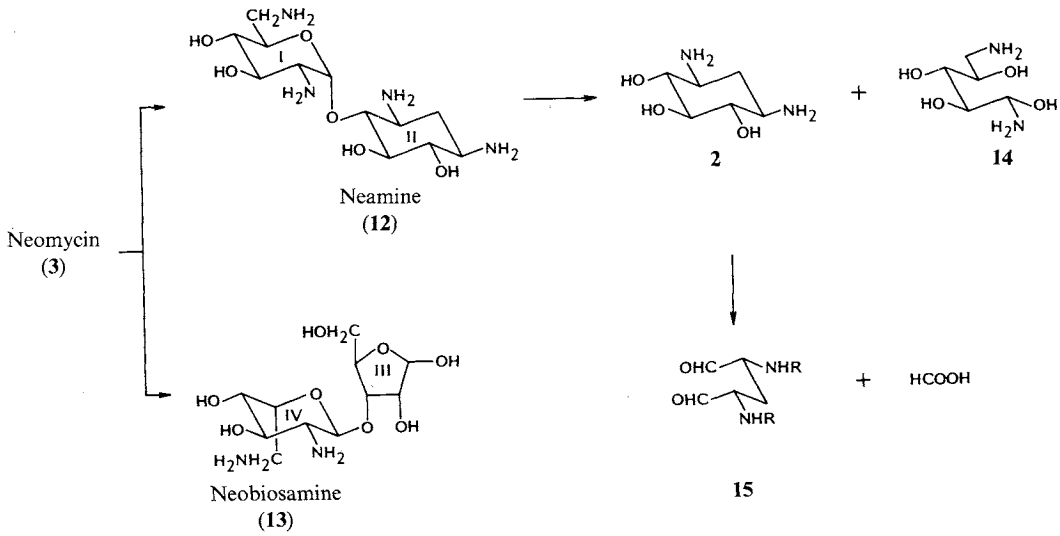
Samples of each single labelled 6-deoxy sugar were incubated with culture of *S. fradiae* (9×10 ml) for 96 hours. The antibiotic produced as a mixture of neomycins B and C (in the ratio of 9 : 1) was isolated and purified by ion exchange chromatography[10].

Table 2. The incorporation of a mixture of [6-^{3}H]6-deoxy-5-keto-D-glucose and [*U*-^{14}C]D-glucose into neomycins and the location of the two isotopes in the various subunits of the antibiotic.

Compound	^3H - ^{14}C
[6-^{3}H]6-Deoxy-5-keto-D-glucose + [*U*-^{14}C]D-glucose	6.8
Neomycins	1.8
Neamine	3.2
Neobiosamine	0.5
2-Deoxystreptamine	6.6
Neosamine C	0.0

The antibiotic biosynthesised from the above precursors as in Table 1 was degraded to furnish the various subunits according to the sequence of Scheme 5.

Scheme 5. The degradation of neomycins B and C.



The mixture was hydrolysed to give the common neamine unit **12** which was further degraded to **14** and **15**.

is glucose. This is because glucose is known to label the four rings of the antibiotic about equally while 6-deoxy-5-ketoglucose was expected to be incorporated specifically only into the 2-deoxystreptamine ring.

In order to shed further light on the labelling pattern the neomycin was hydrolysed first to neamine (Ring I-Ring II) plus neobiosamine (Ring III-Ring IV) and neamine then further converted into neosaminol C (**14**) and 2-deoxystreptamine (**2**, Scheme 5). The radiochemical data in Table 2 clearly show that ^3H was located only in 2-deoxystreptamine and the ^3H - ^{14}C ratio of the latter was identical to that of the original mixture. From the data, without making any allowance for the loss of tritium accompanying the conversion of the methyl group of 6-deoxy-5-ketoglucose into the methylene carbon of 2-deoxystreptamine, it appears that the two sugars (6-deoxy-5-ketoglucose (**10**) and glucose) are comparable precursors of the aminocyclitol.

For comparison, feeding experiments were also performed with another deoxy sugar, 6-deoxyglucose (**11**), and it was found that this precursor was incorporated in an 0.03% radiochemical yield into neomycins. Since in the biosynthesis of 2-deoxystreptamine the 5-^{3}H of [5,6-$^3H_2$]6-deoxyglucose will be eliminated we estimate that the latter sugar is 1/5 as efficient a precursor of the aminocyclitol as is its 5-keto counterpart. The relative efficiency of the incorporation suggests but does not prove that 6-deoxyglucose is incorporated into 2-deoxystreptamine *via* the 5-keto derivative. Irrespective of the validity of this assumption the results described in Tables 1 and 2 strongly suggest that in the biosynthesis of 2-deoxystreptamine the removal of the C-6 substituent of glucose precedes cyclisation. We now turn to the feeding experiments with samples of glucose containing ^{3}H at C-3, C-4 and C-5. The extent of ^{3}H retention or loss in the biosynthetic experiments performed with these labelled precursors was quantified using a double labelling approach in which [*U*-^{14}C]glucose served as an internal reference.

