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, thus indicating that the remaining one-third was in the streptose moiety. 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-1-14C 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 conclude 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. Serine-3-14C, D-glucose-1-14C, D-glucose-2-14C, D-glucose-3,4-14C, and D-glucose-6-14C were administered individually to streptomycin-producing cultures of S. griseus. The streptose moiety of the labeled streptomycin synthesized was degraded, and isotope distribution in all 6 carbons was determined. Pattern of 14C labeling found was consistent only with a mechanism involving the utilization of all carbons of glucose in cleavage and rearrangement at carbon atoms 3 and 4 of glucose to yield the branched sugar, streptose.

EXPERIMENTAL PROCEDURE

Culture Techniques

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

† The culture of S. griseus was the kind gift of Charles Pfizer Company, Inc.
0.063% CaCl₂·2H₂O, 0.002% Fe₂O₃·7H₂O, 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 p-glucose was administered at 5 days; 24 hours later, the broth was separated by filtration. The amount of streptomycin synthesized was determined by a ferric maltol assay (5).

**Labeled Materials**
p-glucose-1₄C (5.1 × 10⁷ cpm per µmole), p-glucose-2₄C (3.4 × 10⁷ cpm per µmole), and p-glucose-6₄C (3.4 × 10⁷ cpm per µmole) were obtained from Nuclear-Chicago, and p-glucose-1₄C (9.2 × 10⁷ cpm per µmole) and L-serine-3₄C (2.2 × 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 trireineckate derivative. After recrystallization, streptomycin trireineckate 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 then removed by filtration. After removal of the acetone under reduced pressure from the filtrate, the solution was freed of sulfate with barium ion. Following removal of barium sulfate, the solution was lyophilized.

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 5 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]D +105° (c, 0.96, in H₂O). The compound gave the following analysis.

C₁₆H₁₈N₂O₁₉·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]D +105°. The compound gave the following analysis.

C₁₆H₁₈N₂O₁₉·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 Acetaldehyde 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°. Acetaldehyde 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 KH₂PO₄ 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°. Acetaldehyde was prepared by addition of 50 ml of 0.4% dimedon to the contents of the adsorption tube. This was adjusted to pH 4.6, and was then allowed to stand overnight. Acetaldehyde 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 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-glucosamine—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-acetyldihydrostreptobiosamine. 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 incipient
precipitation, and the solution was then refrigerated for 24 hours at 5°. Calcium glycerate, which separated, was recrystallized from ethanol-water, m.p. 137–139°, [α]$_D^{20}$ –13.0 (c 2.6, in water). N-Methyl-L-glucosamine was eluted from the above column with 30 ml of 4 N NaOH, followed by 30 ml of water. The combined eluates were dried under reduced pressure. The N-methyl-L-glucosamine was converted to the penta-O-acetyl derivative (1) for radioassay, m.p. 154–156°, with decomposition.

6. Degradation of Calcium Glycerate—Calcium glycerate (100 mg) was dissolved in 5 ml of water and adjusted to pH 4.0. The solution was freed of Ca$^2+$ with a column (6 X 0.8 cm) of Dowex 50 (H$^+$).

The glyceric acid was oxidized with 2 ml of 0.1 M periodic acid at 47° for 4 hours. Carbon dioxide was separated as the p-bromophenylacyl derivative (14). 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 98–104% 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-L-glucosamine, confirming the findings of Wolfe and DeWalt (10).

Tracer Experiments—In all experiments, the labeled compounds to $S$. griseus were administered Streptomycin Streptidine Streptosea L-glucosaminide

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>Amount administered</th>
<th>Isolated streptomycin and degradation products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpn X 10$^{-7}$</td>
<td>cpn/10$^{-10}$</td>
</tr>
<tr>
<td>L-Serine-3-14C</td>
<td>5.4</td>
<td>2620</td>
</tr>
<tr>
<td>d-Glucose-1-14C</td>
<td>4.9</td>
<td>780</td>
</tr>
<tr>
<td>d-Glucose-2,14C</td>
<td>3.1</td>
<td>800</td>
</tr>
<tr>
<td>d-Glucose-3,4,14C</td>
<td>3.3</td>
<td>744</td>
</tr>
<tr>
<td>d-Glucose-6,14C</td>
<td>2.8</td>
<td>644</td>
</tr>
</tbody>
</table>

a Difference between the specific activities, expressed on a molar basis, of the N-acetyl tetrahydrostreptobiosamine and the corresponding penta-O-acety]-N-methyl-L-glucosamine.

Radioassay Procedures

All 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 determinations of dimened, idoform, and p-bromophenylacetyl derivatives and Hyamine carbonate consisted of 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis-[2-(5-phenyloxazolyl)]benzene in toluene. For other derivatives, the medium used was 0.5% 2,5-diphenyloxazole, 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 μmoles of each sample were 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 amounts of 14C.

