Mechanism of the Formation of the Two Epimeric Diamino-hexose Rings of Neomycin B

Sayed K. Goda, Walid Al-Feel, and Muhammad Akhtar *
Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU

By using variously tritiated hexoses and *Streptomyces fradiae* (a neomycin-producer), the mechanism and stereochemistry of the formation of various subunits of neomycin B were investigated. The results of the incorporation of (6*RS*)-D-[6-^{3}H]glucose into neomycin B followed by specific degradation showed that during the formation of the aminomethyl group of the neosamine B ring one of the 6-^{3}H atoms of the precursor was removed. The ^{3}H remaining at C-6 of the neosamine B ring was shown to be located in the H_{Si} orientation through the isolation of the chiral centre as glycine and the analysis of the latter by using an exchange reaction catalysed by serine hydroxymethyltransferase. These results suggested that, as has been previously shown for neosamine C, the aminomethyl group of neosamine B is formed by an oxidation-transamination process; $-CH_2OH \longrightarrow -CHO \longrightarrow -CH_2NH_2$

D-[5-^{3}H]Glucose was prepared by an unambiguous method and incorporated into neomycin B. The radiochemical data on the various subunits of the antibiotic could be interpreted to suggest that the L-idose configuration at C-5 of the neosamine B ring is produced *via* an enolisation process involving a carbonyl group at either C-4 or C-6. The involvement of C-4 was then eliminated by feeding experiments in which it was shown that the hydrogen atom at C-4 of D-glucose was undisturbed during the incorporation of this position into the C-4 of neosamine B or C.

Aminocyclitol antibiotics constitute an important and expanding group of therapeutically important compounds. The antibiotics of this class contain a variety of modified or rearranged sugar units,[1a] whose biosynthesis from primary precursors, such as D-glucose, seems to involve the participation of novel enzymic reactions. This is exemplified by the structural feature of neomycin B (Scheme 1, structure 1) which, in addition to a ribosyl subunit, contains three unusual rings. Two of these rings, which for historical reasons are known as neosamine C and neosamine B may be regarded as dideoxydiamino derivatives of D-glucose and L-idose, respectively (Rings I and IV, structure 1).

It has been established that both these rings arise from D-glucose *via* D-glucosamine[1b] and recent work has shown that the C-6 aminomethyl group of the neosamine C subunit of neomycins[2] and of a related antibiotic ribostamycin[3] arises from an oxidation-transamination sequence of the type shown in Scheme 2. Our previous approach[2] has now been extended to the study of the biosynthesis of the neosamine B moiety of neomycin B. The formation of the neosamine B ring from D-glucosamine involves not only the elaboration of C-6 but also the epimerization of C-5. The chemistry of the processes underlying these changes may be studied using D-glucose or glucosamine containing labelled hydrogen either at C-6 or C-5.

Results and Discussion

Feeding and Degradation Experiments.—Before any mechanistic work could be undertaken, it was necessary to define the growth conditions for the maximum production of the antibiotics and establish a feeding regime to optimise the incorporation of the labelled precursors into neomycin B and C. A systematic study, a detailed account of which is described elsewhere,[4] showed that *Streptomyces fradiae* (A.T.C.C. 10745) produced about 700 μg ml^{-1} of a mixture of neomycin B and C during a total incubation period of 120 h. The optimal incorporation conditions involved a protocol in which *S.fradiae* was grown in a defined medium[5] until the cell density was about 90% (usually 30—40 h) of that expected for the stationary phase and then the radioactive precursor (Table 1) under investigation was administered and the growth continued for further 12 h. The antibiotics produced under these conditions consisted of a mixture of neomycin B and C in the ratio 9:1. In such an

Table 1. Incorporation of doubly labelled precursors into neomycin B

Precursors	^{3}H:^{14}C Ratio of precursors	Amount of precursor added: Weight (mg)	Amount of precursor added: Radioactivity ^{14}C d.p.m.	Yield of neomycins (mg)	Total counts in neomycin B ^{14}C d.p.m.	^{3}H:^{14}C ratio of neomycin B	% Incorporation (^{14}C)
(6*RS*)-D-[1-^{14}C,6-^{3}H]glucosamine[a]	6	2.18	73.8 × 10^6	19.2	1.59 × 10^6	3.4	2.1
(6*RS*)-D-[6-^{14}C,6-^{3}H]glucose[a]	30	1.8	117 × 10^6	18.2	0.5 × 10^6		0.4
D-[U-^{14}C,5-^{3}H]glucose[b]	11.6	0.024	86 × 10^6	20	0.55 × 10^6	5.4	0.63
D-[U-^{14}C,3,4-$^3H_2$]glucose[b]	7.6	0.01	36 × 10^6	25	0.34 × 10^6	5.5	0.96

