The Biosynthesis of Streptomycin

THE ORIGIN OF THE C-FORMYL GROUP OF STREPTOSE

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1. The biosynthesis of streptomycin in *Streptomyces griseus* has been studied by adding D-[3,4- $^{14}\text{C}_2$]glucose or D-[1,3- $^{14}\text{C}_2$]glucose to the growth medium and degrading the streptomycin produced. 2. The results suggest that the C-3' branch carbon atom of L-streptose arises from C-3 of D-glucose. 3. The mechanism of biosynthesis of streptose from glucose is discussed. It probably involves an intramolecular rearrangement of a 6-deoxy-4-oxylhexose derivative, and it is suggested that the nucleoside diphosphate sugar derivative hitherto recognized as an intermediate in the biosynthesis of L-rhamnose might participate in such a rearrangement.

In recent studies on the biosynthesis of the L-streptose moiety of streptomycin (Candy, Blumson & Baddiley, 1964) it was shown that C-1 of streptose arises from C-1 of D-glucose and that C-5 of streptose arises from C-6 of glucose. It was suggested that the formation of streptose takes place such that either C-3 or C-4 of hexose becomes the C-3' branch carbon atom of streptose. To examine further the mechanism of biosynthesis of streptose experiments have been carried out on the incorporation of radioactivity from D-[3,4- $^{14}\text{C}_2$]glucose and D-[1,3- $^{14}\text{C}_2$]glucose into streptomycin.

MATERIALS AND METHODS

Production of streptomycin. *Streptomyces griseus* was grown in the presence of labelled glucose, and streptomycin was subsequently isolated as described by Candy *et al.* (1964). For the D-[3,4- $^{14}\text{C}_2$]glucose experiment 0.02 mc was used, and for the D-[1,3- $^{14}\text{C}_2$]glucose experiment approx. 0.005 mc was used.

Degradation of streptomycin. Maltol was prepared from streptomycin as described by Candy *et al.* (1964). Maltol is derived from the streptose moiety of streptomycin and contains all of (and only) the carbon atoms originally present in the streptose.

The carbon of the C-formyl group of streptomycin was obtained as formic acid by selective oxidation of streptomycin with periodate. A solution containing 0.47 m-mole of streptomycin in 30 ml. of water was mixed with a solution of 0.47 m-mole of sodium metaperiodate in 250 ml. of water and the oxidation was allowed to proceed for 30 min. at 20° in the dark. The mixture was rapidly passed through a column containing 20 g. wet wt. of Amberlite IR-120

(H⁺ form) resin, then through a column containing 20 g. wet wt. of Amberlite IR-4B (OH⁻ form) resin. The column of Amberlite IR-4B resin was washed with 500 ml. of water and the formic acid was eluted slowly with 25 ml. of 0.25 M-Ba(OH)₂. The eluate was acidified with orthophosphoric acid and a slight colour due to iodine was destroyed with the minimum volume of dilute sodium thiosulphate. The solution was subjected to steam-distillation, the distillate made just alkaline with NaOH, evaporated to about 1 ml. under reduced pressure, acidified with orthophosphoric acid and again subjected to steam-distillation from a small volume. The distillate contained 0.225 m-mole of formic acid in 6.5 ml. of water. Formic acid was determined by the method of Wood & Gest (1957).

Measurement of radioactivity. Radioactive measurements were made either with a Packard Tri-Carb scintillation counter model 3314 or with a Nuclear-Chicago liquid-scintillation system 725. The scintillator consisted of: 7 g. of 2,5-diphenyloxazole, 0.3 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 100 g. of naphthalene and 1 l. of dioxan. Corrections were made for self-absorption effects by adding to each sample after counting 0.1 ml. (2840 disintegrations/min.) of a solution of [^{14}C]glycerol in butan-1-ol. Self-absorption was inversely proportional to the counting rate given by the glycerol standard. Counting was normally carried out to a standard error of 1%.

Labelled materials. D-[3,4- $^{14}\text{C}_2$]Glucose (1.33 $\mu\text{C}/\text{mg.}$) was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. [1,3- $^{14}\text{C}_2$]Glycerol (51 $\mu\text{C}/\text{mg.}$) was obtained from The Radiochemical Centre, Amersham, Bucks.

