Biosynthesis of Streptomycin

I. ORIGIN OF THE GUANIDINE GROUP*

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Although the antibiotic streptomycin was isolated and characterized a number of years ago, little knowledge has accumulated concerning its synthesis by Streptomyces griseus. In 1954 Numerof, Gordon, Virgona, and O'Brien (1) administered glycine and acetate labeled in either the carboxyl or the alpha carbon to cultures of S. griseus. A low extent of incorporation of 14C into the streptomycin synthesized was observed in each case. The incorporated 14C was found to be predominantly concentrated in the guanidine carbons. Considerable quantities of the 14C-glycine and -acetate were degraded by the organism to 14CO2. During the same year, Hunter, Herbert, and Hockenhull (2) grew S. griseus in an 14CO2 atmosphere and found the streptomycin produced to be labeled with 14C exclusively in the guanidine side chains. This finding suggested that incorporation of the carbon atoms of acetate and glycine might proceed via a pathway involving CO2 as an intermediate (3). Addition of L-arginine to the medium was observed to depress 14CO2 incorporation into the antibiotic, thus indicating a probable role for this amino acid in the synthesis of streptomycin guanidine groups from 14CO2. During the same year, Hunter, Herbert, and Hockenhull (2) grew S. griseus in an 14CO2 atmosphere and found the streptomycin produced to be labeled with 14C exclusively in the guanidine side chains. This finding suggested that incorporation of the carbon atoms of acetate and glycine might proceed via a pathway involving CO2 as an intermediate (3). Addition of L-arginine to the medium was observed to depress 14CO2 incorporation into the antibiotic, thus indicating a probable role for this amino acid in the synthesis of streptomycin guanidine groups from 14CO2.

In the present study, direct evidence is presented that the guanidine carbons of streptomycin arise by transamination. Administration of L-arginine-guanidino-14C to S. griseus cultures resulted in marked incorporation of isotope into the guanidine side chains. Bicarbonate-14C administered under similar conditions displayed little incorporation. Furthermore, a correlation between transaminase activity of the mycelia and streptomycin production was found.

EXPERIMENTAL PROCEDURE

Culture Techniques—S. griseus was cultivated on agar slants composed of 1% glucose, 0.05% L-asparagine, and 0.05% KH2PO4 in distilled water (6) until well sporulated. The spores were transferred to a small volume of medium (5 to 15 ml), and after thorough mixing, usually 1.0 ml was used to inoculate each flask of medium. The liquid medium used, unless otherwise stated, was that used by Silverman and Rieder (6), which consists of 1% glucose, 1% tryptone, 0.6% beef extract, and 1% NaCl in distilled water. For each tracer study, 50 ml of medium per flask were used, and for transaminase studies, 500 ml per flask. Cultures were grown in Erlenmeyer flasks on a rotary shaker incubator at 28° and at 75 oscillations per minute. Maximal growth for this strain was achieved at 3 days. Maximal streptomycin production was realized in 7 days and ranged from 0.65 to 0.98 mg per ml of medium as assayed by the maltol procedure of St. John, Flick, and Tepe (7). The 14C compounds used were administered, unless otherwise stated, at 96 hours after spore inoculation, and the streptomycin was isolated from the broth at 168 hours. The streptomycin synthesized after isoenzyme administration was determined by the difference between the total quantity produced and that produced at the time of administration of 14C compounds.

14C-Labeled Compounds—L-Arginine-guanidino-14C and L-homoarginine-guanidino-14C were purchased from California Corporation for Biochemical Research, and NaH14CO3, from Volk Radiochemical Company.

Isolation of Streptomycin—At the termination of experiments 7 days after inoculation, the broth was separated from the mycelium by centrifugation and the mycelium was washed once with 10 ml of water. One gram of streptomycin sulfate carrier was added to the combined broth and washing, and streptomycin was isolated as the trireineckate derivative essentially by the method of Hunter et al. (2). The reineckates were recrystallized twice. Additional recrystallization did not change the specific activity.