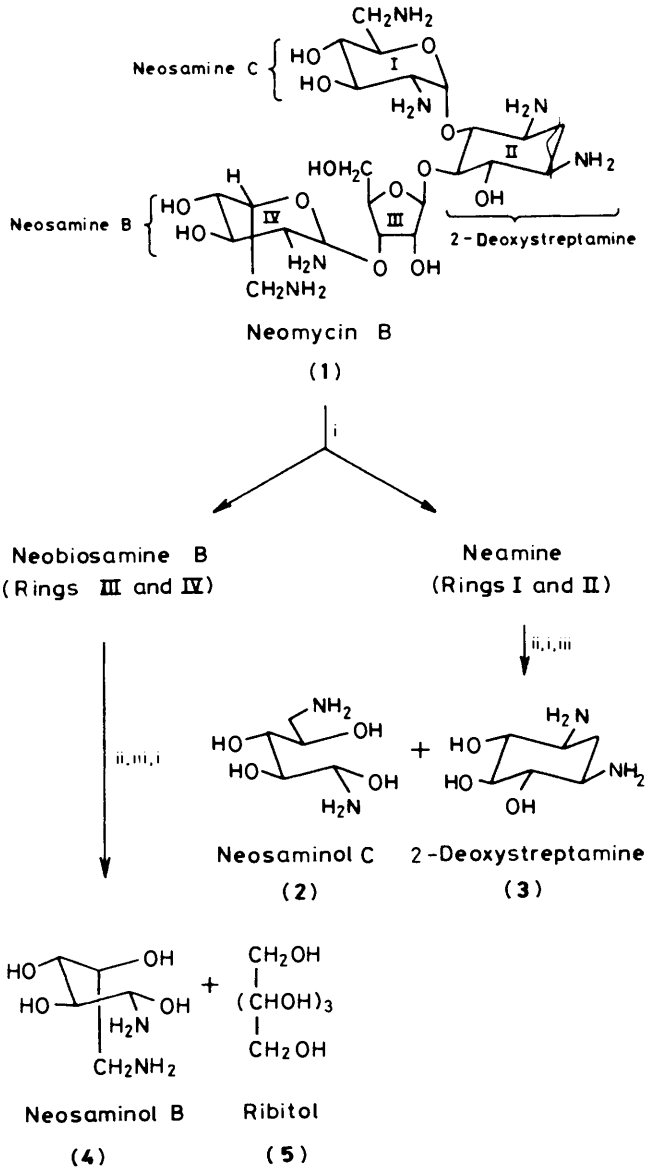
Samples of doubly labelled hexoses were incubated with cultures of *S.fradiae* (10—20 × 10 ml) for 12 h. The antibiotic was isolated and purified by ion exchange chromatography. The percentage incorporation in the last column is based on the ^{14}C content of the precursor. (For details, see Experimental section).

[a] Added to 10 flasks. [b] Added to 20 flasks.

Table 2. Radiochemical data on the precursors used to obtain the samples of labelled neomycin B and of the degradation products derived from the latter

	Experiment 1 (6*RS*)-D-[1-^{14}C,6-^{3}H]glucosamine ^{3}H:^{14}C and atomic ratios		Experiment 2 (6*RS*)-D-[6-^{14}C,6-^{3}H]glucose ^{3}H:^{14}C and atomic ratios		Experiment 3 D-[U-^{14}C,5-^{3}H]glucose ^{3}H:^{14}C and atomic ratios		Experiment 4 D-[U-^{14}C,3,4-^{3}H$_2$]glucose ^{3}H:^{14}C and atomic ratios	
Precursor	6.0	2:1	30	2:1	11.6	1:6	7.6	2:6
Neosaminol C	3.0	1:1	14.2	0.94:1	5.8	0.5:6	7.1	1.92:6
2-Deoxystreptamine	5.93	1.98:1	28.5	1.9:1	1.4	0.1:6		
Ribitol					13.1	0.94:5		
Neosaminol B	3.16	1.05:1	15.1	1:1	1.4	0.1:6	7.1	1.92:6

Samples of labelled neomycin B were prepared from variously labelled hexoses as shown in Table 1. A comparison of the ^{3}H:^{14}C and atomic (parenthesis) ratios of each sample of the hexose with the 4 sub-units of the antibiotic may be made along vertical columns. The details of the degradation procedures are described in the Experimental section.



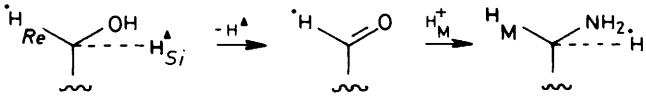
Scheme 1. *Reagents:* i, H^+; ii, Ac_2O; iii, $NaBH_4$

experiment 0.4—2.1% of the ^{14}C associated with D-glucose or glucosamine was incorporated into the two species which were separated by ion exchange column chomatography. Neomycin B was then partially hydrolysed to give its rings I and II as neamine and rings III and IV as neobiosamine B (Scheme 1). These two disaccharides were further processed to obtain the ring I of the antibiotic as neosaminol C, ring II as 2′-deoxystreptamine and ring IV as neosaminol B.

Table 3. Determination of the stereochemistry of ^{3}H at C-6 of neosaminol B

Time of incubation (min)	^{3}H:^{14}C ratios and loss of ^{3}H (%) Glycine from neosaminol B	(2*RS*)-[2-^{14}C,2-^{3}H] glycine
0	15.2 (0)	4.8 (0)
30	2.8 (81.57)	2.3 (47.9)
60	0.5 (96.7)	2.25 (46.8)

Neosaminol B (^{3}H:^{14}C = 15.1) from experiment 2 (Table 2) was degraded and the resulting glycine and serine were found to have ^{3}H:^{14}C ratios of 14 and ∞, respectively. The presence of ^{14}C in glycine and its complete absence from serine proves that the precursor was incorporated into the antibiotic intact. The doubly labelled glycine was incubated with serine hydroxymethyltransferase and at various time intervals samples of the incubation mixture was removed, processed for the preparation of benzyloxycarbonylglycine which was then subjected to the determination of radioactivity. In a control experiment, it was established that a sample of (2*RS*)-[2-^{14}C,2-^{3}H]glycine lost half of its radioactivity during a 60 min incubation period. The complete exchange of ^{3}H from glycine obtained from the labelled neosaminol B shows that in the former, the ^{3}H was in the H_{Si} position.



Scheme 2.