Samples of [*U*-^{14}C, 3,4-$^3H_2$]glucose, [*U*-^{14}C, 3-^{3}H]glucose or [*U*-^{14}C, 5-^{3}H]glucose were fed to growing cultures of *S. fradiae* and neomycin was isolated, purified and degraded to obtain its four rings. Based on the ^{14}C radioactivity originally used, the incorporations of glucose into the antibiotic in these experiments were about 0.9% (Table 3). The incorporation of ^{3}H from [5-^{3}H]glucose into various subunits of neomycin has been reported previously[10]. In the present context suffice to draw attention to the fact that the ribose ring of neomycin was formed retaining the original C-5 tritium to the extent of 80% which proved that the glucose skeleton was incorporated intact into the antibiotic. The 2-deoxystreptamine ring, however, contained only 10% of the ^{3}H originally present in the precursor glucose. The C-5 of glucose occupies C-3 in 2-deoxystreptamine and since the amino group in this position (C-3) is expected to arise *via* a carbonyl group, the C-5 tritium will be removed in the attendant reaction. In the experiment in which [*U*-^{14}C, 3-^{3}H]- and [*U*-^{14}C, 3,4-$^3H_2$]glucose samples were used as precursors the ^{3}H - ^{14}C atomic ratios of the derived neosaminol (Ring I) and ribitol (Ring III) showed the complete retention of the original ^{3}H. These results provide the important information that under the incubation conditions used in the present study the exogenously added glucose was incorporated into the antibiotic without the labilisation of ^{3}H or the rearrangement of the carbon skeleton. With this information in hand it is justified to assume that the ^{3}H content of 2-deoxystreptamine genuinely reflects the mechanism(s) through which its carbocyclic ring is produced from glucose. Table 4 shows that ^{3}H - ^{14}C ratio of 2-deoxystreptamine was half that of glucose when the latter precursor was labelled with ^{3}H at C-3 as well as C-4 thus suggesting that in the biosynthesis one of the hydrogen atoms of the glucose is removed. On the other hand [*U*-^{14}C, 3-^{3}H]-D-glucose was incorporated into 2-deoxystreptamine with complete maintenance of its ^{3}H - ^{14}C ratio which proves that it must be the C-4 tritium that is labilised in the synthesis from 3,4-tritiated glucose. That in

Table 3. The incorporation of doubly labelled D-glucose samples into neomycin B.

Precursors	^{3}H - ^{14}C ratio of precursors	Amount of precursor added		^{3}H - ^{14}C ratio of neomycin B	Percentage incorporation ^{14}C
		Weight (mg)	Radioactivity ^{14}C dpm		
[5-^{3}H, *U*-^{14}C]D-Glucose	11.6	0.024	86×10^6	5.4	0.63
[3,4-$^3H_2$, *U*-^{14}C]D-Glucose	7.6	0.01	36×10^6	5.5	0.96
[3-^{3}H, *U*-^{14}C]D-Glucose	7.9	0.04	32×10^6	7.8	1.2

Samples of doubly labelled hexoses were incubated with cultures of *S. fradiae* (20×10 ml) for 12 hours. The antibiotic was isolated and purified in Table 1. The percentage incorporation in the last column is based on the ^{14}C content of the precursor.

Table 4. Radiochemical data on ^{3}H-^{14}C ratios of variously labelled glucose and of the degradation products derived from the biosynthetic neomycin.

