

## The Role of the Pseudo-Disaccharide Neamine as an Intermediate in the Biosynthesis of Neomycin

By CEDRIC J. PEARCE, JOHN E. G. BARNETT, CHRISTOPHER ANTHONY  
and MUHAMMAD AKHTAR

*Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.*

and STEPHAN D. GERO

*Institut de Chimie des Substances Naturelles, C.N.R.S., 91190 Gif-sur-Yvette, France*

(Received 1 June 1976)

By using wild-type and deoxystreptamine-negative mutants of *Streptomyces fradiae* grown in media containing [6-<sup>3</sup>H]glucose or [U-<sup>14</sup>C]glucose, and by subsequent hydrolysis of the labelled neomycin produced, neamines labelled with <sup>3</sup>H in both rings I and II, but with <sup>14</sup>C in ring I only, were prepared. A mixture of these two forms of neamine was converted by deoxystreptamine-negative *Streptomyces rimosus* forma *paromomycinus* into neomycin (not paromomycin) with a 30% yield. The <sup>3</sup>H:<sup>14</sup>C ratio in this neomycin was the same as that measured in neamine produced by hydrolysis of the neomycin, and in unused neamine reisolated from the incubation medium. The <sup>3</sup>H:<sup>14</sup>C ratio in the neomycin was not affected by the presence of unlabelled deoxystreptamine during the incubation. The radioactivity in the neomycin was associated with rings I and II only. It is concluded that the added neamine is incorporated into antibiotic intact, without initial hydrolysis, and that the probable first step in the subunit assembly of neomycin is the formation of neamine.

The antibiotic neomycin is a pseudo-tetrasaccharide consisting of four carbocyclic rings joined by glycosidic linkages (Fig. 1). Although several features of the biosynthesis of neomycin have received attention (Falkner, 1969; Rinehart *et al.*, 1974; Shier *et al.*, 1974), the sequence in which the various rings of the antibiotic are assembled to produce the final molecule is not yet known. Mutants of *Streptomyces fradiae* and *Streptomyces rimosus* forma *paromomycinus* have been discovered (Shier *et al.*, 1969, 1972) which synthesize neomycin or the related antibiotic paromomycin, only when supplemented with 2-deoxystreptamine.

The present paper describes a study of the utilization of potential precursors of these antibiotics in an attempt to elucidate the sequence of assembly.

### Experimental

#### Materials

Standard laboratory chemicals used were of reagent grade and were from BDH Chemicals, Poole, Dorset, U.K. D-[6-<sup>3</sup>H<sub>2</sub>]Glucose and D-[U-<sup>14</sup>C]glucose were from The Radiochemical Centre, Amersham, Bucks., U.K. Diagnostic Sensitivity Test agar base (DST agar), nutrient agar and nutrient broth were from Oxoid, London S.E.1, U.K. Neomycin sulphate was from Boehringer Corp. (London), London W.5, U.K.

Neomycin B sulphate was prepared from neomycin sulphate by using paper chromatography developed with 2-methylpropan-2-ol/butan-2-one/methanol/6.5M-NH<sub>3</sub> solution (3:16:1:6, by vol.). Neamine was prepared from neomycin sulphate by the method of Dutcher & Donin (1952).

The scintillation fluid contained: xylene, 2 litres; Synperonic NXP (obtained from The Cargo Fleet Chemical Co., Eaglescliffe Industrial Estate, Stockton, U.K.), 1 litre; 2,5-diphenyloxazole, 12g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.9g.

#### Organisms and culture methods

*Streptomyces fradiae* (A.T.C.C. 10745) and the deoxystreptamine-negative mutant of this organism (A.T.C.C. 21401) were grown in either the liquid seed medium or the liquid complex medium of Sebek (1955). The deoxystreptamine-negative mutant of *S. rimosus* forma *paromomycinus* (A.T.C.C. 21484) was grown in liquid medium of the following composition: glucose, 1%; soya-bean meal, 1%; casein hydrolysate, 0.25%; CaCO<sub>3</sub>, 0.5%; NaCl, 0.5%; NH<sub>4</sub>Cl, 0.167%; made up in water with the final pH adjusted with NaOH to 7.5 and sterilized in an autoclave at 121°C for 15 min.

