Biosynthesis of Streptomycin

III. ORIGIN OF THE CARBON ATOMS OF STREPTOSE*

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SUMMARY

The pathway of synthesis by Streptomyces griseus of the streptose moiety of streptomycin from D-glucose has been studied. L-Serine-3-14C, D-glucose-1-14C, D-glucose-2-14C, D-glucose-3,4-14C, and D-glucose-6-14C were given to streptomycin-producing cultures. A 40 to 46% incorporation of 14C into the streptose moiety of the isolated streptomycin was found. Procedures for the total degradation of streptose were developed and utilized to determine the 14C-label pattern in this moiety. Examination of the isotope distribution data for the 6 carbons of streptose after administration of the above labeled compounds to the organism shows that streptose arises from a carbon-carbon rearrangement at carbon atoms 3 and 4 of glucose to form the branched sugar, streptose.

Streptomycin, an antibiotic produced by Streptomyces griseus, is composed of three moieties: streptidine, streptose, and N-methyl-L-glucosamine, joined by glycosidic bonds. Streptose, the central moiety, is a C-3-formyl derivative of 5-deoxy-L-lyxose (Fig. 1).

In 1955, Hunter and Hockenhull (1) showed that D-glucose is a precursor of streptose. These investigators gave uniformly labeled D-glucose-14C to cultures of S. griseus. Labeled streptomycin was isolated and degraded. Isotope incorporated into the streptidine and N-methyl-L-glucosamine moieties accounted for approximately two-thirds of the radioactivity of the streptomycin. Streptose is one of the few branched sugars occurring in nature, and, therefore, the mechanism of its formation is intriguing. Investigation of its biosynthesis has been difficult because of the marked lability of this moiety in streptomycin when the antibiotic is subjected to chemical manipulation.

Recently, Candy, Blumson, and Baddiley (2) gave D-glucose-14C and D-glucose-6-14C to cultures of S. griseus. The streptomycin synthesized was separated, and the streptose was isolated as maltol, an alkaline degradation product of the antibiotic which arises by an undefined reaction mechanism. The maltol was partially degraded by chemical means. From examination of the 14C distribution in the maltol, these investigators concluded that their data suggested a mechanism of streptose biosynthesis involving a carbon-carbon rearrangement of glucose to form streptose.

The purpose of this report is to show that streptose arises from an intramolecular rearrangement involving carbon atoms 3 and 4 of D-glucose to yield the branched sugar, streptose.

EXPERIMENTAL PROCEDURE

Culture Techniques

S. griseus1 was grown on a medium containing 1.0% mannitol, 1.5% L-proline, 0.5% NaCl, 0.2% K2HPO4, 0.1% MgSO4·7H2O

1 The culture of S. griseus was the kind gift of Charles Pfizer Company, Inc.
0.003% CaCl₂, 2H₂O, 0.002% FeSO₄·7H₂O, and 0.001% ZnSO₄·7H₂O (4), supplemented with 0.5% myo-inositol and 0.02% L-arginine. For each experiment, 50 ml of this medium were inoculated with a small inoculum of a vegetative culture and were shaken at 120 oscillations per min in an incubator shaker at 28°. Each of the labeled compounds plus 28 μmoles of unlabeled D-glucose was administered at 5 days; 24 hours later, the broth was separated by filtration. The amount of streptomycin synthesized was determined by a ferrie maltol assay (5).

Labeled Materials

D-Glucose-1-¹⁴C (5.1 × 10⁷ cpm per μmole), D-glucose-2-¹⁴C (3.4 × 10⁷ cpm per μmole), and D-glucose-6-¹⁴C (3.4 × 10⁷ cpm per μmole) were obtained from Volk.

Isolation of Streptomycin

Streptomycin sulfate carrier (1.0 g) was added to each broth. The streptomycin was isolated essentially by the method of Hunter, Herbert, and Hockenhull (6), which consists of removal of the streptomycin from the medium with Amberlite IRC-50 (Na⁺), elution with acid, and isolation of streptomycin in the eluate as the trireinektate derivative. After recrystallization, streptomycin treinektate was converted to the sulfate salt for radioassay and degradation.

