4-METHYL-3-HYDROXYANTHRANILIC ACID, AN INTERMEDIATE IN ACTINOMYCIN BIOSYNTHESIS

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The chromophore (actinocin) of the peptide antibiotic actinomycin can be formed chemically (Brockmann and Muxfeldt, 1958) and enzymatically (Weissbach and Katz, 1961; Katz and Weissbach, 1962) by the condensation of 2 moles of 4-methyl-3-hydroxyanthranilic acid (4MHAA). The phenoxazinone condensing enzyme will also form phenoxazinones from anthranyloyl peptides (Salzman et al., unpublished data) and a mechanism for the synthesis of the antibiotic can be formulated in which 4MHAA is converted to a 4MHAA pentapeptide which then condenses to yield the antibiotic (Fig. 1). This scheme suggests that the methylation of the phenoxazinone precursor occurs before condensation and that 4MHAA is a free intermediate in the biosynthetic pathway. The present communication describes experiments obtained with washed mycelium of Streptomyces antibioticus, which demonstrate that 4MHAA is an intermediate in the conversion of ^{14}C-DL-tryptophan to actinomycin.

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1. National Institute of General Medical Sciences predoctoral fellow.

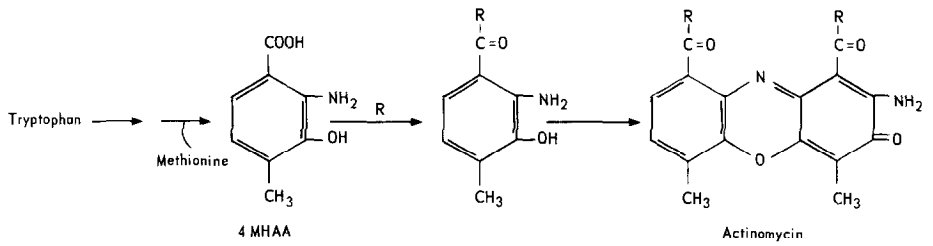

Fig. 1. Proposed pathway for actinomycin biosynthesis.

S. antibioticus was grown as described previously (Katz et al., 1958; Katz and Goss, 1958). After 72 hours, at which time the actinomycin titer was approximately 60 μg/ml, the mycelium was harvested by filtration on a suction flask, washed with saline and resuspended in 0.1 M PO_4 buffer, pH 7.4, to 30% of the original volume of medium.

Radioactive compounds were purchased from New England Nuclear Corporation, with the exception of benzene-ring labeled DL-tryptophan which was obtained from Nuclear-Chicago Corporation. The 4MHAA was obtained from Dr. A. Mauger, National Institutes of Health. Incubations were performed for periods up to 30 minutes at 30° with shaking in 20 ml beakers on a Dubnoff metabolic shaker. The incubation mixture, in a total volume of 5 ml, contained 4.8 ml of washed mycelial suspension, 500 mμmoles L-valine-1-^{14}C or DL-tryptophan-^{14}C, uniformly labeled in the benzene ring (200 cpm/mμmole of the L-isomer), 500 mμmoles each of L-threonine, L-proline, sarcosine, L-methionine, and either L-valine or L-tryptophan depending on which radioisotope was employed. Any further additions are mentioned below. After an

incubation the contents of a beaker were filtered through glass wool to remove mycelium, and radioactive actinomycin was extracted into 3 ml of ethyl acetate from a 2 ml aliquot of the filtrate. Two ml of the ethyl acetate fraction were counted in a naphthalene-dioxane solution (Bray, 1960) using a scintillation spectrometer. Total extractable ^{14}C-metabolites (including actinomycin) were obtained by acidifying 1 ml of the filtrate to pH 1.0, extracting with 3 ml of ethyl acetate, and measurement of the radioactivity in 2 ml of the ethyl acetate phase. Previous experiments (Katz and Weissbach, 1962a; 1963) have shown that, under the conditions used, the radioactivity in the neutral ethyl acetate extract is present almost entirely in actinomycin.

Preliminary experiments showed that incorporation of amino acids into actinomycin with washed mycelium, proceeded at about

Table I

Incorporation of L-Valine-^{14}C into Actinomycin

System	mμmoles Valine Incorporated I	II
No additions	10.5	13.2
+ Kynurenine	12.0	9.0
+ 3-OH-kynurenine	23.0	25.0
+ HAA	24.3	26.0
+ 4MHAA	20.5	24.3

The basic system (5 ml) contained washed mycelium, an amino acid mix and 500 mμmoles L-valine-^{14}C as described in the text. Five hundred mμmoles of the other components were added as indicated.

2/3 the rate observed with cells in the glutamate-galactose medium. The ability to replace the complete medium with buffer, however, facilitated testing the effect of possible intermediates on actinomycin synthesis. As shown in Table I the incorporation of L-valine-^{14}C into actinomycin was stimulated significantly by the addition of 3-hydroxy-DL-kynurenine, 3-hydroxyanthranilic acid (HAA), or 4MHAA. Addition of kynurenine, and, in other experiments, tryptophan, had little influence. The stimulation of valine incorporation into actinomycin by HAA or 4MHAA suggested that the cells ability to make the chromophore precursor was rate limiting, and that HAA and 4MHAA might be intermediates in actinomycin synthesis. This would be in agreement with previous observations that tryptophan is the precursor of the phenoxazinone moiety of the antibiotic (Katz, 1960; Sivak, et al., 1962; Katz and Weissbach, 1962a, 1963).