D-[1,3- $^{14}\text{C}_2$]Glucose was prepared by the method represented in Scheme 1. A mixture of 21.3 μmoles of [1,3- $^{14}\text{C}_2$]glycerol (0.1 mc), 60 μmoles of ATP, 0.05 ml. of glycerol kinase (Sigma Chemical Co., St Louis, Mo., U.S.A.; catalysed the phosphorylation of 120 μmoles of glycerol/min./ml. of enzyme at 37° and pH 9.8) and 3 ml. of buffer (150 μmoles of glycine, 15 μmoles of MgCl_2 , 0.3 mg. of serum albumin, adjusted to pH 9.8 with NaOH) were incubated for 30 min. at 37°. To the reaction mixture were added 5 ml. of buffer,

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Scheme 1. Preparation of D-[1,3-¹⁴C₂]glucose. Experimental details are given in the text.

pH 7.2 (350 μ moles of Na₂HPO₄, 150 μ moles of KH₂PO₄), 10 μ moles of NAD, 100 μ moles of glyceraldehyde 3-phosphate, 0.2 ml. of glycerol phosphate dehydrogenase (Sigma; catalysed the reduction of 240 μ moles of dihydroxyacetone phosphate/min./ml. of enzyme at 25°, 0.4 ml. of 'diaphorase' (Sigma; catalysed the conversion of 240 μ moles of NADH₂ into NAD/min./ml. of enzyme at 25° and pH 7), 0.1 ml. of aldolase (Sigma; '100 μ mole units/ml.') and 0.05 mg. of methylene blue. The mixture was incubated with shaking for 2 hr. at 37°. Paper chromatography of a sample of the reaction mixture in ethanol-m-ammonium acetate buffer, pH 3.8 (5:2, v/v), showed two main radioactive peaks: one represented approx. 65% of the radioactivity and had *R_F* corresponding to that of glycerol 1-phosphate; the other represented approx. 35% of the radioactivity and had *R_F* corresponding to that of fructose 1,6-diphosphate.

The reaction mixture was added to a column (diam. 1 cm.) containing 25 g. wet wt. of Dowex 1 (X2; Cl⁻ form) resin. The column was washed with 1 l. of 0.01N-HCl; this eluted the glycerol 1-phosphate. The column was then washed with 1 l. of 0.05N-HCl followed by 200 ml. of 0.5N-HCl. The fructose diphosphate was eluted by the 0.5N-HCl. The fractions containing the radioactivity were combined and the fructose 1,6-diphosphate was hydrolysed to fructose 6-phosphate with N-HCl at 37° for 5 days (Neuberg, Lustig & Rothenburg, 1944). The HCl was removed by repeated evaporation with toluene under reduced pressure and the residue was dissolved in 10 ml. of 0.2M-tris. The solution was adjusted to pH 8 with NaOH and diluted to 50 ml. Then 0.1 ml. of phosphohexose isomerase (Sigma; catalysed the conversion of 2000 μ moles of glucose 6-phosphate into fructose 6-phosphate/min./ml. of enzyme at 37° and pH 7.4) was added and the mixture was incubated for 1 hr. at 37°.

The reaction mixture was then adjusted to pH 10 with NaOH, MgCl₂ was added to give a concentration of 1 mM, 5 mg. of alkali phosphatase (Sigma) was added and the mixture was incubated for 1 hr. at 37°. The products were separated by paper chromatography in propan-1-ol-ethyl acetate-water (7:1:2, by vol.) on acid-washed Whatman no. 3MM paper. The radioactive material corresponding to glucose (approx. 30% of the total radioactivity) was eluted, diluted with 1 g. of carrier glucose and recrystallized twice from methanol-ethyl acetate.

Degradation of D-[1,3-¹⁴C₂]glucose. Radioactivity in glucose produced by the above procedure should ideally be confined to C-1 and C-3, and should be equally divided between them. To check the distribution of radioactivity in the product, a sample was degraded by an adaptation of the method of Abercrombie & Jones (1960). First, 0.1 g. of the [¹⁴C]glucose was converted into methyl α -D-glucoside, diluted with 1 g. of carrier methyl α -D-glucoside and recrystallized twice (the product had m.p. 166°). Then 0.6 g. of methyl α -D-[¹⁴C]glucoside was oxidized with sodium metaperiodate and the resulting formic acid and dialdehyde were separated as described by Abercrombie & Jones (1960). The formic acid (derived from C-3 of glucose) was purified by steam-distillation as described above for the formic acid from streptomycin. The dialdehyde was reduced with sodium borohydride, hydrolysed to glycerol and glycolaldehyde, and the glycolaldehyde was reduced to ethylene glycol (Abercrombie & Jones, 1960). The solution was treated with an excess of Amberlite IR-120 (H⁺ form) resin and repeatedly evaporated under reduced pressure with methanol to remove boric acid. The residue was dissolved in water, treated with a mixture of Amberlite IR-120 (H⁺ form) and Amberlite IR-4B (OH⁻ form)

Table 1. *Distribution of radioactivity in enzymically prepared D-[1,3-¹⁴C₂]glucose*

Experimental details are given in the text.

	Carbon atoms of glucose	Specific activity (counts/min./μmole)	Specific activity (% of glucose)
Methyl α-D-glucoside	All	334	100
Glycerol tri- <i>p</i> -nitrobenzoate	4, 5 and 6	6.6	2.0
Glycol di- <i>p</i> -nitrobenzoate	1 and 2	165	49.4
Formic acid	3	163	48.8

Table 2. *Distribution of radioactivity in streptose from streptomycin produced by S. griseus grown in the presence of D-[3,4-¹⁴C₂]glucose and D-[1,3-¹⁴C₂]glucose*

Experimental details are given in the text.