Compound	^{3}H-^{14}C and (Atomic) ratios		
	[3,4-$^{3}H_2$, *U*-^{14}C]D-Glucose	[3-^{3}H, *U*-^{14}C]D-Glucose	[5-^{3}H, *U*-^{14}C]D-Glucose
Glucose	7.7 (2:6)	7.8 (1:6)	9.3 (1:6)
Neosaminol C	7.5 (1.9:6)	7.9 (1.01:6)	
2-Deoxystreptamine	3.7 (0.97:6)	7.9 (1.01:6)	0.93 (0.1:6)
Ribose (measured as ribitol)			9 (0.81:5)
Dialdehyde derivative	0 (0:5)	0 (0:5)	
Cyclic derivative of the dialdehyde	0 (0:5)	0 (0:5)	
Formic acid	20.4 (0.88:1)	37 (0.8:1)	

A comparison of the ^{3}H-^{14}C and atomic ratios of neomycin subunits with those of the precursor glucose is shown. The details of the incubation are the same as in Table 3 and those of degradation are described in the experimental section and elsewhere[7,10~12].

both cases the tritium remaining in 2-deoxystreptamine was resident at its C-5 (which corresponds to C-3 of glucose) was shown through the isolation of the C-5 as formate that contained all the ^{3}H radioactivity of the ring while the aldehyde (**15**, Scheme 5) containing the remaining carbon skeleton (C-1, C-2, C-3, C-4 and C-6) of 2-deoxystreptamine was completely devoid of any ^{3}H.

These results unambiguously prove that the formation of the 2-deoxystreptamine ring of neomycin is attended by the loss of the C-4 hydrogen atom of the precursor glucose. This could occur either by the direct oxidation at this position to produce a 4-keto intermediate or indirectly through oxidation at C-5 followed by a prototropic rearrangement yielding an enediol intermediate which rearranges to the 4-ketospecies (**5**). The latter contains the electronic arrangement to undergo a facile elimination reaction, **5**→**6**. The stage at which the C-4 carbonyl function of **6** is reduced cannot be deduced from the experiments of Table 4. However, when the results are taken in conjunction with the observation that 6-deoxy-5-ketoglucose is a good precursor of 2-deoxystreptamine then an argument may be developed that the C-4 carbonyl group of the intermediate of the type **6** is reduced prior to cyclisation, thus favouring the sequence **6**→**7**→**8**→**2** rather than **6**→**9**→**8**→**2**. The rationale underpinning the conclusion is that the sequence **6**→**7**→**8**→**2** when extended to the non-physiological conversion of 6-deoxy-5-ketoglucose into 2-deoxystreptamine requires only one additional reaction, (*i.e.* the enolization of **10**→**7**), that is not involved in the physiological pathway. However, much greater catalytic versatility is required to explain the cyclisation of 6-deoxy-5-ketoglucose if the normal pathway from glucose followed the sequence **6**→**9**→**8**→**2**. Furthermore, the reduction of the C-4 carbonyl group prior to cyclisation will have the added advantage of making the C-6 of the enol (**7**) electron rich enabling this position to add to the C-1 carbonyl group. On the other hand the C-6 position in the enedione (**6**) due to the electron withdrawing property of the C-4 carbonyl group will be deactivated for such a condensation reaction. The electronic considerations thus make the sequence **6**→**7**→**8**→**2** preferable over the alternative **6**→**9**→**8**→**2**, also see ref 13.

Several biological transformations of monosaccharides which are attended by an internal redox reaction are known. The examples of these are the formation of deoxy and epimeric sugars[14~16] In general, the enzymes catalysing these transformations contain a tightly bound nucleotide cofactor NAD or NADP, which participates in the oxidation of the C-4 hydroxyl group of the hexose. At a latter stage in the

sequence the hydride from the reduced cofactor is intramolecularly transferred to a suitable electrophilic centre in the substrate. The loss of the C-4 hydrogen atom of glucose demonstrated in the present work is at variance with the general behaviour displayed by other related enzymic involved in monosaccharide metabolism and may be the consequence of the fact that with the example under study the cofactor is readily dissociated. This means that the NAD(P)H molecule involved in the reduction reaction, **6**→**7**, is different from that produced in the original oxidation, **4**→**5**. If it were not for this property of the enzyme the involvement of the position -4 of glucose in the formation of 2-deoxystreptamine could not have been signalled.

Another experimental finding which needs to be explained is that the two C-6 pro-chiral hydrogen atoms of glucose are incorporated stereospecifically[7,13] into 2-deoxystreptamine and these, H_{Re} and H_{Si}, occupy the axial and equatorial orientations, respectively at C-2 of the aminocyclitol ring as shown in Scheme 4. It is attractive to assume that the stereochemistry of the two hydrogen atoms when these become the part of an unstable enolic system is maintained by fixing the geometry of the double bond through the formation of a pyronose ring (**6a** and **7a**) which is opened only at the juncture of the cyclisation reaction.