Degradation of Streptomycin

1. Hydrolysis to Streptidine and Streptobiosamine—Streptomycin sulfate was dissolved in 1 N sulfuric acid (0.12 g per ml) and allowed to stand at 37° for 48 hours; it was then refrigerated for several hours. Insoluble streptidine sulfate was removed by filtration. An equal volume of acetone was added to the filtrate to precipitate remaining streptidine sulfate, which was removed by filtration. After removal of the acetone under reduced pressure from the filtrate, the solution was freed of water, and allowed to stand at 37° for 48 hours; a procedure similar to that of McGilveray and Herbert, and Hockenhull (6), which consists of removal of the streptomycin from the medium with Amberlite IRC-50 (Na⁺), elution with acid, and isolation of streptomycin in the eluate as the trireinektate derivative. After recrystallization, streptomycin treinektate was converted to the sulfate salt for radioassay and degradation.

2. Tetrahydrostreptobiosamine—The streptobiosamine prepared above was immediately converted to tetrahydrostreptobiosamine by reduction in a manner analogous to that employed by Frush and Isbell for reduction of other carbohydrates (7). Streptobiosamine (350 mg) was added to 5 ml of Amberlite IR-120 (H⁺) (medium porosity) and 20 ml of 0.05 M boric acid in a flask and cooled in ice. While it was stirred, 20 ml of freshly prepared 0.3 M sodium borohydride were added dropwise over 3 min. Stirring was continued for 30 min, and then the solution was treated again in a similar manner with 20 ml of the borohydride solution. After 30 min, the mixture was adjusted to pH 9.0 with NaOH, and the solution was refrigerated overnight. The solution was passed through a column (12 cm × 0.8 cm) of Amberlite IR-120 (H⁺). The column was washed with water, and the tetrahydrostreptobiosamine was eluted with 4 N NH₄OH (0.5 ml per min). The eluate was concentrated to a small volume under reduced pressure at 35°, 20 ml of water were then added, and the solution was again concentrated to 5 ml. This was lyophilized to yield hygroscopic crystalline tetrahydrostreptobiosamine, m.p. 81-85°, with decomposition, [α]₂Ο⁻¹⁰⁵° (c, 0.96, in H₂O). The compound gave the following analysis.

C₁₄H₂₈N₄O₈·H₂O
Calculated: C 43.4, H 7.53
Found: C 43.2, H 7.42

The compound gave a positive test for N-methyl-L-glucosamine (8) and a negative copper reduction test. Periodate oxidation gave 1 mole formaldehyde per mole of compound by a chromotropic acid assay (9).

3. N-Acetyltetrahydrostreptobiosamine—Tetrahydrostreptobiosamine (200 mg) was dissolved in 25 ml of absolute methanol containing 0.6 ml of acetic anhydride. After 4 hours at room temperature, the solvent was removed under reduced pressure. The residue was recrystallized from ethanol-ether, m.p. 79-81°, [α]₂Ο⁻¹⁰⁵° (10). The compound gave the following analysis.

C₁₄H₂₈N₄O₁₉·H₂O
Calculated: C 44.9, H 7.78, N 3.49
Found: C 44.3, H 7.99, N 3.44

Periodate consumption, measured by a spectrophotometric assay (11), revealed that 2 moles of periodate were consumed per mole of compound in agreement with reported values (10).

4. Periodate Oxidation of N-Acetyltetrahydrostreptobiosamine; Isolation of Aceldehyde and Formaldehyde—A 5-ml solution containing 150 mg of N-acetyltetrahydrostreptobiosamine and 160 mg of sodium metaperiodate was allowed to stand for 4 hours at room temperature; excess sodium arsenite was then added. Five milliliters of 1 M NaHCO₃ (12) were then added, and the reaction mixture was heated to 60°. Aceldehyde was aerated out under a stream of CO₂ (1 liter per min) for 1 hour into 2.0% sodium bisulfite. This bisulfite solution was stored and used for subsequent acetaldehyde isolation and degradation. For preparation of acetaldehyde, 2 g of K₂HPO₄ were added to one-half of the bisulfite solution, and the temperature was elevated to 100°; acetaldehyde was aerated out with a stream of N₂ into water at 0°. Aceldehyde was prepared by addition of 50 ml of 0.4% dimedon to the contents of the absorption tube. This was adjusted to pH 4.5, and was then allowed to stand overnight. Aceldehyde formed was recrystallized from water, m.p. 142-143°.