Cultures in Erlenmeyer flasks were incubated at 30°C on a rotary shaker. *Escherichia coli* Bristol strain was used for the antibiotic assay.

### Synthesis of [ring I-<sup>3</sup>H, ring II-<sup>3</sup>H]neamine

*S. fradiae* (wild-type) (A.T.C.C. 10745) was grown in Sebek (1955) seed medium for 3 days. Then 2.5 ml of this was added to 50 ml of Sebek (1955) complex medium in a 500 ml Erlenmeyer flask. After 3 days' incubation, 50  $\mu$ Ci of D-[6-<sup>3</sup>H]glucose was added to the culture, and after a further 2 days, when antibiotic production was maximal, neomycin generally labelled in rings I to IV was isolated. Incorporation of <sup>3</sup>H into neomycin was 3–4%.

[ring I-<sup>3</sup>H, ring II-<sup>3</sup>H]Neamine was prepared from the generally labelled [ring I-<sup>3</sup>H, ring II-<sup>3</sup>H, ring III-<sup>3</sup>H, ring IV-<sup>3</sup>H]neomycin by the method of Dutcher & Donin (1952), which involves methanolysis in methanolic HCl. The reaction mixture was concentrated under vacuum and the residue applied to Whatman 3MM chromatography paper. This was developed with methanol/aq. NH<sub>3</sub> (sp.gr. 0.88) (4:1, v/v). Neamine was eluted with water. By the method of synthesis, the neamine was <sup>3</sup>H-labelled in both rings I and II and is designated [ring I-<sup>3</sup>H, ring II-<sup>3</sup>H]-neamine.

### Synthesis of [ring I-<sup>14</sup>C]neamine

The deoxystreptamine-negative mutant (A.T.C.C. 21401) of the neomycin-producing organism was grown in Sebek (1955) seed medium for 3 days. Then 10 ml of this culture was transferred to a 2-litre Erlenmeyer flask, containing 200 ml of Sebek (1955) complex medium. After 3 days' incubation a solution containing 50 mg of deoxystreptamine, which was sterilized for 15 min at 15 lb/in<sup>2</sup> (103 kPa) in a pressure cooker, and 50  $\mu$ Ci of D-[U-<sup>14</sup>C]glucose was added to the culture. This was incubated for a further 2 days and the neomycin extracted.

Neamine was prepared from the neomycin as described above. By the method of synthesis this species of neamine is considered to contain <sup>14</sup>C only in ring I, and is designated [ring I-<sup>14</sup>C]-neamine.

### Incorporation of neamine into an antibiotic

The deoxystreptamine-negative *S. rimosus* forma *paromomycinus* (A.T.C.C. 21484) was grown in liquid medium for 3 days. Then 2.5 ml of this culture was used to inoculate 50 ml of the same medium in a 500 ml Erlenmeyer flask to which was added 2.5 mg of neamine.

Antibiotic production was optimal after 5 days' incubation and it was isolated by using the cation-exchange-resin technique described below.

### Antibiotic extraction and purification

Neomycins were extracted from centrifuged (30 min at 30000g) broth cultures by adsorption of the supernatant fluid (50 ml) on to an Amberlite

IRC 50 (NH<sub>4</sub><sup>+</sup> form) column (10 cm  $\times$  2.5 cm). This was washed with water and the antibiotic eluted with 2M-NH<sub>3</sub>. The neomycin in the eluates was concentrated under vacuum and further purified by paper chromatography.

The extracts were applied to Whatman 3MM paper and developed with methanol/aq. NH<sub>3</sub> (sp.gr. 0.88) (4:1, v/v) or, in the case of neamine-incorporation experiments, with 2-methylpropan-2-ol/butan-2-one/6.5M-NH<sub>3</sub>/methanol (3:16:6:1, by vol.) (Majumdar & Majumdar, 1967).

