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.

STREPTOMYCIN

Fig. 1. The structure of streptomycin

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, 0.5% K₂HPO₄, 0.1% MgSO₄·7H₂O

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the amount available for subsequent degradation.

were diluted with corresponding nonradioactive material to in-

ume under reduced pressure at 35 ° , 20 ml of water were then

and the tetrahydrostreptobiosamine was eluted with 4 ml of

Amberlite IR-120 (H+). The column was washed with water,

was treated again in a similar manner with 20 ml of the boro-

hydride solution. After 30 min, the mixture was adjusted to

was added, and the solution was again concentrated to 5 ml. This

by filtration. An equal volume of acetone was added to the

for several hours. Insoluble streptidine sulfate was removed

and the column was washed with 30 ml of water. The eluate

oxide, decolorized with charcoal, and filtered. The filtrate was

and washing were combined, adjusted to pH 7.0 with calcium

and the reaction mixture was heated to 60 ° . Acetaldehyde

were added to one-half of the bisulfite solution, and the tem-

methane 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.

for formaldehyde isolation, 50 ml of 0.4% dimedon were

added to one-half of the bisulfite solution, and the tem-

were added to the periodate oxidation reaction flask; the solution was

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

was added to the periodate oxidation reaction flask; the solution was

and the solution was again concentrated to 5 ml. This

was lyophilized to yield hygroscopic crystalline tetrahydrostrep-

obiosamine, m.p. 81-85 ° , with decomposition, [α]25° +105° (c, 0.96, in H2O). The compound gave the following analysis.

*C6H6N2O6 . H2O

Calculated: C 43.4, H 7.53

Found: C 43.2, H 7.42

The compound gave a positive test for N-methyl-D-glucosamine

(8) and a negative copper reduction test. Periodate oxidation
gave 1 mole formaldehyde per mole of compound by a chromo-
tropic acid assay (9).

3. *N*-Acetyltetrahydrostreptobiosamine—Tetrahydrostreptobio-
samine (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]25° +105° (10). The compound gave the following analysis.

*C6H8N2O6 . 1/2H2O

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 degra-

dation. For preparation of acetaldemethone, 2 g of K2HPO4

were added to one-half of the bisulfite solution, and the temper-

ature was elevated to 100 ° ; acetaldehyde was aerated out

with a stream of N2 into water at 0 ° . Acetaldemethone was

prepared by addition of 50 ml of 0.4% dinedon to the contents

of the absorption tube. This was adjusted to pH 4.5, and was

then allowed to stand overnight. Acetaldemethone formed was

recrystallized from water, m.p. 142-143 ° .

For formaldehyde isolation, 50 ml of 0.4% dinedon were

added to the periodate oxidation reaction flask; the solution was

adjusted to pH 4.5 and allowed to stand overnight. Formalde-

methone formed was recrystallized from water, m.p. 190-191 ° .

Excess dinedon was then removed from the mother liquor by ether

extraction, and the aqueous phase was saved.

5. Hydrolysis of *L*-Glyceric Acid-*,Naetyl-*N*-methyl-*L*-glucosa-

mine; 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*-acytyldihydrotreptobio-

samine. This hydrolysate was adjusted to pH 5.0 with silver

oxide, decolorized with charcoal, and filtered. The filtrate was

passed through a column (12 x 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

some compounds isolated during the degradation procedure

were diluted with corresponding nonradioactive material to in-

crease the amount available for subsequent degradation.
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 10 –13.0 (c, 2.6, in water). N-Methyl-L-glucosamine was eluted from the above column with 30 ml of 4 N NH₄OH, followed by 30 ml of water. The combined eluates were dried under reduced pressure. The N-methyl-L-glucosamine 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⁺⁺ with a column (6 × 0.8 cm) of Dowex 50 (H⁺). The glycerate was oxidized with 2 ml of 0.1 M periodic acid at 47° for 4 hours. Carbon dioxide was aerated out of the reaction mixture into 0.1 N NaOH. An aliquot of the NaOH was assayed for carbon dioxide by titration. Carbon dioxide was released from another aliquot by acidification and was absorbed in Hyamine. The Hyamine carbonate was then assayed for carbon dioxide by titration. Carbon dioxide was recovered from the NaOH as CO₂ (C-3)