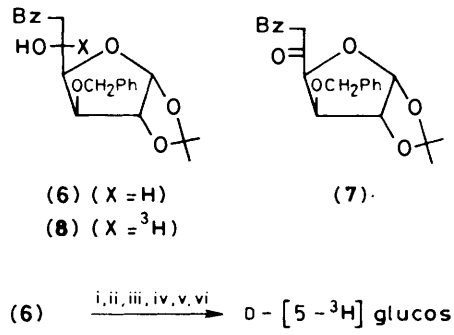
Incorporation of (6*RS*)-[6-^{3}H]*Hexoses into the Various Rings of Neomycin B.*—Table 2 gives the radiochemical data comparing the ^{3}H:^{14}C ratios of the precursors, (6*RS*)-D-[1-^{14}C,6-^{3}H]glucosamine or (6*RS*)-D-[6-^{14}C,6-^{3}H]glucose with those of the four derivatives representing the various rings of the antibiotic. The interpretation of the isotopic data obtained in this study is based on the premise that the carbon skeleton of the precursor is incorporated into the antibiotic intact and without any labilisation of the C–H bonds of the precursor due to side reactions unrelated to antibiotic biosynthesis. That these two criteria were fulfilled in the present study was respectively shown by the fact that (i) C-6 of glucose was exclusively located at C-6 of the two neosamines [2,6] (also see legend to Table 3) and (ii) (6*RS*)-D-[6-^{14}C,6-^{3}H]glucose was incorporated into the 2-deoxystreptamine ring with complete retention of its two C-6 hydrogen atoms (Table 2, expts. 1 and 2).

We have previously shown [6] that the two C-6 hydrogen atoms

of D-glucose which are retained are exclusively located at C-2 of the 2-deoxystreptamine ring and have exploited this observation to consider a novel mechanism through which the latter ring is biosynthesised from D-glucose.[6] The data in Table 2 also confirm another observation,[2] that in the formation of the neosamine C ring from either D-glucosamine or D-glucose, one of the two C-6 hydrogen atoms of the precursor is eliminated. It has been shown elsewhere[2] that the hydrogen remaining at this position occupies the H_{Si} orientation in the final product. The latter stereochemical conclusion[2] was subsequently confirmed in an independent[3] study in which it was shown that in the biosynthesis of the neosamine C ring of a related antibiotic, ribostamycin, by *S.ribosidificus*, one of the C-6 hydrogen atoms of the precursor was also present in the 6-H_{Si} position of the neosamine C ring. In another study[3] the same laboratory had established that it is the H_{Si} at C-6 of the D-glucose that is labilised in the biosynthesis. If it is assumed that the basic mechanism and stereochemistry of the aminomethylation process in the biosynthesis of the two antibiotics are similar then the cumulative results from these studies may be combined to suggest that the aminomethyl group of the neosamine C ring of the two antibiotics is produced though an oxidation-transamination process involving the intermediacy of an aldehyde and that the overall process occurs with inversion of configuration, as shown in Scheme 2.

In the present work the new finding, however, is that $^3H:^{14}C$ ratio of neosaminol B was also half that of (6*RS*)-D-[1-^{14}C,6-^{3}H]glucosamine or (6*RS*)-D-[6-^{14}C,6-^{3}H]glucose (Table 2) which shows that the formation of the neosamine B ring is achieved by the removal of one of the C-6 hydrogen atoms of D-glucose. The stereochemistry of the ^3H remaining at C-6 of neosaminol B was determined by degradation to obtain the C(5)–C(6) moiety as glycine. The incubation of the latter with serine hydroxymethyltransferase led to the almost complete loss of ^3H, thus proving that the isotopic hydrogen at C-2 of the glycine and hence at C-6 of the precursor neosamine ring was exclusively located at the H_{Si} position. These experiments taken together show that, despite stereochemical differences at C-5, the overall mechanism and stereochemistry of the formation of the C-6 aminomethyl group of the two neosamine rings are identical and that these features may be represented by the common sequence of Scheme 2.

Synthesis of D-[5-^{3}H]*Glucose and its Incorporation into Neomycin B.*—In order to investigate the mechanism though which the L-configuration of the neosamine B ring is created, we undertook a study using D-[5-^{3}H]glucose that is readily available commercially. We performed several incorporation experiments using three different batches of D-[5-^{3}H]glucose supplied by Amersham International, but when the samples of the labelled antibiotic were carried though the laborious degradations of Scheme 1, the $^3H:^{14}C$ ratio data of the products were unexpected, thus casting doubt on the regiospecificity of the ^3H label in the precursor glucose. Sodium periodate degradation of the original samples of glucose revealed that in every case the ^3H was located *not* at C-5 but predominantly at C-6.* Consequently, we were led to the development of a new method for the synthesis of D-[5-^{3}H]glucose.



(6) (X = H) (7).
(8) (X = ^{3}H)

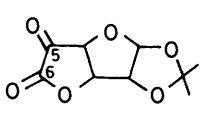
(6) $\xrightarrow{i,ii,iii,iv,v,vi}$ D-[5-^{3}H] glucose

Scheme 3. *Reagents:* i, Me_2SO–P_2O_5; ii, NaB^3H_4; iii, t.l.c.; iv, KOH; v, Pt–H_2; vi H^+