Antibiotics were located by using the KI/starch method of Pan & Dutcher (1956). Antibacterial activity was detected by using a bioautogram in which a strip of the chromatogram was placed on the surface of an *E. coli*-seeded nutrient-agar plate, which was incubated overnight at 37°C and then examined for inhibition of growth around the strip. Antibiotics were eluted from the paper chromatogram with water.

### Antibiotic assays

*E. coli* Bristol strain was grown overnight at 37°C in nutrient broth. A 1% inoculum of this was added to molten DST agar and plates were poured. Sterile paper discs were placed on the surface of the plate and 25  $\mu$ l of the solution of antibiotic to be assayed was applied to the disc. This was incubated at 37°C overnight and the zone of growth inhibition measured. A plot of zone of inhibition against log (concentration of antibiotics) gave a straight line.

When the concentration of antibiotic was less than 50  $\mu$ g/ml, wells were cut into the agar plate, with a cork-borer, and 0.1 ml of the solution to be assayed was added. These were treated in the same way as plates used in the disc technique.

Antibiotic standards were prepared in 0.1M-potassium phosphate buffer, pH 7.9.

### Results

The neomycins differ from the paromomycins only at C-6 of ring I, which has an amino group in the former and a hydroxyl group in the latter (Fig. 1).

Initial tests showed that neamine was not incorporated into either antibiotic by a deoxystreptamine-negative mutant of *S. fradiae*. However, when a 2-deoxystreptamine-negative mutant of the paromomycin-producing organism *Streptomyces rimosus* forma *paromomycinus* was incubated for 5 days in liquid medium containing 50  $\mu$ g of neamine/ml, the antibiotic activity of the broth was approximately equivalent to 20  $\mu$ g of neomycin/ml or 40  $\mu$ g of paromomycin/ml. The antibiotic activity was isolated from the incubation medium by using ion-exchange resin and further purified by paper chromatography with two different solvent systems. The antibacterial activities were present in two areas,

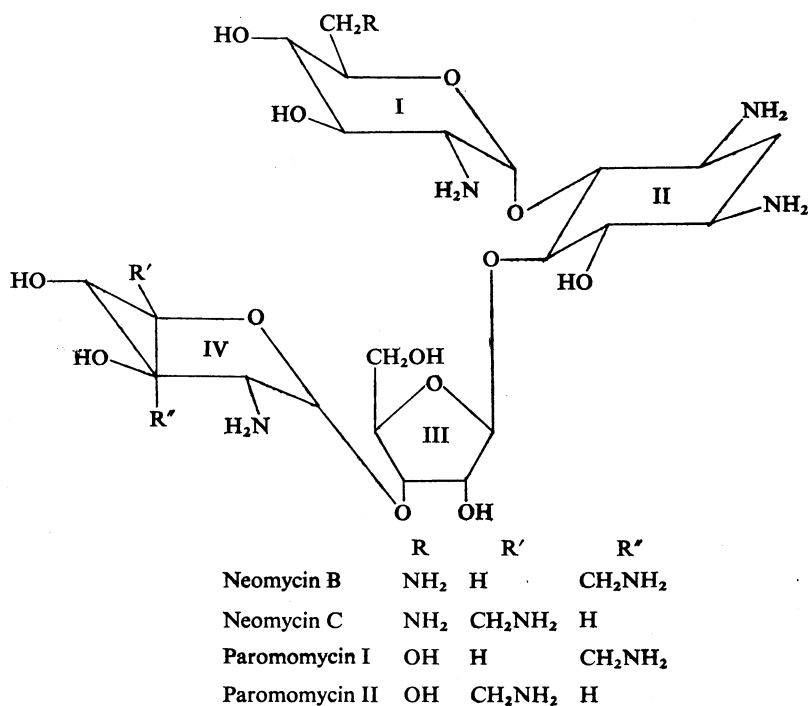


Fig. 1. Structure of neomycins and paromomycins

Table 1. Labelling of neomycin from [<sup>3</sup>H,<sup>14</sup>C]neamine

[ring I-<sup>14</sup>C, ring II-<sup>3</sup>H]Neamine was incubated with the deoxystreptamine-negative mutant of the paromomycin-producing organism. Neomycins and unchanged neamine were isolated from the culture by using Amberlite IRC 50 and purified on Whatman 3MM paper in the solvent system 2-methylpropan-2-ol/butan-2-one/methanol/6.5M-NH<sub>3</sub> solution (3:16:1:6, by vol.). For the determination of specific radioactivities, neomycin and neamine were assayed by the procedure described in the Experimental section; purified neomycin B was used as standard.