For formaldehyde isolation, 50 ml of 0.4% dimedon were added to the periodate oxidation reaction flask; the solution was adjusted to pH 4.5 and allowed to stand overnight. Formaldehyde was stored and used for subsequent acetaldehyde isolation and degradation. For preparation of acetaldehyde, 2 g of K₂HPO₄ were added to one-half of the bisulfite solution, and the temperature was elevated to 100°; acetaldehyde was aerated out with a stream of N₂ into water at 0°. Aceldehyde was prepared by addition of 50 ml of 0.4% dimedon to the contents of the absorption tube. This was adjusted to pH 4.5, and was then allowed to stand overnight. Aceldehyde formed was recrystallized from water, m.p. 190-191°. Excess dimedon was then removed from the mother liquor by ether extraction, and the aqueous phase was saved.

5. Hydrolysis of L-Glyceric Acid-N-Acetyl-N-methyl-L-glucosaminide; Isolation of Glyceric Acid and N-Methyl-L-glucosamine—The aqueous solution remaining from the formaldehyde isolation was adjusted to pH 1.0 with HCl and was allowed to stand at 47° for 48 hours, a procedure similar to that of McGilveray and Stenlake (13) for the hydrolysis of N-acetylxyihydrotreptobiosamine. This hydrolysate was adjusted to pH 5.0 with silver oxide, decolorized with charcoal, and filtered. The filtrate was passed through a column (12 × 0.8 cm) of Amberlite 120 (H⁺), and the column was washed with 30 ml of water. The eluate and washing were combined, adjusted to pH 7.0 with calcium hydroxide, and filtered. The filtrate was concentrated under reduced pressure to 10 ml. Ethanol was added to incipiently

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Radioactivity measurements were made with a Nuclear-Chicago model 725 liquid scintillation spectrometer. Quench corrections were made either by the channels ratio method or by internal standardization. The scintillation medium used for activity determination was 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis-[2-(5-phenyloxazolyl)]benzene and 5% naphthalene in a solvent composed of 80% p-dioxane and 20% ethylene glycol monomethyl ether. At least 10 μmole of each sample was used for radioassay. The standard error of all determinations was held within 2%, except for degradation products derived from carbons of streptose containing only tritium. No attempts were made to correct for quenching.

**RESULTS**

**Streptomycin Production**—The synthesis of streptomycin by this strain of *S. griseus* grown in the media and under the conditions described in these experiments reached a maximum range between days 5 and 6. The total quantity of streptomycin synthesized per flask during the 6 days of incubation ranged from 14.1 to 31.1 μmoles. The amount of streptomycin produced per flask during the 24-hour period following administration of the 14C-labeled compounds ranged from 6.4 to 13.3 μmoles. The quantity of mannosidostreptomycin present following 6 days of cultures grown under these conditions was found to be less than 20% by chemical assay (5).

**Degradation Procedure**—Streptomycin was hydrolyzed to streptidine and streptobiosamine with acid. These conditions (as "Experimental Procedure") would also hydrolyze the glycosidic bond between the mannose and N-methyl-N-glucosamine moieties of any mannosidostreptomycin present (16). N-Acetyltetrahydrostreptobiosamine was prepared from the streptobiosamine by a procedure different from that employed for the first preparation of this derivative (10). Analytical data indicated that the derivative was pure when prepared by this procedure. In addition, the recovery of 14C as the sum of the streptidine and N-acetyl tetrahydrostreptobiosamine specific activities amounted to 98.7-103.0% of that of the isolated streptomycin (Table I). Further, cleavage of 1 mole of this compound with 2 moles of periodate, followed by hydrolysis, resulted in the recovery of essentially 1 mole each of formaldehyde, acetaldehyde, L-glyceric acid, and N-methyl-N-glucosamine, confirming the findings of Wolff and DeWalt (10).