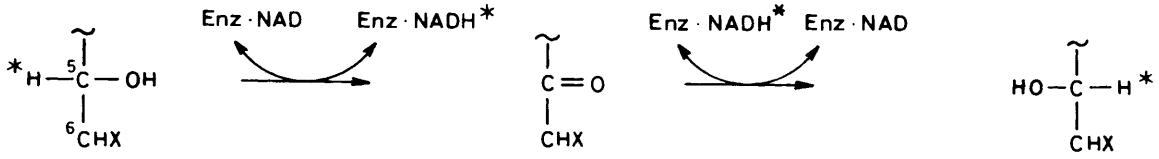
3-*O*-Benzyl-1,2-*O*-isopropylidene-D-glucofuranose[7] was selectively mono-benzoylated to give the 6-benzoate [(6) in Scheme 3] which was oxidised to the 5-oxo derivative (7). Reduction of compound (7) with NaB^2H_4, gave a mixture of two C-5 epimeric alcohols, corresponding to the configuration of L-idose and D-glucose in the ratio of 3:2 respectively. These were separated by t.l.c. D-[5-^{3}H]Glucose, obtained from compound (6) as shown in Scheme 3 was mixed with D-[U-^{14}C]-glucose and the doubly labelled samples incorporated into neomycin B, as above. The antibiotic was then hydrolysed to obtain its rings I, II, III and IV as neosaminol C, 2′-deoxystreptamine, ribitol, and neosaminol B, respectively, (Scheme 1). The similarity between the $^3H:^{14}C$ ratio of the original glucose and the neomycin B-derived ribitol (Table 2, expt. 3) proves that the precursor was incorporated into the antibiotic intact and without prior labilisation of ^3H, thus fulfilling an important prerequisite for a rational interpretation of the radiochemical data. The predominant loss of the 5-^{3}H in the formation of the 2-deoxystreptamine ring is consistent with the notion that C-5 of D-glucose is converted into the C-3 amino group of 2-deoxystreptamine *via* a carbonyl intermediate.

The Fate of the 5-^{3}H *Atom of Glucose During the Elaboration of Neosamine B and C and Consideration of the Various Mechanisms for the Epimerisation Process.*—In order to evaluate the significance of the radiochemical data on the two neosaminols (Table 2, expt. 3), we first considered the various theoretical mechanistic pathways through which sugars of D-configuration may be epimerised to produce their L-counterparts. The epimerisation may occur either by an internal redox reaction at C-5 (*cf.* Scheme 4), or *via* a transient enol generated from the tautomerism of an appropriate carbonyl intermediate (Scheme 5). The former type of mechanism operates in the interconversion of UDP-glucose and UDP-galactose and occurs through the participation of an enzyme-bound pyridine nucleotide.[8] This mechanistic principle when extended to the formation of neosamine B ring requires the complete retention of ^3H during its biosynthesis from D-[5-^{3}H]glucose. The radiochemical data of Table 2 showing almost 90% loss of ^3H from neosaminol B thus argue against the participation of the mechanism of the type shown in Scheme 4 for the creation of the epimeric centre at C-5 of the neosamine B ring. It should though be stressed that on the basis of this evidence alone a redox mechanism cannot be entirely eliminated since there is no *a priori* reason why an enzymic reaction operating through the reaction sequence of Scheme 4 must use a tightly bound co-

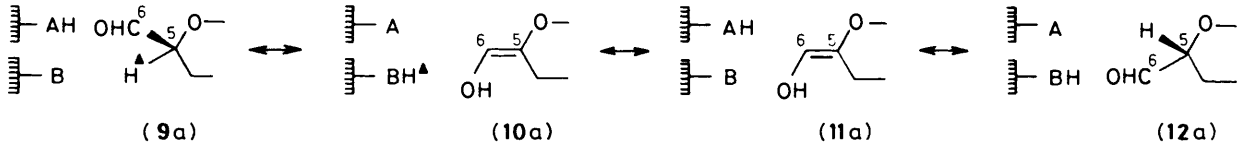
* The commercial preparation of D-[5-^{3}H]glucose is achieved[17] using the keto lactone; which is first converted to the corresponding keto acid



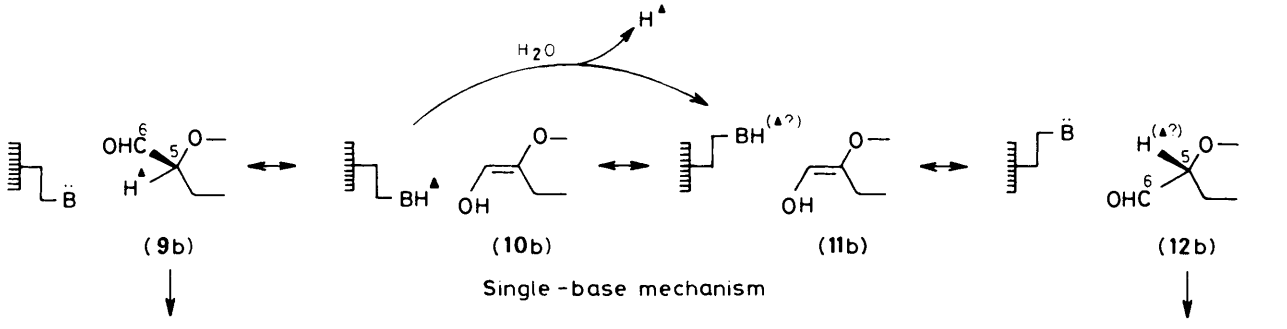
and then ^3H is introduced at C-5 by the reduction of the carbonyl group with NaB^3H_4. Relactonisation, followed by reduction with unlabelled $NaBH_4$ generates the primary hydroxy group at C-6. If at the first stage of the above sequence the lactone ring is incompletely opened, then the ^3H will be introduced predominently at C-6 as was found in the samples supplied to us by Amersham International.