Compound isolated	Radioactivity (d.p.m.)		<sup>3</sup> H: <sup>14</sup> C ratio	Specific radioactivity (d.p.m./μmol)	
	<sup>3</sup> H	<sup>14</sup> C		<sup>14</sup> C	<sup>3</sup> H
Neamine	9290	510	18.2	490	8930
Neomycin B	4790	300	16	520	8300
Neomycin C	2220	125	17.7	—	—

which corresponded to the positions of neomycins B and C (for structures see Fig. 1). It should be noted that the amount of antibiotic produced when neamine was used as precursor was the same as was obtained with deoxystreptamine; this indicates

that the incorporation of neamine into neomycin is not by a minor alternative pathway.

The possibility was considered that antibacterial activity in these regions was due to an antibiotic, other than neomycin, formed by the hydrolysis of the added neamine and the subsequent incorporation of the deoxystreptamine moiety into paromomycin or a related compound. This possibility was excluded by repeating the experiment with [ring I-<sup>14</sup>C, ring II-<sup>3</sup>H]neamine.

The double-labelled neamine was incubated with the 2-deoxystreptamine-negative mutant of the paromomycin-producing organism for 5 days in liquid culture, and at the end of this period the antibiotics were extracted from the broth and separated by using paper chromatography. The areas corresponding to neamine, neomycin B and neomycin C were eluted and the radioactivity and antibiotic activity determined in each sample. The results in Table 1 show that neamine, neomycin B and neomycin C all have similar <sup>3</sup>H:<sup>14</sup>C ratios.

In another experiment antibiotic was biosynthesized from a mixture containing equal amounts of the double-labelled neamine and non-radioactive 2-deoxystreptamine. Once again the <sup>3</sup>H:<sup>14</sup>C ratios of the precursor neamine and the product, neomycin, were almost identical (Table 2).

Table 2. Labelling of neomycin from [ $^{14}\text{C}$ , $^3\text{H}$ ]neamine in the presence of deoxystreptamine

[ring I- $^{14}\text{C}$ , $^3\text{H}$ , ring II- $^3\text{H}$ ]Neamine and non-radioactive deoxystreptamine were incubated with the deoxystreptamine-negative mutant of the paromomycin-producing organism. Neomycins and unchanged neamine were isolated from the culture by using Amberlite IRC 50 resin and purified further on Whatman 3MM paper in the solvent system 2-methylpropan-2-ol/butan-2-one/methanol/6.5M-NH<sub>3</sub> solution (3:16:1:6, by vol.).

Compound isolated	Radioactivity (d.p.m.)		$^3\text{H}:$ $^{14}\text{C}$ ratio
	$^3\text{H}$	$^{14}\text{C}$	
Neamine	7290	530	14
Neomycin B	7290	620	16
Neomycin C	3160	270	12

Table 3. Radioactivity associated with aminoglycosides during a synthesis and degradation cycle

[ring I- $^{14}\text{C}$ , $^3\text{H}$ , ring II- $^3\text{H}$ ]Neamine was incubated in the presence of the deoxystreptamine-negative paromomycin-producing organism. The neomycin that was produced during this incubation and the unchanged neamine were isolated. The neomycin was hydrolysed in methanolic HCl to give neamine, which was purified by using paper chromatography; the solvent was methanol/aq. NH<sub>3</sub> (sp.gr. 0.88) (4:1, v/v). The Table shows the  $^3\text{H}:$  $^{14}\text{C}$  ratio of neamines and neomycin during the synthesis and degradation cycle.