**Tracer Experiments**—In all experiments, the labeled com-
Distribution of $^{14}$C in streptose moiety of streptomycin from S. griseus given L-serine-$^{14}$C

<table>
<thead>
<tr>
<th>Degradation products</th>
<th>Carbon atoms derived from streptose</th>
<th>Specific activity</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/10 #moles</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Streptose</td>
<td>All</td>
<td>1109</td>
<td>100</td>
</tr>
<tr>
<td>Glycic acid</td>
<td>1,2,3</td>
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<td>16</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1,3</td>
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<td>Formaldehyde</td>
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<td>333</td>
<td></td>
</tr>
<tr>
<td>Iodoform</td>
<td>5</td>
<td>384</td>
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</table>

* Value not determined.

Distribution of $^{14}$C in streptose moiety of streptomycin from S. griseus given D-glucose labeled with $^{14}$C in various carbons

<table>
<thead>
<tr>
<th>Degradation products</th>
<th>Carbon atoms derived from streptose</th>
<th>Labeled D-glucose administered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/10 #moles</td>
<td>%</td>
</tr>
<tr>
<td>Streptose</td>
<td>All</td>
<td>355</td>
</tr>
<tr>
<td>Glycic acid</td>
<td>1,2,3</td>
<td>337</td>
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<td>Formaldehyde</td>
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<tr>
<td>Formaldehyde</td>
<td>5</td>
<td>190</td>
</tr>
</tbody>
</table>

* Value not determined.

Some randomization of label occurred after the administration of all labeled glucoses, as would be expected.

**DISCUSSION**

Studies of the label distributions in streptomycin following administration of specifically labeled, suspected precursors to S. griseus have yielded valuable information concerning metabolic pathways for synthesis of this antibiotic. However, application of tracer methodology to the elucidation of streptose biosynthesis has been difficult. Although recently synthesized (19), streptose has, to date, defied attempts at its isolation from streptomycin. Thus, any extensive degradation of the antibiotic to examine streptose requires prior conversion of this moiety to a stable derivative. Recently, Candy et al. (2) stabilized this moiety by taking advantage of the known ability of streptomycin streptose to be converted to maltol in an alkaline medium (3). However, because the mechanism of the carbon-carbon rearrangement of the streptose moiety to form maltol is still uncertain, a complete carbon label pattern cannot be formulated. The activities of at least carbon atoms 3 and 3' of streptose cannot be assessed by the procedure of Candy et al. (2).

We have developed a total degradation of the streptose moiety based on the conversion of streptobiosamine (streptose-N-methyl-L-glucosamine), obtained from mild hydrolysis of streptomycin, to the previously well characterized N-acetyltaetahydrorstreptobiosamine (10), and the subjection of this derivative to extensive degradation.

Streptose is unique in being a branched deoxypentose, and, therefore, the mechanism of formation of the formyl side chain at carbon 3 is of considerable interest. Three obvious pathways can be postulated: (a) formyl addition at carbon 3 of a pentose precursor, (b) an intramolecular rearrangement of glucose either after or prior to reduction at carbon 6, and (c) condensation of a 2- and 4-carbon unit in such a manner as to form a formyl side chain.

The possibility of a pentose formylation reaction has now been eliminated. Significant incorporation of $^{14}$C into streptomycin streptose was not found after the administration of potential formyl group precursors, namely formate-$^{14}$C and l-methionine-methyl-$^{14}$C, to cultures (2, 20). Likewise, as reported here, the administration of L-serine-$^{14}$C, another formyl group precursor, did not result in selective labeling of the formyl carbon of streptose.

Table III gives the $^{14}$C incorporation pattern in the carbons of streptose following the administration of variously labeled D-glucoses.
In view of the lack of evidence for this mechanism, the possibility of an intramolecular rearrangement of glucose was investigated. Glucoses labeled specifically with $^{14}$C in positions 1, 2, 3 and 4, or 6 were administered individually to S. griseus cultures. The distribution of label in the streptose of the streptomycin synthesized was found to be exactly as predicted for a carbon-carbon rearrangement of carbon atoms 3 and 4 of glucose to form the formyl side chain.