Scheme 4. X = HOH, HNH_2 or O



Two-base mechanism



Scheme 5.

enzyme leading to the conversation of ^3H at the epimeric centre, nonetheless, such a process is rendered less likely because of the precedent cited. The second mechanism (Scheme 5) to rationalise the epimerisation process invokes the intermediacy of an enol formed from a 6-aldehyde of the type (**9**). The first step in the mechanism is the removal of a C-5 hydrogen to produce a planar enolic species (**10**), that upon proton addition from the side opposite to the one from which the hydrogen was originally removed, will produce an epimeric product. If the proton abstraction and additon steps occur using two different ionisable groups on the enzyme, then the overall process will be attended by the loss of the hydrogen originally resident at the centre undergoing epimerisation (see the two-base mechanism, Scheme 5). The epimerisation can also occur using a single-base mechanism in which case two possibilities exist with respect to the fate of ^3H at C-5 (see Scheme 5). If the conjugated acid of the enzyme base in the complex (**10b**) is completely shielded from the medium, then the hydrogen removed in the deprotonation reaction is faithfully transferred back to C-5. In such an event, there would be no significant loss of ^3H from C-5 of the precursor during the formation of the neosamine B ring of the antibiotic and, hence, the difference between the $^3H:^{14}C$ ratios of the [5-^3H] precursor and neosamine B will be small or negligible. In an alternative pathway, the group BH in the complex is exposed to the medium and exchanges with the surrounding water molecules faster than the rate of collapse of the enol intermediate, in which case the C-5 hydrogen atom in the product will originate from the protons of the medium and, therefore, a significant drop in the $^3H:^{14}C$ ratios between the progenitor and neosamine B would be observed. The radiochemical data (Table 2) showing the high loss of ^3H from neosaminol B, suggest that the epimeric centre at C-5 of the neosamine B ring is produced though an enolisation process involving either a single-base exchange-mediated mechanism, or the use of two different catalytic groups located on each face of the planar enol. It would appear that such mechanistic principles are also applicable to many other epimerases and racemases, namely ribulose 5-phosphate 3-epimerase,[9] methylmalonyl-CoA racemase,[10] and hydroxyproline[11] and proline racemases.[12] The fact that 45—50% of the ^3H was also lost in the incorporation of D-[5-^3H]glucose into the neosamine C ring is in accord with both the variants of the enolisation mechanism since it can be readily seen that pathway leading to the formation of the two epimeric neosamines is linked by a set of reversible reactions which could catalyse the exchange of the original C-5 hydrogen atom of the intermediate (**9**) with the protons of the medium. The amount of ^3H eventually removed from C-5 during the formation of the neosamine C ring will depend upon the rate at which the aldehyde of L-configuration (**12**; Scheme 5) is removed from the equilibrium by the amination step.

The high labilisation of the C-5 hydrogen during the incorporation of D-[5-^3H]glucose into the neosamine B ring will also be consistent with the intermediacy of an alternative enol formed *via* a 4-keto species. This possibility is, however, eliminated by the demonstration that when neomycin B was biosynthesised from D-[U-^{14}C;3,4-$^3H_2$]glucose the $^3H:^{14}C$ ratio of neosaminol B as well as C were identical to that of the precursor (Table 2, expt. 4), thus emphasising that position 4 of glucose may not be involved in the epimerisation process. In the light of the results and arguments presented above, the epimerisation step in the biosynthesis of the neosamine B may be best

rationalised by the mechanism of Scheme 5. This mechanism may be further favoured because of the economy it offers of using a C-6 aldehydic species (**9**) that, from the result obtained using (6*RS*)-D-[6-^{3}H]glucose, has already been suggested as an intermediate in the C-6 amination process.

Experimental

The laboratory chemicals were ex-stock from BDH or Koch-Light. D-[U-^{14}C]Glucose (296 mCi mmol^{-1}), (6*RS*)-[6-^{3}H]-D-glucose (33 Ci mmol^{-1}) and D-[1-^{14}C]glucosamine (54 mCi mmol^{-1}) were obtained from Amersham International p.l.c. [3,4-$^3H_2$]-D-glucose (44.6 Ci mmol^{-1}) was obtained from New England Nuclear, Dreieich, West Germany, and D-[5-^{3}H]-glucose was prepared as described below.

Nutrient agar and nutrient broth were obtained from Oxoid, London, SE1. Tryptic Soya broth, bacto-inorganic salts were obtained from Difco Laboratories, Detroit, Michigan, USA. Chemicals for scintillation counting were obtained from G & G Chemicals, South Ascot, Berks.

The neomycins producer, *Streptomyces fradiae* wild type (A.T.C.C. 10745) was obtained from the American Type Culture Collection (A.T.C.C.), C16.C.

Radiochemical Analysis.—Tritiated D-*Glucose.* Although the chomatographic method recommended by Amersham International to assess the purity of labelled glucose was found satisfactory for D-[^{14}C]glucose, this method gave misleading results with samples of (6*RS*)-D-[6-^{3}H]glucose that had been stored for a few months. A systematic study revealed that (6*RS*)-D-[6-^{3}H]glucose, which is produced as a high specific radioactivity product, undergoes extensive radiolysis during storage resulting in the formation of isomeric hexoses which co-chomatograph with D-[^{3}H]glucose. Since these hexose isomers will be expected to be discriminated by an enzyme that is normally specific for D-glucose, an enzymic method to estimate the purity of D-[^{3}H]glucose samples was developed. The method involved mixing the samples of D-[^{3}H]glucose under test with D-[^{14}C]glucose and the conversion of the doubly labelled sample using hexokinase and ATP into glucose 6-phosphate. The latter was then separated by chomatography and used for the determination of its ^{3}H:^{14}C ratio.

Labelled neomycin B and its degradation products. For the determination of the ^{3}H:^{14}C ratio of neomycin B and the degradation products derived from it, the material previously purified by ion-exchange chomatography was subjected to t.l.c. and it was ensured that the radioactivity was associated only with a single zone corresponding to the position of the expected compound. The silica gel from the region containing the radioactive material was scraped and placed in a scintillation vial. After the addition of water (1 ml) and Tritoscint [10 ml; prepared from xylene (2 l)–synperonic NXP (1 l)–2,5-diphenyloxazole (12 g)–1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (1.5 g)] the suspension was allowed to stand in the dark for 1 h and then counted in a Philips liquid scintillation counter (PW 4700) using an automatic quench correction program.