	Carbon atoms derived from L-streptose	Specific activity (counts/min./μmole)	Specific activity (% of streptose)
D-[3,4- ¹⁴ C ₂]Glucose experiment			
Maltol	All	18.3	100
Formic acid	C-3'	5.73	31.3
D-[1,3- ¹⁴ C ₂]Glucose experiment			
Maltol	All	2.69	100
Formic acid	C-3'	1.065	39.6

resins and run on a paper chromatogram with acid-washed Whatman no. 3MM paper and butan-1-ol-ethanol-water (3:1:1, by vol.) as developing solvent. The areas corresponding to ethylene glycol and glycerol were eluted from the paper. The glycol (representing C-1-2 of glucose) was converted into its di-*p*-nitrobenzoate (yield 51mg., m.p. 145°) and the glycerol (representing C-4-6 of glucose) was converted into its tri-*p*-nitrobenzoate (yield 150mg., m.p. 198°) (Smith & Van Cleve, 1955). The radioactivity of samples of the glycerol derivative, the glycol derivative, formic acid and methyl α-D-glucoside was determined. The results in Table 1 show that, although about 2% contamination at C-4-6 of the D-[1,3-¹⁴C₂]glucose had occurred, most of the radioactivity was distributed equally between C-1-2 and C-3. As the glucose was prepared from [1,3-¹⁴C₂]glycerol, it is reasonable to assume that most of the radioactivity of C-1-2 was in C-1. Certainly, the product was satisfactory for the present purpose, which was to distinguish between C-3 and C-4 of glucose as precursor of C-3' of streptose.

RESULTS AND DISCUSSION

The distribution of ¹⁴C in streptomycin produced by *S. griseus* grown in the presence of D-[3,4-¹⁴C₂]glucose and of D-[1,3-¹⁴C₂]glucose is shown in Table 2. The results of the incorporation of radioactivity from D-[3,4-¹⁴C₂]glucose into L-streptose show that 31.3% of the labelling appears in the C-3' branch carbon atom. Although this is lower than the theoretical value of 50%, the incorporation is sufficiently high to suggest that the C-3' carbon of streptose is derived from either C-3 or C-4 of

glucose. Certainly, if C-3' is derived from any other carbon atom of glucose then the incorporation would be expected to be considerably smaller. The results obtained with D-[1,3-¹⁴C₂]glucose strongly suggest that it is C-3 of D-glucose that ultimately appears as C-3' of L-streptose.

A possible explanation of the 'dilution' of the labelling of C-3' of streptose is as follows. There is evidence that the pentose phosphate shunt pathway is of quantitative importance in the oxidation of glucose by *Streptomyces* (Cochrane, Peck & Harrison, 1953; Ganguly & Roy, 1956; Silverman & Rieder, 1960). Recycling of glucose through the pentose phosphate shunt pathway would be expected to produce dilution of the labelling at C-3, but not at C-4. Katz & Wood (1960) have calculated the quantitative effects of such a process on the specific activities of the carbon atoms of labelled glucose. If it is arbitrarily assumed that 80% of the total glucose is metabolized through the pentose phosphate cycle, then calculation (with the values given by Katz & Wood, 1960) shows that [3,4-¹⁴C₂]glucose gives a product that contains 30.3% of its total radioactivity in C-3; similarly, [1,3-¹⁴C₂]glucose gives a product that contains 40% of its total radioactivity in C-3. These values correspond to those actually found for C-3' of streptose. It is not suggested that the actual metabolism of glucose in *S. griseus* is as simple as for the above theoretical model. Nevertheless, the operation of the pentose phosphate cycle as a

Scheme 2. Suggested biosynthesis of L-streptose from an intermediate in L-rhamnose biosynthesis. The numbers in parentheses refer to numbering of C atoms in D-glucose.

major pathway of glucose metabolism provides a reasonable explanation of the labelling patterns found in the streptose.

From our results it is suggested that the formation of L-streptose takes place by a rearrangement of the hexose carbon skeleton such that C-3 becomes the C-3' branch carbon of the product. It has already been suggested (Blumsom & Baddiley, 1961; Candy *et al.* 1964) that L-streptose might be derived from D-glucose at the nucleoside diphosphate sugar level. Thus an intramolecular rearrangement involving thymidine diphosphate sugar intermediates on the pathway from D-glucose to L-rhamnose has been postulated. It is known that rhamnose is formed in this way in *S. griseus* (Blumsom & Baddiley, 1961), and the previous isotope studies together with the present evidence strongly suggest that ring contraction and branching occur in a simultaneous process as shown in Scheme 2; the 6-deoxy-4-oxy sugar derivative of dTDP has already been suggested as an intermediate in the pathway of L-rhamnose synthesis. Attack by C-2 of the carbonyl carbon at C-4 may be assisted by association between the carbonyl oxygen and a positive centre in the enzyme protein.

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