In the light of these cumulative considerations we predict that the immediate product of cyclisation of D-glucose is 2-deoxyinosose and thus the enzyme involved in the process may be designated a 2-deoxyinosose synthase. The intermediacy of 2-deoxy-*scyllo*-inosose and its aminated counterpart in the biosynthesis of 2-deoxystreptamine has been demonstrated previously[17~20]. It is interesting to draw attention to the fact that the mechanism considered in this paper for the formation of the carbocyclic ring of 2-deoxystreptamine is reminiscent of the reaction catalysed by dehydroquinate synthase[21].

Materials and Methods

The laboratory chemicals were ex-stock from BDH or Kochlight. D-[*U*-^{14}C]Glucose (296 mCi mmol^{-1}) was obtained from Amersham International PLC. D-[3,4-$^{3}H_2$]Glucose (44.6 Ci mmol^{-1}), D-[3-^{3}H]glucose were obtained from New England Nuclear, Dreieich, West Germany and D-[5-^{3}H]glucose prepared as described previously[10]. Nutrient agar and nutrient broth were obtained from Oxoid, London, SE1. Tryptic soya broth, Bacto-organic salts were obtained from Difco Laboratories, Detroit, Michigan, U.S.A. Chemicals for scintillation counting were obtained from G&G Chemicals, South Ascot, Berks. The neomycin producer, *Streptomyces fradiae* wild type (ATCC 10745), was obtained from the American Type Culture Collection (ATCC) C16C. Thin layer chromatography (TLC) was performed using plates coated with silica gel.

Preparation of Labelled 6-Deoxy Sugars

Methyl 2,3,4-Tri-*O*-acetyl-6-iodo-α-glycopyranoside

Methyl 2,3,4-tri-*O*-acetyl-6-*O*-methylsulfonyl-α-glycopyranoside[22] (10 g, 20 mmol) and sodium iodide (10 g, 600 mmol) were dissolved in dry acetone (80 ml). The solution was refluxed for 2 hours whereupon a white precipitate of methyl sodium sulfonate was formed. The solution was filtered, and the residue washed several times with acetone. The filtrate was evaporated under reduced pressure to a residue which was dissolved in chloroform and washed with water. The chloroform solution was evaporated under reduced pressure, the crude material was crystallised from methanol to give the title compound (7 g, 60%). ^{1}H NMR (60 MHz, DMSO-d_6) δ 1.9 (3H, s, CH_3), 2.0 (3H, s, CH_3), 2.1 (3H, s, CH_3), 3.0 (3H, s, CH_3), 3.2 (2H, d, $-CH_2$).

D-[6-^{3}H]6-Deoxy-5-ketoglucose

The preceding compound was converted to methyl pyranoside 5,6-α-glucosene as described

elsewhere[23]. The latter (170 mg) was dissolved in a solution (1 ml) prepared from trifluoroacetic acid (0.8 ml) and tritiated water (0.2 ml, 1Ci of ^3H). The reaction mixture was left for about one hour at room temperature. The solvents were removed under reduced pressure, the residue was then dissolved in water followed by evaporation *in vacuo*. The preceding operation was repeated 5 times. TLC of the residue showed the presence of three bands, the desired compound being the major product (Rf 0.3, 70%).

For purification 9 mg of the crude residue containing 1.9×10^8 dpm of ^3H was spotted on a 10 cm × 20 cm TLC plate which was developed in an ascending tank with chloroform - methanol (4 : 1, v/v). The plate was dried and scanned for radioactivity. After detection the product was eluted with methanol and the solution evaporated to yield D-[6-$^3H_3$]6-deoxy-5-ketoglucose as an oil. $\delta_H(^2H_2O)$ 2.3 (3H); $(M+1)^+$ of the compound as *tris*-trimethylsilyl-*bis*-methoxyoxime has *m/z* at 437.2252. $C_{17}O_5H_{40}Si_3N_2+H$ requires 437.2323.

D-[5,6-^3H]6-Deoxyglucose

PtO_2 (20 mg) was suspended in ethyl acetate (20 ml) and shaken in an atmosphere of tritium gas generated by the reaction ($2^3H_2O+2Li \rightarrow 2Li(OH)+{}^3H_2$) at room temperature. Methyl pyranoside of 2,3,4-tri-*O*-acetyl, 5,6-α-glucosene[23] (50 mg in 1 ml of ethyl acetate) was then added, and the suspension shaken with tritium until the absorption of the gas ceased. The time required for the reduction was about 2 hours. Removal of the catalyst and concentration under pressure afforded a mixture which was resolved by TLC into two isomers, the glucose derivative 15% (Rf 0.7) and the idose derivative 85% (Rf 0.73).