7. Degradation of Acetaldehyde—The remainder of the acetaldehyde bisulfite solution from Section 4 was used for degradation of acetaldehyde to iodoform and formic acid (15). The formic acid was isolated 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 to 104% of that of the isolated streptomycin (Table I). Further more, 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 compound administered was hydrolyzed to streptomycin 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-Acetylthretostreptobiosamine 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 to 104% of that of the isolated streptomycin (Table I). Further more, 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).

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 dexamene, iodoform, and N-bromophenylacetyl derivatives and Hyamine carbonate consisted of 0.5% 2,5-diphenylloxazole 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 for all determinations was held within 2%, except for degradation products derived from carbons of streptose containing only traces 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-Acetylthretostreptobiosamine 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 to 104% of that of the isolated streptomycin (Table I). Further more, 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).

Table I

<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>cpm X 10⁻²</td>
<td>cpm/10 μmoles</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>

* Difference between the specific activities, expressed on molar basis, of the N-acetyl tetrahydrostreptobiosamine and th of its corresponding penta-O-acetyl-N-methyl-L-glucosamine.
The incorporation of $^{14}$C in the carbons of the streptose moiety of streptomycin from S. griseus given L-serine-$^{14}$C.

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

Disregarding penta-O-acetyl-N-methyl-D-glucosamine.

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-D-glucosamine), obtained from mild hydrolysis of streptomycin, to the previously well characterized N-acetyltetrahydrostreptobiosamine (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) 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-$^{14}$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. 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.

Table II: 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>Specific activity</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobic acid</td>
<td>1-2-3</td>
<td>25</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>1-2-3</td>
<td>28</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1-2-3</td>
<td>33</td>
</tr>
<tr>
<td>Formic acid</td>
<td>1-2-3</td>
<td>45</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>1-2-3</td>
<td>50</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1-2-3</td>
<td>50</td>
</tr>
<tr>
<td>Aconitate</td>
<td>1-2-3</td>
<td>50</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>1-2-3</td>
<td>50</td>
</tr>
<tr>
<td>Formic acid</td>
<td>1-2-3</td>
<td>50</td>
</tr>
<tr>
<td>Iodoform</td>
<td>1-2-3</td>
<td>50</td>
</tr>
</tbody>
</table>

* Value not determined.

**Table III**

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>Labeled D-glucose administered</th>
<th>Carbon atoms derived from streptose</th>
<th>1-4C</th>
<th>2-4C</th>
<th>3, 4-4C</th>
<th>6-4C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobic acid</td>
<td>1-2-3</td>
<td>25</td>
<td>33</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>1-2-3</td>
<td>25</td>
<td>33</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1-2-3</td>
<td>25</td>
<td>33</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Formic acid</td>
<td>1-2-3</td>
<td>25</td>
<td>33</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>1-2-3</td>
<td>25</td>
<td>33</td>
<td>50</td>
<td>75</td>
<td>100</td>
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<td>Formaldehyde</td>
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<td>Aconitate</td>
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<td>Formic acid</td>
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<td>25</td>
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<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Iodoform</td>
<td>1-2-3</td>
<td>25</td>
<td>33</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
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</table>

* Value not determined.
ose (Table I). 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 asymmetric 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 $^{14}$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 the intramolecular rearrangement occurs prior to the reaction removing the oxygen at position 6 of the glucose. The natural occurrence of hydroxystreptomycin (22), which contains a 3-C-formyl-1-lyxose (5-hydroxystreptose) moiety in its molecule instead of 5-deoxy-3-C-formyl-1-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 the 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, that 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, 30.6% was concentrated. Since the activity ratio of the $^{14}$C-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 in the administered glucoses 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 through the pentose shunt pathway was observed. In addition, 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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