Water-insoluble compounds. For the determination of the radioactivity of compounds soluble only in organic solvents, the scintillation fluid used was Toluene–butyl PBD (10 ml) prepared from butyl PBD* (8.0 g) in toluene (1 l).

Preparation of D-[5-^{3}H]*Glucose.*—3-O-*Benzyl*-1,2-O-*isopropylidene*-D-*glucofuranose.* 3-*O*-Benzyl-1,2:5,6-di-*O*-isopropylidene-D-glucofuranose[7] (5 g) in methanol (50 ml) and 0.8% (v/v) aqueous sulphuric acid was allowed to stand overnight at room temperature. After the neutralisation of the acid by the addition of saturated potassium carbonate, the reaction mixture was extracted with three 200 ml portions of chloroform and the combined chloroform extracts were washed with water and dried (anhydrous Na_2SO_4). Removal of the solvent under reduced pressure gave the above compound as an oil (2.8 g, 63%) which in chloroform–methanol (9:1, v/v) had an R_F value of 0.6 [detected by methanol–H_2SO_4 (4:1)]. The preceding diol was further purified by conversion into its diacetyl derivative[7] m.p. 117—118 °C (lit.,[7] m.p. 119—120 °C) followed by hydrolysis.

6-O-*Benzoyl*-3-O-*benzyl*-1,2-O-*isopropylidene*-5-*oxo*-D-*gluco-furanose* (**7**). A mixture of 6-*O*-benzoyl-3-*O*-benzyl-1,2-*O*-isopropylidene-D-glucofuranose (**6**) (200 mg) prepared from the preceding diol,[7] and phosphorus pentaoxide (200 mg) in dimethyl sulphoxide (2.3 ml) was stirred for 2 h at 50 °C and then for 24 h at room temperature. The reaction mixture was carefully diluted with ice–water and then with chloroform. The organic layer was washed, dried (anhydrous Na_2SO_4) and evaporated to dryness under reduced pressure to give an oil (80 mg) which on t.l.c. analysis had an R_F value of 0.6 [benzene–ethyl acetate (4:1, v/v)].

6-O-*Benzoyl*-3-O-*benzyl*-1,2-O-*isopropylidene*-D-[5-^{3}H]-*glucofuranose* (**8**) *and the idose counterpart.* To the compound (**7**) (100 mg) in methanol (2 ml) was added NaB^3H_4 (1—2 mg, *ca.* 250 mCi mmol^{-1}) and the reaction mixture was left at room temperature for 10 min. After the addition of unlabelled $NaBH_4$ (40 mg) the mixture was left for a further 20 min and then acidified with 1M-HCl and finally the solvent was removed under reduced pressure to give an oil which was treated with 10% methanolic KOH (10 ml) at room temperature for 15 h. After the removal of methanol under reduced pressure, the residue was dissolved in chloroform (50 ml), the organic layer washed several times with water, dried (anhydrous Na_2SO_4) and evaporated under reduced pressure. The residue (20 mg, 400 × 10^6 d.p.m.) in chloroform was applied to a silica gel t.l.c. plate (20 × 10 cm) which was developed in benzene–ethyl acetate (3:2, v/v). The plate was dried and redeveloped two more times in the same solvent. The plate was scanned for the presence of radioactivity and the two peaks were obtained with R_F 0.4 and 0.3 corresponding to the derivatives of D-glucose (8 mg, 160 × 10^6 d.p.m.) and L-idose (12 mg 240 × 10^6 d.p.m.), respectively. These bands were separately scraped, and after the elution of labelled material with methanol (20 ml) the derivative of glucose was shaken over PtO_2 (20 mg) in an atmosphere of H_2 until the absorption of the gas ceased (2 h). After the removal of the catalyst, the solvent was removed under reduced pressure and the residue on t.l.c. analysis gave a single zone of radioactivity in the region corresponding to the position of the authentic 1,2-*O*-isopropylidene-D-glucofuranose, R_F 0.38 [chloroform–methanol (7:3, v/v)].

D-[5-^{3}H]*Glucose.* The preceding residue after dilution with unlabelled material (100 mg) was treated with 4M-HCl (10 ml) for 24 h. After the removal of the acid under reduced pressure, the residue was applied to a silica gel plate which was developed in chloroform–methanol (1:1, v/v), and the radioactive band in the position of D-glucose removed and eluted with methanol. After the removal of the solvent the residue was taken up in distilled water and stored at −20 °C. In order to establish the purity of the D-glucose and show the regiospecificity of ^{3}H, a sample of the preceding solution was removed and mixed with D-[U-^{14}C]glucose to give a ^{3}H:^{14}C ratio of 11.5. This material was converted to glucose 6-phosphate which had a ^{3}H:^{14}C ratio of 11:6. Another doubly labelled glucose sample with ^{3}H:^{14}C = 5.5 when oxidised with $NaIO_4$ and the C-6 isolated as formaldehyde dimedone was found to have a ^{3}H:^{14}C ratio of 0.8. This showed that less than 3% of the ^{3}H present in the

* 5-biphenyl-4-yl-2-(4-t-butylphenyl)-1,3,4-oxadiazole.

sample of glucose was located at C-6. As expected, the carbon atoms 1—5 isolated together as a sodium formate had ^3H: ^{14}C ratio of 6.2.

Ion Exchange and T.l.c. Separation of Neomycin and its Degradation Products.—Throughout the work described below, column chromatography was performed using Amberlite CG-50 NH_4^+ form. The resin was placed in a column (2.5 × 70 cm) and, before the application of the sample, washed with a volume of water that was twice that of the packed resin-bed. T.l.c. was performed using silica gel plates and unless otherwise mentioned, developed in ammonia–methanol (2:5 v/v) and the compounds detected by ninhydrin.