The preceding mixture (4 mg, 2.5×10^8 dpm), was deacylated[23] and spotted on a 10 cm × 20 cm TLC plate which was developed in an ascending tank with chloroform - methanol (4 : 1, v/v). The plate was dried and was scanned for radioactivity. The glucose derivative, which was identified by co-migration with an authentic sample, was eluted with methanol and the material was subjected to two further rounds of chromatography to obtain a homogeneous sample.

A solution of the preceding compound, methyl D-6-deoxy-glucopyranoside (1.6 mg, 90×10^6 dpm), in 1 M hydrochloric acid (10 ml) was heated under reflux for 4 hours. The cooled solution was evaporated under reduced pressure. Analytical TLC developed in chloroform - methanol (4 : 1, v/v) showed one spot (Rf 0.3) corresponding to the position of authentic 6-deoxy-α-D-glucose.

The Incorporation of Labelled Precursors into Neomycins B and C

Streptomyces fradiae (ATCC 10745) spores were produced on Bacto-inorganic salt agar, transferred to a Tryptic soya broth medium (10 ml, prepared according to manufacturer's instructions) and incubated for 40 hours at 28°C. The fully grown culture (1.0 ml) and a 30% (w/v) solution of maltose (0.5 ml) were then added to a defined medium[10] (9.0 ml) contained in a 125-ml conical flask. Several such flasks were normally prepared and incubated together at 28°C on a rotary shaker (150 revolutions/minute) for 48 hours until the cell density reached 80~90% of that expected for the stationary phase. The labelled precursors whose amounts and radiochemical characteristics are defined in Tables 1, 2 and 3 in water (2 ml) were added to the cultures and the incubation continued for 96 hours for the experiments of Tables 1 and 2, and 12 hours for those in Table 3. The antibiotic produced under these conditions consists of neomycins B and C in the ratio of 9 : 1. In the present study the antibiotic was isolated and purified as a mixture of the two isomers and degraded to neamine, neobiosamine, 2-deoxystreptamine and neosaminol C as described previously[10]. The further degradation of 2-deoxystreptamine is detailed below.

Degradation of 2-Deoxystreptamine

The labelled 2-deoxystreptamine was mixed with carrier and the resulting material (25 mg) was dissolved in hot methanol (50 ml). Dowex 1-X2 resin (carbonate form washed with methanol prior to use, 20 ml) was added to the preceding solution and the mixture stirred for 5 minutes (pH 8.0) then benzoyl chloride (0.1 ml) was added dropwise over 10 minutes with stirring.

The mixture was kept at room temperature for 45 minutes with occasional stirring and the pH was maintained between 6 and 7 by adding Dowex 1-X2 (CO_3^-) and then filtered. The resin was washed several times with MeOH - H_2O (7 : 3, v/v). The filtrate was treated with 10 ml of Dowex 50W-X8 (H^+), stirred for 5 minutes and then filtered. The resin was washed with MeOH - H_2O (7 : 3, v/v) five times. The combined solvent mixture was evaporated to dryness using a rotary evaporator. The residue was washed several

times with diethyl ether to remove traces of benzoic acid, and yielded *N,N'*-dibenzoyl-2-deoxystreptamine as a white powder (25 mg). Analysis by TLC (using ethyl acetate - methanol, 4 : 6) showed one spot at Rf 0.6; visualised under UV light.

The preceding *N,N'*-dibenzoyl-2-deoxystreptamine (20 mg) was dissolved in 50 ml of MeOH - H_2O (7 : 3, v/v) with stirring for 20 minutes and then treated with a solution of sodium periodate (150 mg in 5 ml water) which was added dropwise over a 10-minute period. The stirring was continued for another 5 minutes. The reaction (clear solution) was then left in the dark at room temperature without stirring for 24 hours. The pH of the solution was adjusted to 10 with sodium hydroxide and the solvent removed using a rotary evaporator. The dialdehyde and its cyclic derivative were extracted with ethyl acetate (5 × 5 ml). The extracts were combined and the solvent was evaporated to dryness *in vacuo* to give a white solid residue (16 mg). Analysis of the product by TLC using ethyl acetate and detection by UV light showed the presence of two spots of Rf, 0.55 and 0.4 attributed to 2,4-diamino-*N,N'*-dibenzoylglutardialdehyde and cyclic structure (the dialdehyde combined with one molecule of water), respectively. The pH of the residue left after ethyl acetate extraction which contained sodium formate was adjusted to 2 by orthophosphoric acid and the solution freeze-dried. The formic acid was collected, the pH adjusted to 7.5 with NaOH and the sample freeze-dried. The resulting sodium formate was analysed for its ^{3}H - ^{14}C ratio.

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