Degradation of Streptomycin—Streptomycin trireineckate was converted to the sulfate salt. Streptidine sulfate was isolated after hydrolysis of the streptomycin sulfate (3). A portion of

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1 Cultures of Streptomyces griseus 3754 (Rutger's strain) were kindly provided by Dr. Allen H. Heim, Department of Microbiology, Georgetown University.
the streptidine sulfate was converted to the dipicrate dihydrate salt (8), and this was recrystallized twice from water before radioassay. Another portion of the streptidine sulfate was oxidized with permanganate, and guanidine was isolated from the oxidation products as the picrate salt (9). The picrate was recrystallized at least once from water before assay. The purity of the picrates of streptidine and guanidine was established by nitrogen analysis (Dumas). All analyzed samples were found to be pure within 2% of theory.

Radiobicarbonate Experiments—$^{14}$CO$_2$ was isolated from the broth by acidification of the media and aeration of the gas into 1 N NaOH.

Assay of Radioactivity—Radioactivity was measured by solid sample counting of the reineckate and picrate derivatives. $^{14}$CO$_2$ was assayed as Ba$^{14}$CO$_3$. All measurements were made with a Nuclear-Chicago gas flow detector tube.

Transamidinase Activity—The enzyme assay developed employs canavanine and ornithine as substrates (10). Specific activity is expressed as millimicromoles of arginine synthesized per 2 hours per mmole of mycelium nitrogen. One gram of mycelia (wet weight) was homogenized with 10 ml of 0.1 M phosphate buffer, pH 7.5, in a Kontes all-glass homogenizer. A 1-ml aliquot was added to 3.5 ml of a buffer-substrate solution (1 part of 0.1 M phosphate buffer, pH 7.5, plus 3 parts each of 2.4 mM solutions of L-canavanine sulfate and L-ornithine hydrochloride adjusted to pH 7.5) which was first incubated in a 25-ml Erlenmeyer flask for 20 minutes at 37°. After a 2-hour incubation in a Dubnoff shaking incubator, 2 ml of 30% trichloroacetic acid were added, and the mixture was centrifuged. Color development for arginine was carried out on a 0.5-ml aliquot of supernatant liquid according to the Sakaguchi procedure of L-Homoarginine-14C.

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Homogenate Nitrogen Analyses—Nitrogen analyses of homogenates were done by a micro-Kjeldahl procedure (12).

RESULTS

L-Arginine-14C Experiments—In all experiments, the amount of streptomycin synthesized during the period after 14C compound administration ranged from 16.0 to 27.6 mg. Greater incorporation of isotope was found when 14C-arginine was given at 96 hours rather than at 72 hours. These results are depicted in Table I. Table II indicates the incorporation of homoarginine and of arginine in various amounts to S. griseus cultures. Homoarginine did not function as an amidine donor. Isolation control experiments were performed by adding 14C-arginine and carrier streptomycin to broths from a 7-day culture and subsequently isolating the streptomycin. No radioactivity was detected in the streptomycin reineckates from these isolation controls after one recrystallization.

Degradation Studies—In several experiments, streptomycin was degraded to streptidine, which was subsequently degraded to guanidine. The specific activities of the streptomycin, streptidine, and guanidine moieties are given in Table III.

Bicarbonate Experiments—The extent of incorporation of 14C from NaH14CO$_3$ into streptomycin is shown in Table IV. The time of the addition of the isotope was identical with that of the 14C-arginine experiments. At 7 days, approximately one-fifth of the administered bicarbonate was found in the media.

Transamidinase—Table V shows a comparison of cell weight, transamidinase activity, and streptomycin production by the organism at various times in the growth cycle when grown on two different media. An approximately 3-fold increase in enzyme concentration was noted for the glucose-tryptone