As can be seen from Fig. 3, carbon atoms 1 and 5 of streptose are derived from carbon atoms 1 and 6, respectively, of glucose, in agreement with the conclusion of Candy et al. (2). In addition, our experiments showed that carbon 2 of streptose arises from carbon 2 of glucose, and that carbon atoms 3 and 3' of streptose arise from carbon atoms 3 and 4 of glucose. It is important to note that an essentially equal label distribution is seen in carbon atoms 3 and 3' of streptose synthesized following glucose-3,4-$^{14}$C. This distribution ratio is similar to that of the administered labeled glucose, indicating that extensive asymmetrical dilution of label at position 3 or 4 had not occurred. The distribution of the label in the carbons of streptose shows that some randomization occurred even when favorable conditions were used, i.e., administration of isotopic compounds at the time of maximum streptomycin synthesis and to a glucose-free medium. Inspection of the data in Table III discloses that this randomization resulted in very low levels of $^{14}$C incorporation in the various carbons involved. As a consequence, the statistical counting error was increased. These values should be considered only as reasonable approximations of a low but detectable $^{14}$C activity in those carbons.

Finally, a mechanism involving fragmentation of the carbon chain of glucose followed by recombination of the fragments to form streptose is possible. However, the marked incorporation of isotope into specific carbons of the streptose moiety after administration of the glucoses labeled in specific positions to cultures renders this type of mechanism most unlikely.

The intermediate steps in the conversion of glucose to streptose are of prime interest. Baddiley et al. (21) have postulated a mechanism for the formation of streptose from thymidine diphosphate rhamnose, since the organism has been shown to synthesize TDP-rhamnose from TDP-glucose (21), and rhamnose has the necessary methyl group in position 6. Evidence for this hypothesis however, has not been reported. Although numerous pathways can be postulated, it is conceivable that an intramolecular rearrangement occurs prior to the reaction removing the oxygen at position 6 of the glucose. The natural occurrence of hydroxystreptomyein (22), which contains a 3-C-formyl-L-lyxose (5-hydroxystreptose) moiety in its molecule instead of 5-deoxy-3-C-formyl-L-lyxose (streptose), is suggestive. Our laboratory is currently engaged in a study of the intermediates involved in the synthesis of streptose from glucose.

While this manuscript was in preparation, Candy and Baddiley (23) reported that the formyl carbon of streptose was derived from carbon 3 of glucose. Glucose-3,4-$^{14}$C and glucose-1,3-$^{14}$C (prepared by an elegant enzymatic synthesis) were given to S. griseus, and the $^{14}$C-streptomycin formed was isolated. The specific activity of the streptose moiety was determined from a maltol derivative. The activity of the formyl carbon was determined as formic acid derived from periodate oxidation of streptomycin, in such a manner, as claimed by these investigators, the only the formyl carbon of streptose would be liberated as formic acid. However, experimental proof of this selective oxidation was not presented. After administration of glucose-3,4-$^{14}$C 31.3% of the $^{14}$C of streptose was concentrated in the formyl carbon; after glucose-1,3-$^{14}$C, 39.6% was concentrated. Since the activity ratio of the $^{14}$C-labeled carbons in the administered labeled glucoses was essentially 1:1, these investigators expect 50% of the streptose label in the formyl carbon. The label found which was less than 50%, was interpreted as an asymmetric "dilution" of the specific activities of carbon atoms 1 and 3 of streptose. The administration of labeled glucose, indicating that extensive asymmetrical dilution of label at position 3 or 4 had not occurred. The distribution of the label in the carbons of streptose shows that some randomization occurred even when favorable conditions were used, i.e., administration of isotopic compounds at the time of maximum streptomycin synthesis and to a glucose-free medium. Inspection of the data in Table III discloses that this randomization resulted in very low levels of $^{14}$C incorporation in the various carbons involved. As a consequence, the statistical counting error was increased. These values should be considered only as reasonable approximations of a low but detectable $^{14}$C activity in those carbons.

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