Compound isolated	Radioactivity (d.p.m.)		$^3\text{H}:$ $^{14}\text{C}$ ratio
	$^3\text{H}$	$^{14}\text{C}$	
Neamine isolated from culture	4560	3820	1.19
Neomycin isolated from culture	1520	1400	1.08
Neamine produced from the hydrolysis of isolated neomycin	4600	3900	1.18

Finally, the double-labelled neamine was incubated with the deoxystreptamine-negative mutant, and the resultant antibiotic isolated and purified. A sample of the latter was subject to methanolysis followed by chromatography. The majority of the  $^{14}\text{C}$  and  $^3\text{H}$  isolated from the chromatogram was associated with neamine (Fig. 2). Also, during this biosynthesis and degradation sequence, no change in the  $^3\text{H}:$  $^{14}\text{C}$  ratio of the precursor neamine and that obtained from the degradation of neomycin was observed (Table 3).

## Discussion

The ability of deoxystreptamine-negative mutants of aminoglycoside-producing *Streptomyces* to incorporate added aminocyclitols suggests that in the normal biosynthetic sequence deoxystreptamine is formed first as the free cyclitol and that the other

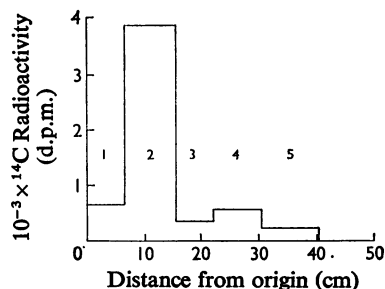


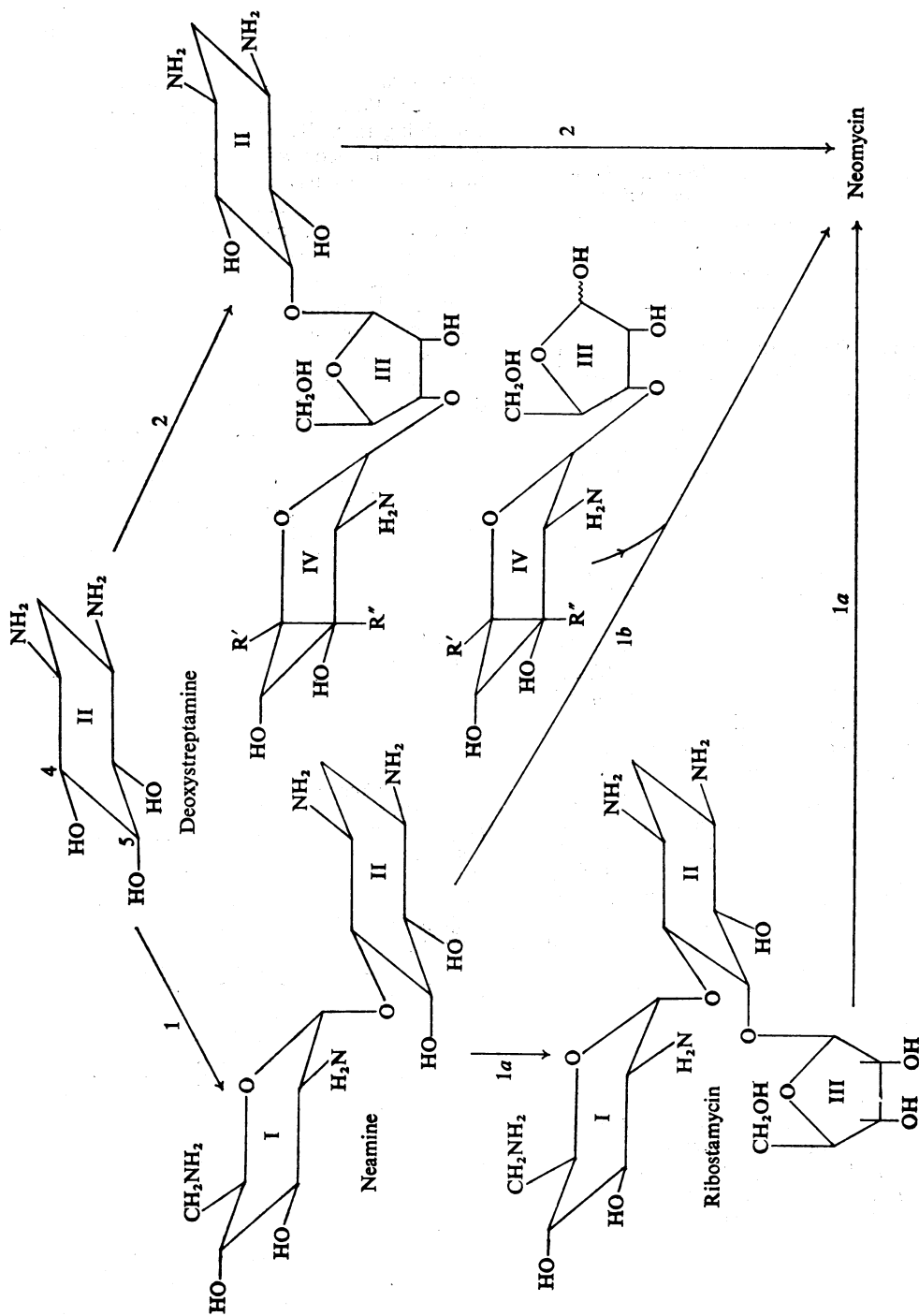
Fig. 2. Distribution of radioactivity in neomycin biosynthesized from radioactive neamine

