Biosynthesis of Highly Labeled Actinomycins

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SUMMARY

The synthesis of tritiated actinomycin having a specific activity of 80,000 cpm/μg has been accomplished by growing *Streptomyces antibioticus* in the presence of methyl labeled L-methionine.

Recent interest in the mechanism of action of actinomycin as an inhibitor of the DNA-dependent RNA synthesis (1, 2), and the use of the compound as an antitumor agent (3, 4), have suggested that actinomycin of high specific activity would be of use in a variety of biochemical and pharmacologic studies. The present report, based on earlier studies on amino acid incorporation into actinomycin (5–7), describes the synthesis of highly labeled actinomycin (80,000 cpm/μg) from L-methionine, labeled with tritium in the methyl group.

L-Methionine was selected as precursor since it is the source of the methyl groups of sarcosine, *N*-methyl-L-valine and the actinomycin chromophore (5–7). There are 2 moles each of sarcosine and *N*-methyl-L-valine per mole of actinomycin as well as two methyl groups per mole of the chromophore; thus 6 moles of methionine are required for synthesis of 1 mole of antibiotic (Fig. 1).

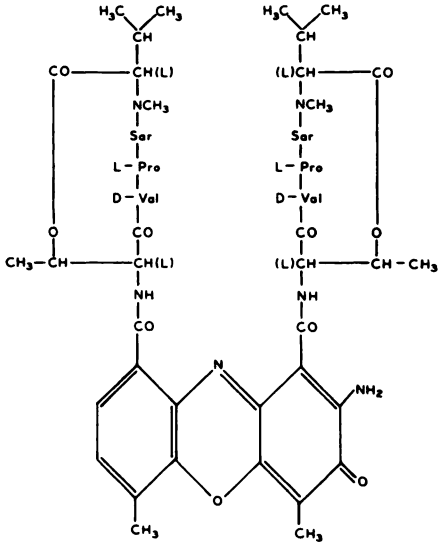

FIG. 1. *Structure of actinomycin IV.*

Actinomycin I contains 1 residue of 4-hydroxy-L-proline in place of 1 residue of L-proline, and actinomycin V contains 1 residue of 4-keto-L-proline in place of 1 residue of L-proline.

Seventy-five millicuries of L-methionine-methyl-^{3}H (250 mC/mmole), New England Nuclear Corporation, in 15 ml of water was diluted to 50 ml with distilled water. All radioactive measurements were performed with a liquid scintillation counter using a naphthalene-dioxane solution (8).

Streptomyces antibioticus strain 3720 was employed. The organism was cultivated for 48 hr in N-Z medium (9, 10), harvested by centrifugation at 6000 rpm for 10 min, and

then washed twice with 100 ml of physiological saline. The mycelium was finally resuspended in 100 ml of saline, and 2 ml of the mycelial suspension was used to inoculate each of 50–250 ml Erlenmeyer flasks containing 100 ml of glutamic acid–galactose–mineral salts medium (9, 10). The organism was incubated for 42 hr at 30° at which time the actinomycin titer was 32 μg per milliliter of medium. Chloramphenicol (Parke, Davis & Co.), 3.0 mg in 2 ml, was then added to each flask to inhibit protein synthesis and to enhance actinomycin formation (6, 7). Immediately afterward 0.5 ml of L-methionine-methyl-^{3}H (750 μC, 3 μmoles) was added to the flasks, and the cultures were reincubated for an additional 2 hr. The same concentration of radioisotope was then supplied again followed by a similar incubation period. This procedure was decided upon as a result of studies which showed that maximum incorporation of the methyl group from methionine can be achieved using chloramphenicol, 30 mμmoles of L-methionine per milliliter of medium and a 2-hr incubation period.