Evidence for the role of 4MHAA as a direct intermediate in the pathway was sought by testing the isotope dilution effect of 4MHAA on the incorporation of DL-^{14}C-tryptophan into the antibiotic. If 4MHAA is an intermediate it would be expected to dilute out the radioactivity incorporated into actinomycin from tryptophan. In a series of experiments with washed mycelium, a 40-80% decrease in ^{14}C-tryptophan incorporation into actinomycin (neutral extractable cpm) was obtained. By contrast the total extractable cpm (acid extractable cpm) was enhanced slightly (Table II). This indicated that the decreased incorporation of ^{14}C-tryptophan into actinomycin, in the presence of 4MHAA, was not due to an unrelated inhibition of tryptophan metabolism or

Table II

Effect of 4MHAA on ^{14}C-Tryptophan Incorporation into Actinomycin

Additions	mμmoles Tryptophan Incorporated: Actinomycin (neutral extraction)	Total metabolites (acid extraction)
None	8.6	26.0
4MHAA	2.1	31.2
HAA	6.6	28.0

The incubation conditions are described in the text. Five hundred mμmoles of 4MHAA or HAA were used.

transport. Paper chromatographic separation of acid extractable metabolites after incubating washed mycelium with ^{14}C-tryptophan in the presence of 4MHAA provided evidence for the formation of 4MHAA. As seen in Figure 2, in the presence of 4MHAA, a large amount of a radioactive component, with chromatographic properties (methanol, propanol, benzene, H_2O, 2:2:1:1) similar to 4MHAA is seen, with some radioactivity also present in the actinomycin area. In a similar experiment (Figure 2) in which unlabeled 4MHAA was not added, no significant radioactivity is seen in the 4MHAA region, and a greater proportion of the radioactivity is present in the actinomycin region. Similar results were obtained with two other solvent systems (butanol, ethanol, H_2O, 2:1:1; methanol, butanol, benzene, H_2O, 2:1:1:1). In addition this ^{14}C-product, formed from ^{14}C-tryptophan in the presence of 4MHAA, also cochromatographed with 4MHAA on a Dowex-1-Cl col-

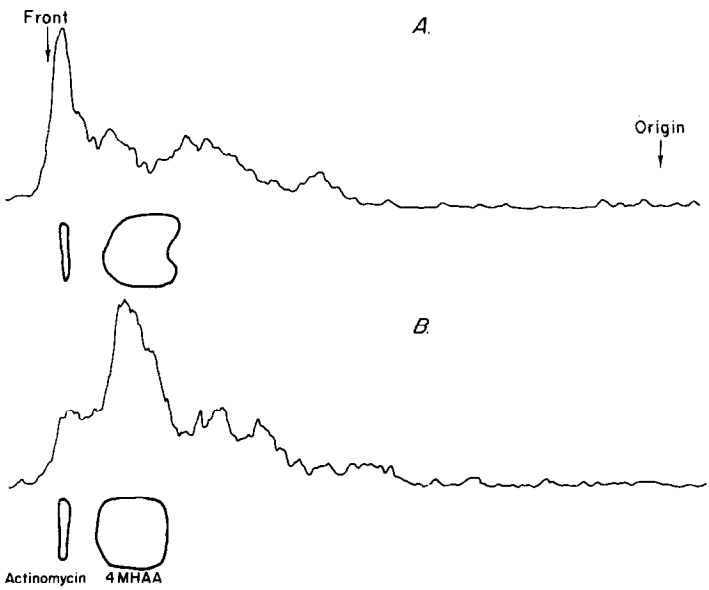

Fig. 2. Paper chromatographic identification of 4MHAA as an intermediate in actinomycin biosynthesis. The bottom curve (B) shows a scan of ethyl acetate extractable radioactive compounds after incubation of ^{14}C-tryptophan in the presence of unlabeled 4MHAA. The top curve (A) is that of a similar experiment in which 4MHAA was omitted. The solvent system used was (methanol, propanol, benzene, H_2O, 2:2:1:1). Ascending chromatography, with Whatman #1 paper, for 16 hrs. was employed.

umn (adsorption from pH 7.0 buffer and elution with 6N acetic acid).

An unexpected, and still unexplained result, was the finding that a similar pool of HAA did not significantly affect (generally less than 20%) ^{14}C-incorporation from tryptophan into actinomycin (Table II). Although the conversion of tryptophan to 4MHAA, is readily pictured as proceeding through HAA, it is conceivable that the latter compound is not a free intermediate in actinomycin biosynthesis. However, it must be stressed that

the present studies were done with whole cells and it may not be possible to elucidate the role of HAA until a cell-free system is obtained which catalyzes the synthesis of the antibiotic.

In summary, 3-hydroxykynurenine, HAA and 4MHAA stimulate L-valine incorporation into actinomycin. Experiments with ^{14}C-DL-tryptophan have provided evidence that 4MHAA is an intermediate in actinomycin synthesis.

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