The neomycin, which was biosynthesized as described in Table 3, was hydrolysed in methanolic HCl and the lysate subjected to paper chromatography in the solvent methanol/NH<sub>3</sub> (sp.gr. 0.88) (4:1, v/v). The chromatogram was divided into five sections and the radioactivity determined in each section. The histogram demonstrates the radioactivity (only  $^{14}\text{C}$  is shown) associated with each section. Section 2 corresponds to neamine (rings I and II) and section 4 to methyl neobiosaminides (methyl glycosides of rings III and IV).

sugars are added to it. This conclusion might also be reached on theoretical grounds, because it seems probable that the aminocyclitol will act as an acceptor for normal glycosylation reactions. The biosynthesis of neomycin from deoxystreptamine may then involve an initial glycosylation at either the C-4 or the C-5 hydroxyl groups, as shown in Scheme 1. The initial product of glycosylation at the C-4 hydroxyl group is neamine, and at C-5 is either the pseudo-trisaccharide containing rings II, III and IV or the pseudo-disaccharide containing rings II and III.

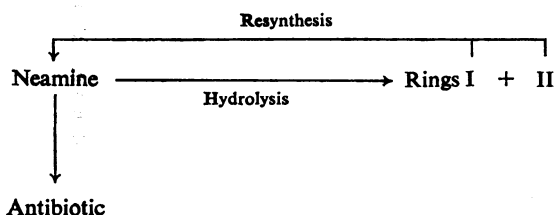
Experiments supporting both routes 1 and 2 of Scheme 1 have been described in the literature. Thus the participation of route 1 is implicated by the isolation of neamine from neomycin-producing cultures (Peck *et al.*, 1949; Perlman & O'Brien, 1953), and of route 2 by the isolation of a pseudo-trisaccharide, containing rings II, III and IV of neomycin, from a commercial sample of neomycin (Claes *et al.*, 1974).

Previous attempts to incorporate neamine (Scheme 1) into neomycin by a deoxystreptamine-negative mutant were unsuccessful (Shier *et al.*, 1974), although both neamine and 3',4'-dideoxynamine have been incorporated into ribostamycin by a deoxystreptamine-negative mutant of *Streptomyces ribosidificus* (Kojima & Satoh, 1973). We have now shown that neamine can be incorporated into neomycin, in up to a 30% yield, by a deoxystreptamine-negative mutant of *S. rimosus* forma *paromomycinus*, which produces paromomycin when grown in the presence of



Scheme 1. Biosynthesis of neomycin from preformed subunits

In pathways 1a and 1b the addition of ring I to the 4-hydroxyl group of ring II is assumed to be the initial reaction, whereas in pathway 2 the addition of either ring III or rings III plus IV to the 5-hydroxyl group is assumed to occur first.