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 rate 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 in 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-L-glucosamine moieties of any mannosidostreptomycin present (16). N-Acetyl tetrahydrostreptobiosamine 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 98–104% 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-L-glucosamine, confirming the findings of Wolfe and DeWalt (10).
pounds were administered at 5 days, and the cultures were harvested 24 hours later. The specific activities of the isolated streptomycin and the three moieties are given in Table I. The data show that the incorporation of radioactivity from glucose, labeled in various positions, into the streptidine moiety ranged between 11 and 25% of the molar specific radioactivity of the intact streptomycin molecule. This incorporation is, in general, somewhat less than seen when crude organic media (1, 17) are used for production, but is in accord with the observations of Majumdar and Kutzner (18) that exogenous myo-inositol depressed incorporation of uniformly labeled 14C-glucose into streptidine. The incorporation of 14C in the streptose moiety of streptomycin ranged from 33 to 44%, and that of the methyl-14C-glucosamine moiety ranged from 40 to 46%, and that of the A-streptidine. The incorporation of 14C in the streptose moiety of streptomycin was 33 to 44%, and that of the A-streptidine was 33 to 46%. The incorporation of 14C in the streptose moiety of streptomycin produced by cultures given L-serine-3-14C, another formyl group precursor, (b) an intramolecular rearrangement of glucose either directly to the formyl side chain. 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 14C into streptomycin was not found after the administration of potential formyl group precursors, namely formate-14C and L-methionine-14C, to cultures (2, 20). Likewise, as reported here, the administration of L-serine-3-14C, another formyl group precursor, did not result in selective labeling of the formyl carbon of strept-

Table III gives the 14C incorporation pattern in the carbons of streptose following the administration of variously labeled D-glucoses. Some randomization of label occurred after the administration of all labeled glucoses, as would be expected.

**Table III**

<table>
<thead>
<tr>
<th>Degradation products</th>
<th>Carbon atoms derived from streptose</th>
<th>Labeled D-glucose administered</th>
<th>1-14C</th>
<th>2-14C</th>
<th>3-14C</th>
<th>4-14C</th>
<th>5-14C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/10 μmoles %</td>
<td>cpm/10 μmoles %</td>
<td>cpm/10 μmoles %</td>
<td>cpm/10 μmoles %</td>
<td>cpm/10 μmoles %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptose</td>
<td>All</td>
<td>355</td>
<td>337</td>
<td>282</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyceric acid</td>
<td>1, 2, 3</td>
<td>80</td>
<td>28</td>
<td>73</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>3'</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aetaledehyde</td>
<td>4, 5</td>
<td>25</td>
<td>150</td>
<td>50</td>
<td>218</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idoform</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Value not determined.

Table II shows the distribution of 14C in the carbons of the streptose moiety of streptomycin produced by cultures given L-serine-3-14C. The isotope incorporated in the streptose was not concentrated in the formyl carbon of the streptose, but was distributed randomly throughout the molecule.

**Table II**

<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 μmoles %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptose</td>
<td>All</td>
<td>1109</td>
<td>100</td>
</tr>
<tr>
<td>Glyceric acid</td>
<td>1, 2, 3</td>
<td>182</td>
<td>16</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>2</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>CO3</td>
<td>3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>3'</td>
<td>136</td>
<td>12</td>
</tr>
<tr>
<td>Aetaledehyde</td>
<td>4, 5</td>
<td>717</td>
<td>65</td>
</tr>
<tr>
<td>Aetaledehyde</td>
<td>4</td>
<td>333</td>
<td></td>
</tr>
<tr>
<td>Idoform</td>
<td>5</td>
<td>384</td>
<td></td>
</tr>
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</table>

* Value not determined.

**Fig. 3.** Structural relationship of D-glucose to L-streptose. Symbols (▲, □, ●, ○) indicate the labeling pattern of streptose of the streptomycin synthesized from S. griseus from specifically labeled glucoses.
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tose (Table 1). In view of the lack of evidence for this mechanism, the possibility of an intramolecular rearrangement of glucose was investigated. Glucose labeled specifically with ^14C 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-^14C. 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 utilized, 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 ^14C 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 ^14C activity in these 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 glucose 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 di-phosphate 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 hydroxystreptomyccin (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-^14C and glucose-1,3-^14C (prepared by an elegant enzymatic synthesis) were given to S. griseus, and the ^14C-streptomycin formed was isolated. The specific activity of the streptose moiety was determined from its 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, 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-^14C 31.3% of the ^14C of streptose was concentrated in the formyl carbon; after glucose-1,3-^14C, 39.6% was concentrated. Since the activity ratio of the ^14C-labeled carbons in the administered labeled glucoses was essentially 1:1, these investigators expected 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 the administered glucose by recycling of these compounds through the pentose shunt pathway. However, in the present experiments, in which isotope was added at a later stage in the growth cycle of the organism, an equal distribution of isotope from D-glucose-3,4-^14C was found in carbon atoms 3 and 3' of streptose (Table III).

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