The incorporation of labelled precursors into neomycin B and C. Streptomyces fradiae spores were produced on bacto-inorganic salt agar and transferred to a tryptic soya broth medium (prepared according to manufacturer's instructions; 10 ml) which was incubated for 40 h 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[5] (9.0 ml) contained in a 125 ml conical flask. Ten such flasks were normally prepared and incubated together at 28 °C on a rotary shaker (150 revolutions min^{-1}) for 48 h until the cell density reached 80—90% of that expected for the stationary phase. The labelled precursor whose amount and radiochemical characteristics are shown in Table 1, in water (2 ml) was then added to the cultures and the incubation continued for an additional 12 h. After acidification to pH 4.5, the cell-mass was removed by centrifugation (20 000 r.p.m. for 10 min) and the supernatant applied to a column (2.5 × 70 cm) containing prewashed resin (0.5 ml settled-resin bed/mg of antibiotic). The resin-bed was first washed with water to remove neutral and acidic components and the antibiotic then eluted with 2M-ammonium hydroxide (100 ml). The eluant was evaporated to dryness under reduced pressure and the residue taken up in water (5 ml) and brought to pH 4.5. A portion of the solution was used to determine the amount of the antibiotic by the ribose assay[13] and usually 250 μg of neomycin B plus C was found to be produced per ml of the medium during a 60 h growth period used in the incorporation experiments. The preceding solution containing 25—30 mg of the mixture of antibiotics was applied to the resin (bed volume 15 ml) and the elution was performed using a linear gradient developed from 1.52 l each of 0.15M- and 0.27M-NH_4OH. Fractions of 18 ml were collected at a flow rate of 100 ml h^{-1}, when neomycin C and B eluted between 2 160—2 706 and 3 330—3 870 ml, respectively. The two peaks were separately pooled and evaporated to dryness. The two components were found by t.l.c. to have R_F 0.23 and 0.18, respectively.

Degradation of Neomycin B to Neamine and Neobiosamine B.—Based upon the method of Falker,[14] neomycin B (25 mg) was dissolved in 1.5M-HCl (20 ml) and the mixture heated at 80 °C for 4.5 h. After the removal of the solvent under reduced pressure the residue was analysed by t.l.c., when three components having R_F values of 0.36 (neamine), 0.45 (neobiosamine B), and 0.75 (unknown impurities) were detected. The preceding residue in water (20 ml) was acidified, applied to the resin (settled volume 15 ml). The mixture was separated using a gradient generated from 2 l each of 0.1M- and 0.25M-ammoniam hydroxide solutions at a flow rate of 300 ml h^{-1}. The fractions (18 ml each) were collected and monitored by t.l.c. Two major peaks were found in volumes 270—810 ml (sample I) and 1 080—1 530 (sample II). Sample I gave two spots at R_F 0.5 (neobiosamine B, 10 mg) and R_F, 0.76 (unknown impurities), whilst sample II showed one spot at R_F, 0.36 (neamine; 12 mg).

Degradation of Neamine.—^{14}Dowex 1-X_2 (CO_3^-) (15 ml) was added to neamine (12 mg) in water (2 ml), and the mixture was stirred for 1 min. Acetic anhydride (0.5 ml) was then added dropwise with stirring, and the mixture was kept at room temperature for 45 min with occasional stirring. The reaction mixture was filtered and the resin washed five times with water. The filtrate was treated with Dowex 50 W-8 (H^+) (3 ml), with stirring for 5 min. The resin was filtered and washed five times with water. The combined filtrate was evaporated to dryness to give tetra-*N*-acetylneamine (17 mg, 94.8%) as a pale yellow solid. Analysis by t.l.c. and visualisation with starch–iodine, gave one spot at R_F 0.8. Tetra-*N*-acetylneamine (15 ml) was dissolved in 4M-HCl (10 ml) and heated to reflux for 2 h. After the solution had cooled, the solvent was evaporated to dryness, and the residue was dissolved in water (10 ml) and the solution was again evaporated to dryness (this was repeated several times).

The preceding residue which contained 2-deoxystreptamine, neosamine C and unchanged neamine in water (2 ml) was brought to pH 8.0 with dilute sodium hydroxide solution and treated with $NaBH_4$ (10 mg). The reaction mixture was stirred at room temperature for further half an hour, then acidified with dilute HCl to pH *ca.* 4.5 and applied to a column of the resin. The elution was performed with a linear gradient, generated from 2 l each of 0.05M- and 0.15M-aqueous ammonium hydroxide, at a flow rate of 300 ml h^{-1} at room temperature. Fractions of 18 ml were collected and anlysed by t.l.c. The fraction between 1 080 and 1 530 ml containing 2-deoxystreptamine were combined and evaporated to dryness under reduced pressure to give a residue 4.8 mg (R_F 0.41). The resin bed was then eluted with a second linear gradient generated from 2 l each of 0.15M- and 1.0M-aqueous ammonium hydroxide under the conditions described above. Volumes 1 980—2 430 ml on evaporation, gave unchanged neamine (1.8 mg, 15%), R_F 0.33, and 3 240—3 870 ml gave neosaminol C (5.2 mg) which on t.l.c. analysis gave one spot at R_F 0.3.