Scheme 2. Alternative explanation for the observed results involving a hydrolysis-resynthesis mechanism

In this scheme the neamine incorporated into the antibiotic is not the compound originally added to the medium, but that formed by the combination of rings I and II.

deoxystreptamine. The formation of neomycin rather than paromomycin by this organism strongly suggests that the neamine is incorporated intact.

If the antibiotic produced from neamine in our experiments was a result of neamine being hydrolysed and the released 2-deoxystreptamine being incorporated into paromomycin, the loss of the  $^{14}\text{C}, ^3\text{H}$ -labelled ring I from [ring I- $^{14}\text{C}, ^3\text{H}$ , ring II- $^3\text{H}$ ]-neamine and its replacement with the natural subunit, 2-amino-2-deoxyglucose, would result in the production of an antibiotic containing no  $^{14}\text{C}$ . The  $^3\text{H}:^{14}\text{C}$  ratio of the antibiotic would therefore approach infinity. However, the results in Table 1 show that the  $^3\text{H}:^{14}\text{C}$  ratio of neamine and isolated antibiotic was the same, indicating that precursor neamine is incorporated intact into neomycin.

The possibility that the species of neamine incorporated into neomycin had been formed by the hydrolysis-resynthesis cycle shown in Scheme 2 was eliminated by growing the deoxystreptamine-negative mutant of the paromomycin-producing organism in the presence of [ring I- $^{14}\text{C}, ^3\text{H}$ , ring II- $^3\text{H}$ ]-neamine and non-radioactive deoxystreptamine. If a synthetic pathway involved hydrolysis of the neamine, then the deoxy[ $^3\text{H}$ ]streptamine produced would be diluted with non-radioactive deoxystreptamine. Consequently, the  $^3\text{H}:^{14}\text{C}$  ratio of the antibiotic produced under these circumstances should decrease. However, the results in Table 2 show that the  $^3\text{H}:^{14}\text{C}$  ratios of the original neamine and the biosynthesized neomycin were identical, thus suggesting that the neamine incorporated into antibiotic is the original species added to the culture.

The observation that neomycin synthesized from [ring I- $^{14}\text{C}, ^3\text{H}$ , ring II- $^3\text{H}$ ]-neamine contains radioactivity associated with the neamine part of the molecule only (Table 3) is also consistent with our conclusions that added neamine is incorporated intact into neomycin.

Two plausible pathways exist for the conversion of neamine into neomycin (1a and 1b of Scheme 1). Support for the pathway 1a, involving a stepwise

addition of rings III and IV to neamine, comes from the fact that a pseudo-trisaccharide, ribostamycin (Scheme 1), exists in Nature and has been isolated from cultures of *S. ribosidificus* (Akita *et al.*, 1970; Shomura *et al.*, 1970). However, on the basis of some indirect results, it has been hypothesized (Falkner, 1969) that neobiosamine, a disaccharide containing rings III and IV (Scheme 1), may be a precursor in neomycin biosynthesis; this suggests that pathway 1b may occur. Thus, although pathway 2 in Scheme I has been eliminated by this work, an unambiguous choice between pathways 1a and 1b in this Scheme is not at present possible.

Initial formation of a neamine-like pseudo-disaccharide may be a common step in the production of all deoxystreptamine antibiotics, because a pathway similar to that proposed for the neomycins (glycosylated at C-4 and C-5) has been demonstrated for the subunit assembly of sisomicin and gentamicin (deoxystreptamine antibiotics glycosylated at C-4 and C-6) (Testa & Tilley, 1975, 1976).

The demonstration in the present work that a deoxystreptamine-negative mutant of a paromomycin-producing organism can incorporate a pseudo-disaccharide into neomycin encourages further investigation into the incorporation of analogues of neamine for the production of new antibiotics.

We thank the Science Research Council and Labaz, Brussels, for financial support.

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