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**Table I**

<table>
<thead>
<tr>
<th>Time of 14C-arginine addition after inoculation</th>
<th>Quantity of 14C-arginine administered</th>
<th>Streptomycin synthesized</th>
<th>14C incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>µmoles</td>
<td>c.p.m. x 10$^{-4}$</td>
<td>c.p.m. x 10$^{-4}$/mg</td>
</tr>
<tr>
<td>72</td>
<td>5</td>
<td>5.24</td>
<td>8.45</td>
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<tr>
<td>72</td>
<td>5</td>
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<td>7.15</td>
</tr>
<tr>
<td>96</td>
<td>5</td>
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<td>10.32</td>
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<tr>
<td>96</td>
<td>5</td>
<td>5.24</td>
<td>16.31</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>14C compound administered</th>
<th>Amount of isotopic compound administered</th>
<th>Streptomycin synthesized</th>
<th>14C incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m. x 10$^{-4}$/mmole</td>
<td>c.p.m. x 10$^{-4}$/mg</td>
<td>%</td>
</tr>
<tr>
<td>L-Arginine-guanidino-14C</td>
<td>5</td>
<td>3.14</td>
<td>6.21</td>
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<tr>
<td></td>
<td>3</td>
<td>10.32</td>
<td>2.85</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.86</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>6.60</td>
<td>1.45</td>
</tr>
<tr>
<td>L-Homoarginine-guanidino-14C</td>
<td>0.4</td>
<td>1.14</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Distribution of 14C in isolated streptomycin after administration of arginine-14C to S. griseus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
</tr>
<tr>
<td>Isolated streptomycin</td>
</tr>
<tr>
<td>c.p.m. x 10$^{-4}$/mmole</td>
</tr>
<tr>
<td>2.1</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>1.1</td>
</tr>
<tr>
<td>0.7</td>
</tr>
</tbody>
</table>

* Since streptidine and streptomycin contain two guanidine groups per molecule, the value for guanidine must be doubled in order to compare its specific activity with that of streptidine or streptomycin.
The low extent of incorporation of $^{14}$CO$_2$ into streptomycin guanidine groups as found by Hunter et al. (2) and noted in these experiments (less than 0.1%), compared to 5 to 8.5% for the amidine carbon of arginine, excludes a major pathway of synthesis from arginine involving $^{14}$CO$_2$ as an intermediate. The results clearly indicate that arginine functions as an important precursor of streptomycin.

Degradation studies show unequivocally that arginine contributes its amidine carbon to the streptidine portion of the molecule. Information concerning the distribution of isotope in the streptidine was then obtained by oxidizing the molecule and isolating the guanidine side chains as guanidine from the oxidation products. The specific activity of the isolated guanidine was one-half that of the streptidine. Since streptidine contains two guanidine groups per molecule, the specific activity of the isolated guanidine must be multiplied by a factor of 2 to compare its specific activity with that of streptidine and streptomycin. The results show that essentially all of the radioactivity in streptidine is concentrated in the guanidine carbons. In addition, it should be noted that the inability of homoarginine to function as an effective precursor is in agreement with what is known about both mammalian (13, 14) and S. griseus transamidinase (4). However, impermeability of the cell membrane to this compound cannot be excluded by this study.

Further insight into mechanism was obtained by comparing transamidinase activity and streptomycin production by the organism at different phases of the growth cycle. Not only did the specific activity of the enzyme parallel streptomycin production, but through the fifth day the enzyme produced also paralleled the synthesis of the antibiotic. This latter statement cannot apply to the seventh day because lysis of the cells had begun.

Since the evidence points to an amidine transfer as the reaction for the synthesis of the guanidine side chains, the nature of the acceptor molecule is of prime importance. Identification of the amidine acceptor in the reaction should yield considerable knowledge concerning the sequential steps in the synthesis of this antibiotic. The characterization of the acceptor molecule is currently being pursued in this laboratory.

**SUMMARY**

A 5 to 8.5% incorporation of L-arginine-guanidine-$^{14}$C into *Streptomyces griseus* was achieved when the labeled compound was administered 96 hours after inoculation of the culture. Degradation of the isolated streptomycin disclosed that essentially all of the $^{14}$C was located in the guanidine side chains of the streptidine portion of the molecule. The increase in total and specific activity of transamidinase was found to parallel the increase of streptomycin production during the first 5 days of the growth cycle of *S. griseus*.

Evidence presented is consistent with the hypothesis that the guanidine groups of streptomycin are synthesized by the process of transamidination.

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**REFERENCES**

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