Degradation of Labelled Neobiosamine B to Obtain Ribitol and Neosaminol B.—Neobiosamine B (8 mg) was converted to *N,N*-diacetylneobiosamine (R_F 0.70), using the method described above for the preparation of tetra-acetylneamine, mixed with D-ribose (10 mg), and then reduced with sodium borohydride as above. After the usual work-up, the residue was taken up in 90% aqueous trifluoroacetic acid (3 ml) and the mixture was allowed to stand at 60 °C for 2 h, then evaporated under reduced pressure and the residue divided into two parts. The first half of the mixture was applied to a 20 × 20 cm t.l.c. plate. The plate was developed in ammonia–methanol (5:2, v/v), dried, and the band corresponding to the position of ribitol was separated, rechomatographed and the material used for radiochemical analysis. The second half of the residue in 2M-HCl (10 ml) was refluxed for 2 h and after the removal of the solvent, the residue was adjusted to pH 8.0 reduced by $NaBH_4$ and applied to the resin (5 ml packed volume). Elution using a linear gradient developed from 1 l each at 0.15 and 1M-NH_4OH gave neosaminol B in fractions eluting between 1 080—1 710 ml. After the removal of the solvent, the residue on t.l.c. analysis gave one spot at R_F 0.25.

Degradation of N,N′*-Diacetylneosaminols to Obtain Serine and Glycine.*—This was carried out by a modification of the method of Argoudelis.[15] To *N,N′*-diacetylenosaminol (4 mg) in water (0.5 ml) was added saturated aqueous sodium hydrogen carbonate (0.55 ml), 0.004M-potassium permanganate (1.2 ml), and 0.08M-sodium metaperiodate (2.5 ml), at 20 °C. After 22 h, a solution of lead acetate (0.5M) was then added dropwise until no further precipitation was observed. After the removal of the precipitate, and the unchanged lead acetate by hydrogen sulphide, the filtrate was dried *in vacuo*. The residue was refluxed with 1M-HCl (10 ml) for 3.5 h, then the solvent was removed

under reduced pressure and the residue dissolved in water (1 ml). T.l.c. using cellulose, developed with pyridine–water (18.5:1, v/v) showed two ninhydrin positive spots, one corresponding to glycine R_F 0.5 and the other to serine R_F 0.4.

Equilibration of the H_{Si} of Glycine with Serine Hydroxymethyl Transferase.—This was achieved using an exchange reaction catalysed by serine hydroxymethyltransferase,[16] as follows. In a final volume of 1.0 ml the incubation mixture contained potassium phosphate buffer, pH 7.1 (80 μmol), pyridoxal phosphate* 0.03 μmol; tetrahydrofolic acid, (2.0 μmol); serine hydroxymethyltransferase, (0.6 units); and glycine, (10 μmol) (labelled with ^3H and ^{14}C). The reaction was started by the addition of glycine, the tubes flushed with nitrogen, sealed, and incubated at 37 °C. At zero time, 30 min and 1 h (see Table 3), 300 μl aliquots of the reaction mixture was removed and after the addition of carrier glycine (100 mg) processed for the preparation of benzyloxycarbonylglycine.

Acknowledgements

S. G. thanks the Arab Republic of Egypt for a Research Scholarship.

* = 4-Formyl-5-hydroxy-4-methyl-3-pyridylmethylphosphoric acid.

References

1 (*a*) K. L. Rinehart Jr. and R. M. Strashane, *J. Antibiot.*, 1976, **29**, 319; (*b*) K. L. Rinehart Jr., J. M. Malik, R. S. Nystrom, R. M. Stroshane, S. T. Truitt, M. Targuchi, J. P. Rolls, W. J. Haak, and B. A. Ruff, *J. Am. Chem. Soc.*, 1974, **96**, 2263.
2 W. Al-Feel, M. J. S. Ewad, C. J. Herbert, and M. Akhtar, *J. Chem. Soc., Chem. Commun.*, 1983, 18.
3 K. Kakinuma, Y. Ogawa, T. Sasuki, H. Seto, and N. Otake, *J. Am. Chem. Soc.*, 1981, **103**, 5614; K. Kakinuma and S. Yamaya, *J. Antibiot.*, 1983, **36**, 749.
4 J. L. Foght, Ph.D Thesis, University of Illinois, Urbana, Illinois, USA, 1963.
5 M. K. Majumdar and S. K. Majumdar, *Biochem. J.*, 1971, **122**, 397.
6 M. J. Ewad, W. Al-Feel, and M. Akhtar, *J. Chem. Soc., Chem. Commun.*, 1983, 20.
7 R. L. Whistler and W. C. Lake, 'Methods in Carbohydrate Chemistry,' eds. R. L. Wistler and J. N. Be. Miller, Academic Press, New York and London, 1972, vol 6, p. 286.
8 O. Gabriel, H. M. Kalckar, and R. A. Darrow, in 'Subunit Enzymes: Biochemistry and Function,' ed. K. Ebner, Dekker, New York, 1975, p. 85.
9 L. Davis, N. Lee, and L. Glaser, *J. Biol. Chem.*, 1977, **247**, 5862.
10 P. Overath, G. M. Kellerman, F. Lynen, H. P. Fritz, and H. J. Keller, *Biochem. Z.*, 1962, **335**, 500.
11 E. Adams and I. L. Norton, *J. Biol. Chem.*, 1964, **239**, 1525.
12 G. J. Cardinale, and R. H. Abeles, *Biochemistry*, 1968, **7**, 3970.
13 J. D. Dutcher, N. Hosanky, and J. D. Sherman, *Antibiot. Chemother.*, (Washington D.C.) 1953, **3**, 534.
14 F. C. Falkner, Ph.D. Thesis, University of Illinois, Urbana, Illinois, USA, 1969.
15 A. D. Argoudelis, Ph.D. Thesis, University of Illinois, Urbana, Illinois, USA, 1959.
16 P. M. Jordan and M. Akhtar, *Biochem. J.*, 1970, **116**, 277; G. F. Barnard and M. Akhtar, *Eur. J. Biochem.*, 1979, **99**, 593.
17 W. Mackie and A. S. Perlin, *Can. J. Chem.*, 1965, **43**, 2921.

Received 13*th September* 1985; *Paper* 5/1572