The culture medium was filtered by gravity flow through glass wool in a funnel, and the mycelium was washed 3 times with 100 ml of distilled water. The culture filtrate (4800 ml) and washings (300 ml) were combined and extracted 2 times with an equal volume of ethyl acetate. The ethyl acetate fractions were combined and then washed with one-half the volume of distilled water. The organic layer was dried with sodium sulfate and then evaporated to dryness under vacuum at 45° to 50°. The actinomycin complex was dissolved in 5 ml of acetone. Spectrophotometric assay of actinomycin (molecular extinction coefficient = 24,800 ± 200) at 443 mμ indicated the presence of 140 mg of the antibiotic mixture. Using a Hamilton syringe this solution was applied as a series of spots on a line 3 cm from one edge of a 20-cm glass plate containing a thin layer (0.75 mm) of silica-gel Gr. The whole sample was divided equally among 22 plates, approximating 6.4 mg of actinomycin per plate. The solvent used for thin-layer chromatography was ethyl acetate:acetone (2:1), which was allowed to run to the upper edge of each plate. Generally the three principal components of the actinomycin complex traveled the following distances: actinomycin I, 4.2 cm; actinomycin IV, 10.5 cm; actinomycin V, 12.5 cm (solvent front at 17.1 cm). After the solvent had evaporated from the plates, the colored zones were scraped off and the individual actinomycins were eluted with methanol. The yield of each of the actinomycins was: actinomycin I, 5.4 mg; actinomycin IV, 44.9 mg; actinomycin V, 49.7 mg. The actinomycins were not obtained crystalline because of the danger of radiodecomposition but were kept in methanol at 4°. No loss in biological activity has been observed during more than 1 year of storage. Homogeneity of the actinomycins was studied by thin-layer chromatography in the same solvent system, using 0.5-mm silica gel plates and a considerably reduced amount of sample. The actinomycin I preparation was contaminated with both actinomycins IV and V due to tailing of zones on the preparative thin-layer chromatography. The actinomycin IV sample was slightly contaminated with actinomycin V; spectrophotometric assay after elution of the zones as described before, yielded an analysis of actinomycin IV, 86%; actinomycin V, 14%. Actinomycin V was found to be a homogeneous preparation. The actinomycin complex originally isolated had the following composition: I, 3.8%; IV, 40.4%; V, 55.8%.

The specific radioactivity of the various actinomycins was in the range of 80,000 cpm/μg.

Previous radioisotopic studies have shown that extraction of the culture medium with ethyl acetate under neutral or slightly alkaline conditions was a highly specific method for the isolation of actinomycin (7). This was established by paper chromatographic and crystallization procedures. The present investigation employed thin-layer chromatography in conjunction with the neutral extraction procedure; the thin-layer technique thus adding further specificity to the

isolation procedure. No radioactive substances other than actinomycins I, IV, and V were detected.

The high specific activity of the material should be of value in elucidating the mechanism of action, tissue distribution, and metabolism of this agent.

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Insensitivity to Valine of Streptomycin-Dependent *Escherichia coli* K12

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SUMMARY

A streptomycin dependent mutant was obtained from wild-type (valine-sensitive) *Escherichia coli* K12. This mutant (*E. coli* DK 12) grew exponentially in minimal medium containing valine (plus dihydrostreptomycin). The inhibition by valine of the acetohydroxy acid synthetase from both dependent and parent cells was quantitatively similar. However, the activity of the acetohydroxy acid synthetase of DK 12 cells was higher than that of the parent cells. It was concluded that the insensitivity to valine of *E. coli* DK 12 was the result of derepression of (acetohydroxy) acid synthetase in this mutant, the role of the antibiotic in dependent cells being that of a "derepressor."

Bonner (1) reported that the growth of a wild-type strain of *Escherichia coli* K12 was inhibited by L-valine and that this inhibition was overcome by L-isoleucine. Leavitt and Umbarger (2) proposed that inhibition of the growth of *E. coli* K12 by valine was a consequence of the relatively high sensitivity to inhibition by valine of the initial condensing enzyme (acetohydroxy acid synthetase) of the isoleucine-leucine-valine pathway. Consequently,